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Microfluidic array for three-dimensional perfusion culture of human mammary epithelial cells

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Abstract The ability to culture cells in three dimensional extracellular matrix (3D ECM) has proven to be an important tool for laboratory biology. Here, we demonstrate a microfluidic perfusion array on a 96-well plate format capable of long term 3D ECM culture within biomimetic microchambers. The array consists of 32 independent flow units, each with a 4 μl open-top culture chamber, and 350 μl inlet and outlet wells. Perfusion is generated using gravity and surface tension forces, allowing the array to be operated without any external pumps. MCF-10A mammary epithelial cells cultured in Matrigel in the microfluidic array exhibit acinus morphology over 9 days consistent with previous literature. We further demonstrated the application of the microfluidic array for *in vitro* anti-cancer drug screening.

Keywords Microfluidics · 3D cell culture · Mammary epithelial cells · MCF-10A

1 Introduction

It is widely accepted that culture of cells in a three-dimensional extracellular matrix (3D ECM) environment promotes many biologically relevant functions not found in 2D monolayers (Cukierman et al. 2001). More widespread use of 3D ECM culture in laboratory and clinical research is limited by the lack of adequate tools combining biological relevance, ease of use, and scalability. Microfluidic technology has been repeatedly used in cell culture applications, demonstrating advantages such as improved nutrient transport, reduction of cell/reagent usage, and automation compatibility (El-Ali et al. 2006; Lee et al. 2006, 2007c; Vickerman et al. 2008). In this work, we designed a microfluidic cell array (MiCA) that is capable of passively localizing 3D ECM within a cell culture chamber without hindering continuous perfusion of medium to the cells. The array consists of 32 independent flow units on a 96 well plate frame, and can be operated using standard liquid handling equipment.

A well-studied example of the benefits of 3D ECM culture is acinus formation of human mammary epithelial cells when cultured in laminin rich 3D ECM. Biological ECM contains an abundance of signaling molecules that are directly related to pathways implicated in cancer cell phenotypes (Plachot et al. 2009). Evidence has shown that benign and malignant mammary epithelial cells exhibit different morphologies in 3D ECM compared to 2D (Feder-Mengus et al. 2008; Gurski et al. 2009). While cancer cells in 2D adopt an unnatural flat morphology, cancer cells in 3D show a clustered and rounded morphology which is reminiscent of tumors *in vivo* (Feder-Mengus et al. 2008; Gurski et al. 2009). Kenny et al. further characterized the mammary epithelial cells into four different types: round, mass, grape-like and stellate, based on their morphology in 3D culture (Kenny et al. 2007). In 2D cultures, these morphologies cannot be distinguished. It has also been shown that the growth rate of cancer cells in 3D better fits mathematical models of tumors *in vivo* than cells in 2D (Chignola et al. 2000). Additionally, the gene expression profiles of 3D cultured cells demonstrated a better

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reflection of cancer cell function in the *in vivo* environment (Kenny et al. 2007). There is also evidence to support that cancer cells cultured in 3D show differences in anti-cancer drug sensitivities when compared to 2D (David et al. 2008; Horning et al. 2008). Furthermore, it is suggested that culturing cancer cells in 3D makes them resistant to some chemotherapeutics and the sensitivity differences seen in 3D culture may be representative of the way cancer cells *in vivo* respond to chemotherapeutic treatment (David et al. 2008; Horning et al. 2008). For these reasons, it is expected that 3D ECM cultures of cancer cells will provide more accurate data regarding anti-cancer drug efficacy *in vivo*.

Current 3D ECM culture techniques rely on an overlay method in standard multi-well plastic dishes (Bissell and Barcellos-Hoff 1987).While this recapitulates the gel environment, this static culture format does not model the perfusion mass transport in tissues. In this work, we developed a microfluidic method that integrates continuous medium perfusion through micro-capillaries located adjacent to the cell/gel culture chamber. We used the benign human mammary epithelial cell line MCF-10A to observe cell growth and differentiation to form distinct basal and apical polarity over 9 days. We also demonstrated that a malignant human mammary epithelial cell line (MCF-7) formed a mass-like morphology as described in the literature (Kenny et al. 2007). We further demonstrated that this platform can be applied to anti-cancer drug screening. This proof-of-concept illustrates how microfluidic engineering can provide a platform for 3D ECM culture in an automation friendly perfusion array.

