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Identifying drug resistant cancer cells using microbubble well arrays

Qihui Pu[1] · **Ryan Spooner**[1] · **Lisa A. DeLouise**[1,2]

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Abstract Drug resistance is a characteristic of tumor initiating cells that can give rise to metastatic disease. In this work we demonstrate the use of microbubble well arrays as a cell culture platform to enumerate and characterize drug resistant cells in a human derived tumorigenic squamous cell carcinoma cell line. The spherical architecture and compliant hydrophobic composition of the microbubble well favors single cell survival, clonal proliferation and formation of spheres that do not grow on standard tissue culture plastic and are resistant to cisplatin. Spheres form in isolation and in microbubble wells containing proliferating cells and to some degree they stain positive for common stem cell markers CD44 and CD133. Spheres are also observed in cellularized primary human tumors cultured in microbubble arrays. This proof-of-concept study illustrates the potential for microbubble array technology to enumerate cancer cells resistant to standard care drugs with the ability to test alternative drug combinations. This capability can be developed for designing patient specific treatment strategies. Recovery of drug-resistant cells will allow a more full characterization of their gene expression profile thereby expanding our fundamental knowledge and ability to develop new targets to fight metastatic disease.

Keywords Microbubble well array · Drug resistant · Sphere cell · Squamous cell carcinoma · Tumor initiating cells · Polydimethylsiloxane

1 Introduction

Despite the existence of precise radiologic and surgical methods and effective therapies for the elimination or remission of primary tumors, most cancer-related deaths result from metastatic disease (Talmadge and Fidler, 2010). Cancer metastasis occurs when malignant cells gain the ability to break away from the primary tumor, enter circulation, extravasate the endothelial barrier, survive the immune system and successfully proliferate in a secondary tissue site (Fidler, 2003). Studies estimate that up to one million cells may be shed per gram of tumor daily (Butler and Gullino, 1975; Chang et al., 2000); however, only a very few of these cells possess the capacity to metastasize (Held et al., 2010; Zhou et al., 2009). For a metastasis to develop, a tumor initiating cell (TIC), often referred to as a cancer stem cell, must interact with and condition its secondary tissue microenvironment. TICs must have the ability to self-renew (clonal proliferation) and differentiate to drive continuous heterogeneous tumor growth. However, TICs within a tumor or micrometastases may exist in a quiescent state or over express efflux pumps, which allows them to survive when patients are treated with chemotherapeutics that target actively dividing cells (Li and Bhatia, 2011; Moore and Lyle, 