2 Materials and methods

2.1 Microfabrication

The microfluidic array was fabricated using soft-lithography technology of PDMS (Sylgard 184, Dow Corning) and replicated molding. A 6-inch silicon wafer was used as the mold substrate for the microfluidic structures. A 4 μm layer (SU8 2002 photoresist, Microchem), 40 μm layer (SU8 2035), and 200 μm layer (SU8 2100) were spin-coated on the silicon wafer and patterned as the perfusion barrier, flow channels, and cell culture chamber, respectively. A sheet of modified PMMA (Clarex) was used to sandwich the PDMS between the PMMA and the mold and cured at 70°C for 4 h. Through-holes were cut with a CO_2 laser (Universal Laser), attached to an open-bottom 96 well plate via UV adhesive (Loctite 3321), and bonded to glass cover slide with oxygen plasma (100 W, 0.5 mbar, 3 min). The channels were primed with PBS solution under pressure to remove air bubbles.

2.2 Cell culture

Non-neoplastic human mammary MCF-10A epithelial cells and human breast adenocarcinoma cell lines MCF-7 were obtained from ATCC. MCF-10A cells were grown in D-MEM/F-12 medium (Gibco) supplemented with 2% horse serum (Gibco), 5 ng/ml EGF (Invitrogen), 0.5 μg/ml Hydrocortisone (Sigma), 100 ng/ml Cholera toxin (Sigma) and 10 μg/ml Insulin (Gibco) in a 5% CO_2, atmosphere at 37°C. MCF-7 cells were grown in DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomysin/amphotericin B antibiotic/antimycotic mixtures (MP Biomedicals, LLC) in a 5% CO_2, atmosphere at 37°C. The microfluidic plates were loaded by first aspirating the priming solution from all wells. For 3D cultures, cell suspension at 2,000–6,000 cells/μl was mixed with Matrigel (BD Biosciences) at a 1:8 ratio and 4 μl of the cell/gel mixture was loaded directly onto the cell culture chambers, followed with incubation at 37°C for 15 min to polymerize the Matrigel. Gravity driven perfusion was initiated by adding 300 μl to the flow inlet well and 30 μl to the flow outlet well. Medium was replenished every 2 days for long term culture.

2.3 Perfusion flow characterization

Dextran (3,000 MW) conjugated fluorescein (Invitrogen) was used for the flow rate measurements on the MiCA plates. A standard curve measuring volume as a function of fluorescence intensity was created using a plate reader (Biotek Synergy). To initiate flow, 300 μl of dextran solution was added to the inlet wells with 30 μl in the outlets and placed in a humidified cell culture incubator. To determine flow rates, volumes of the inlet and outlet wells were measured every 12 h on a plate reader on four wells in the absence or presence of Matrigel in the culture chamber. Diffusion of dextran (3,000 MW) fluorescein into the Matrigel filled chamber was monitored via time-lapsed imaging using an inverted microscope. The chambers and channels were initially filled with PBS, and 300 μl of dye was added to the inlet and 30 μl in the outlet at $t=0$.

2.4 Staining

Cell staining was performed according to a previously published protocol (Lee et al. 2007a). After rinsing with 1X PBS, cells were fixed with 4% paraformaldehyde solution for 20 min. The fixation was stopped by washing cells with PBS for 5 min. The Matrigel was then digested using BD cell recovery solution (BD Biosciences) for 20 min, followed by rinsing with PBS for 5 min. Cells were stained with 0.1% of Hoescht 33342 trihydrochloride (Invitrogen)

in 10% Goat serum (Invitrogen) in PBS and incubated at room temperature overnight. Cells were then washed with PBS for an hour and stained with 3 μl of Alexa Fluor 546 phalloidin (Invitrogen) in 1% BSA in PBS for 2 h and rinsed with PBS for 15 min.

2.5 Cytotoxicity assay

MCF-10A cells were loaded to the cell chambers in the presence or absence of Matrigel in the MiCA plates as described above. After perfusion culture for 4 days, cells were treated with Paclitaxel at the indicated concentrations for 3 days. Cytotoxicity analysis was performed using CytoTox-One Cytotoxicity kit (Invitrogen) according to the manufacturer's protocol. Cytotoxicity in each treatment was normalized to the total numbers of the cells in each chamber. Each treatment was measured in triplicate. Cytotoxicity imaging was performed using Ethidium homodimer-1 solutions from LIVE/DEAD® Cell Vitality Assay Kit (Invitrogen) according to the manufacturer's protocol with nuclear staining by 0.1% of Hoescht 33342 trihydrochloride (Invitrogen).

3 Results and discussion

The design goal of the microfluidic perfusion array was to develop a product with practical application for 3D ECM cell culture screening. To achieve this goal, the key features we incorporated were: 1) cultured cells would recapitulate activities found in traditional 3D ECM formats, 2) mass transport would be achieved via continuous perfusion through the culture chamber, and 3) the format would be amenable to existing screening technologies. Based on our previous work with microfluidic cancer cell arrays in the 2D format (Lee et al. 2007b), a number of key innovations were necessary to meet these needs. The most important advances include utilizing a microfabricated perfusion barrier to localize 3D ECM to the culture chamber (and not the perfusion channels), developing a mass transport model to adequately feed cells for long term culture, and adapting the microfluidics to be operated on a standard 96 well plate format.

The layout of the microfluidic array is depicted in Fig. 1. There are 32 independent units tiled on a standard 96 well plate, with each flow unit occupying 3 wells. The 3 wells correspond to a flow inlet, the cell culture chamber, and a flow outlet. Cells/gels are dispensed directly into the open-access culture chamber (Fig. 2). When using Matrigel, the gel is initially loaded at 4°C, and polymerized within the chamber when placed in a 37°C incubator for 15 min. Cells can be loaded without gel, embedded in gel, or overlayed with gel (see supplementary figure 1). Cell feeding is achieved via continuous perfusion of medium from the flow inlet well, past the microfluidic chamber, and out the flow outlet well. Perfusion flow is driven by gravity when there is a liquid height difference between the inlet and outlet wells. The fluidic resistance is set so that when the inlet well has 300 μl of medium and the outlet has 30 μl, 80 μl will flow through every 24 h. As shown in Fig. 3, the flow rate was not affected by the absence or presence of Matrigel in the culture chamber. The measured flow rate started at 5 μl/h for the first 24 h, then dropped to 3 μl/h for the second 24 h, and equilibrium was reached within 3 days. As long as the inlet is refilled and the outlet emptied every 2 days, the cells will receive continuous flow. We found that in a typical cell culture incubator, the evaporation rate was about 1% over 24 h. The advantage of the gravity flow configuration is that no external pump is necessary to drive perfusion to all 32 flow units. In addition, multiple plates

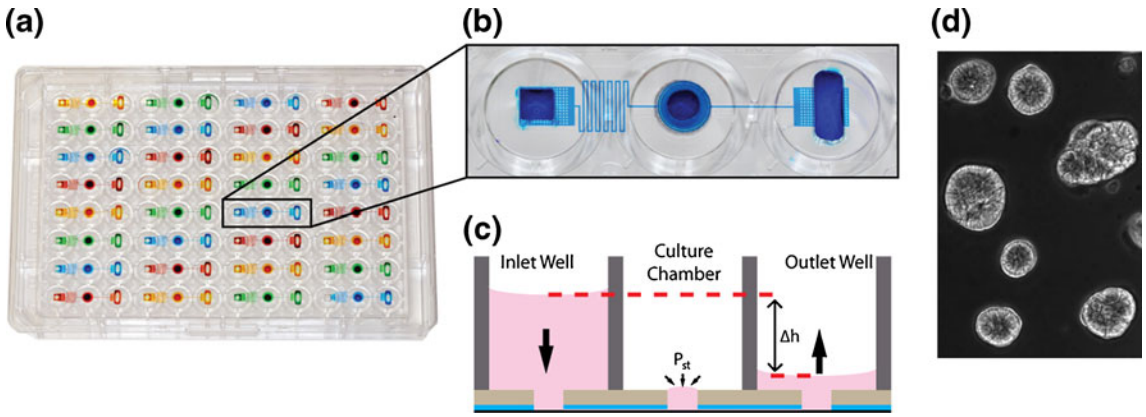

Fig. 1 Design of the 3D microfluidic cell array (MiCA). (**a**) Photo from the bottom of the 32 unit microfluidic perfusion array filled with dyes. (**b**) Image of a single flow unit and (**c**) schematic of cross section. Cells are loaded directly onto the open-access culture chambers. Gravity driven flow is initiated by adding 300 μl of medium to the inlet and 30 μl to the outlet. The medium flows from the inlet through the culture chamber and collects in the outlet. Surface tension pressure (P_{st}) prevents liquid from flowing out of the open chamber. (**d**) MCF-10A cells cultured in Matrigel within the microfluidic chamber adopt a 3D spheroidal morphology

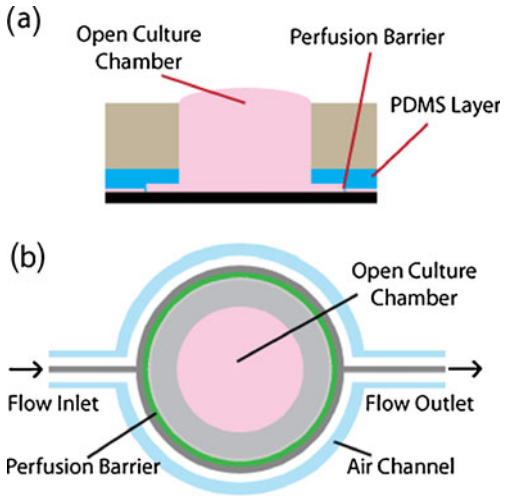

Fig. 2 Microfluidic cell culture chamber design. (**a**) Side view and (**b**) top view schematic of the open top cell culture chamber. The 2 mm diameter chamber has an open top allowing direct dispensing of cells/gels to the chamber. The chamber is connected to the microfluidic perfusion channels. A 4 μm tall perfusion barrier surrounds the culture area to localize the cells/gel to the chamber while enabling diffusion of nutrients and wastes in and out of the chamber

can be run in parallel inside a standard cell culture incubator.

The microfluidic culture unit is designed to localize a polymerizable gel into a defined region without hindering flow of medium to cells in the gel. Separating the cell/gel chamber and the perfusion channel is a microfabricated perfusion barrier—a network of 75×4×4 micron (LxWxH) microfluidic channels surrounding the entire culture region. We found the dimensions of the perfusion barrier reliably prevented the viscous gel from flowing across the barrier, while allowing free diffusion of solutes into the gel (Fig. 3). Using a 3 kDa fluorescent molecule, we found that diffusion into the center of the gel took about 20–30 min, consistent with calculations from the diffusion equation. We designed our chamber dimension to 2 mm diameter to ensure the cells were not nutrient starved.

Next, we tested if human breast epithelial cells could be cultured in the microfluidic array plates to form acinar structures as described in the literature (Debnath et al. 2003). In this work, we utilized Matrigel as a prototypical 3D ECM (Kleinman and Martin 2005). Cell suspension was mixed

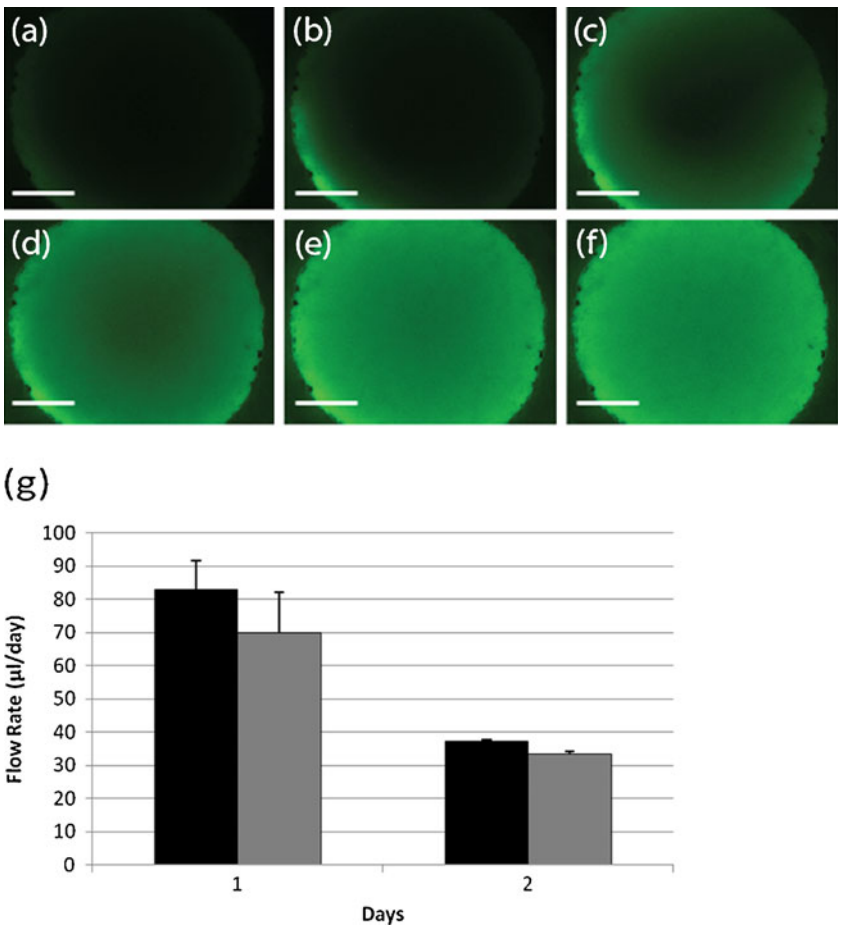

Fig. 3 Media diffusion characterization of the MiCA plates. Diffusion of FITC-Dextran (3 kDa) from the flow channel into the culture chamber after 0 min (**a**), 5 min (**b**), 10 min (**c**), 15 min (**d**), 20 (**e**), 25 (**f**) minutes of gravity driven perfusion. Scale bar=500 μm. (**g**) shows the gravity driven flow volume at 24 and 48 h in the absence (*black*) or presence (*gray*) of Matrigel in the culture chambers

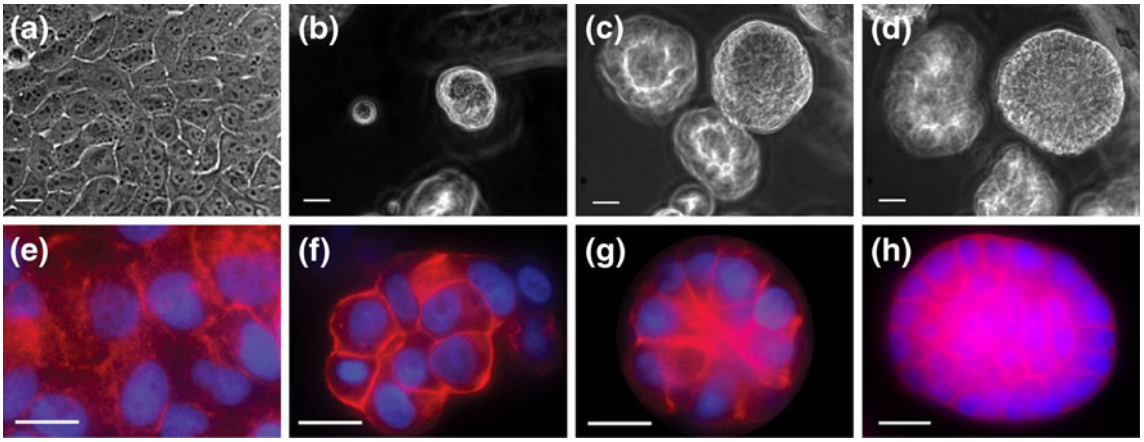

Fig. 4 Culture of breast cancer epithelial cells in the microfluidic chamber. (**a**) MCF-10A cells cultured without gel. MCF-10A cells cultured in Matrigel at 3 (**b**), 6 (**c**), and 9 (**d**) days. Actin (*red*) and nucleus (*blue*) stain of MCF-10A cells grown without gel (**e**), MCF-7 cells in Matrigel after 4 days (**f**), MCF-10A cells in Matrigel after 4 days (**g**), and MCF-10A cells after 9 days (**h**). Scale bar=20 μm in all images

with Matrigel at a 1:8 ratio and loaded into the microfluidic chambers, followed with incubation at 37°C for 15 min to polymerize the Matrigel. Cell culture medium was then added to the inlet wells for gravity perfusion culture, with replacement every 2 days. As shown in Fig. 4, MCF-10A cells underwent cell division and also transformed into a spheroidal morphology. This morphology was not seen on 2D perfusion culture in the microfluidic chamber. This is consistent with previous work (Debnath et al. 2003) that describes MCF-10A cells forming spherical clusters during the early stages of morphogenesis; by days 5–8, forming two populations of cells: a well-polarized outer layer of cells that is in direct contact with the matrix and an inner subset of cells that are poorly polarized; and by day 8, the central, non-polarized cells begin to die by apoptosis, forming a hollow lumen (Debnath et al. 2002). From our experiments, MCF-10A cells cultured in the microfluidic array successfully developed into polarized acini over 9 days.

We then compared MCF-10A and MCF-7 cells in the microfluidic array. Using a live/dead fluorescence assay, we found the viability of both the MCF-10A and MCF-7 cells was well maintained after 12 days of perfusion culture. Actin staining of the cell clusters revealed that MCF-7 cells in 3D ECM formed a mass morphology that is different from the acinar structures formed by the non-neoplastic MCF-10A. More importantly, we observed that MCF-10A cells in direct contact with the matrix assumed basal polarity and that the inner part of the spheroid cluster formed the apical polarity. These findings are fully consistent with previous publications on these two mammary endothelial cell types (Kenny et al. 2007), supporting the claim that a microfluidic perfusion environment can recapitulate relevant biological functions of 3D ECM culture.

To investigate if this platform can be used for anti-cancer drug sensitivity screening, we treated the MCF-10A mammary epithelial cells with Paclitaxel, and measured cytotoxicity with a fluorescence based assay. As shown in Fig. 5, the sensitivity of the cells cultured in 3D and in 2D is significantly different ($P<0.05$). This finding is consistent with the previously reported studies (David et al. 2008; Horning et al. 2008).

Our goal in this work was to present a practical tool that could be utilized by laboratory technicians to culture cancer cells in perfused 3D ECM. Key innovations include the simple operation of the microfluidic array, compatibility with existing automation and analysis instrumentation, and ability to robustly handle small cell/gel samples. Our next steps include the study of various synthetic matrices, comparing morphologies and anti-cancer drug response of

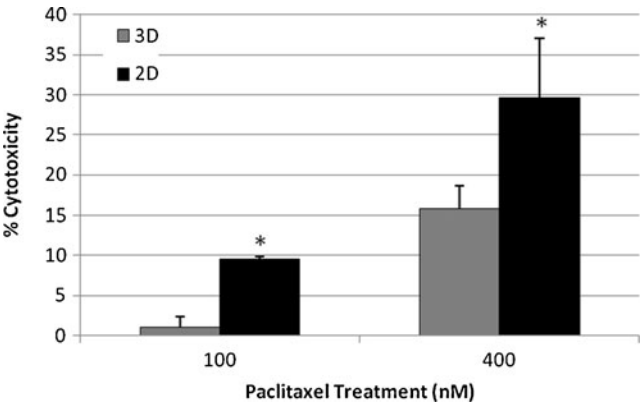

Fig. 5 Cytotoxicity analysis on MCF-10A cells. (**a**) MCF-10A cells were cultured on the MiCA plates for 4 days, followed by treatment with Paclitaxel for 3 days. Continuous perfusion culture in the MiCA plate was used for both 3D (Matrigel) and 2D (no gel). The cytotoxicity analysis was done by using the CytoTox-One Cytotoxicity kit (Invitrogen). The stars indicate the samples with significant differences ($P<0.05$) compared to the 2D values

additional mammary epithelial cell types, and more detailed dissection of biological events triggered in 3D ECM culture. Future application of this type of microfluidic technology may lead to breakthroughs in pharmaceutical drug screening, improved analysis of cancer cell phenotypes, and eventually as a means to diagnose individual patient cancer cells.

References

M.J. Bissell, M.H. Barcellos-Hoff, J. Cell Sci. Suppl. **8**, 327–343 (1987)

R. Chignola, A. Schenetti, G. Andrighetto, E. Chiesa, R. Foroni, S. Sartoris, G. Tridente, D. Liberati, Cell Prolif. **33**, 219–229 (2000)

E. Cukierman, R. Pankov, D.R. Stevens, K.M. Yamada, Science **294**, 1708–1712 (2001)

L. David, V. Dulong, D. Le Cerf, L. Cazin, M. Lamacz, J.P. Vannier, Acta Biomater. **4**, 256–263 (2008)

J. Debnath, K.R. Mills, N.L. Collins, M.J. Reginato, S.K. Muthuswamy, J.S. Brugge, Cell **111**, 29–40 (2002)

J. Debnath, S.K. Muthuswamy, J.S. Brugge, Methods **30**, 256–268 (2003)

J. El-Ali, P.K. Sorger, K.F. Jensen, Cells on chips. Nature **442**, 403–411 (2006)

C. Feder-Mengus, S. Ghosh, A. Reschner, I. Martin, G.C. Spagnoli, Trends Mol. Med. **14**, 333–340 (2008)

L.A. Gurski, A.K. Jha, C. Zhang, X. Jia, M.C. Farach-Carson, Biomaterials **30**, 6076–6085 (2009)

J.L. Horning, S.K. Sahoo, S. Vijayaraghavalu, S. Dimitrijevic, J.K. Vasir, T.K. Jain, A.K. Panda, V. Labhasetwar, Mol. Pharm. **5**, 849–862 (2008)

P.A. Kenny, G.Y. Lee, C.A. Myers, R.M. Neve, J.R. Semeiks, P.T. Spellman, K. Lorenz, E.H. Lee, M.H. Barcellos-Hoff, O.W. Petersen, Mol. Oncol. **1**, 84–96 (2007)

H.K. Kleinman, G.R. Martin, Semin. Cancer Biol. **15**, 378–386 (2005)

P.J. Lee, P.J. Hung, V.M. Rao, L.P. Lee, Biotechnol. Bioeng. **94**, 5–14 (2006)

G.Y. Lee, P.A. Kenny, E.H. Lee, M.J. Bissell, Nat. Methods **4**, 359–365 (2007a)

P.J. Lee, T.A. Gaige, N. Ghorashian, P.J. Hung, Biotechnol. Prog. **23**, 946–951 (2007b)

P.J. Lee, N. Ghorashian, T.A. Gaige, P.J. Hung, JALA Charlottesv Va. **12**, 363–367 (2007c)

C. Plachot, L.S. Chaboub, H.A. Adissu, L. Wang, A. Urazaev, J. Sturgis, E.K. Asem, S.A. Lelievre, BMC Biol. **7**, 77 (2009)

V. Vickerman, J. Blundo, S. Chung, R. Kamm, Lab Chip **8**, 1468–1477 (2008)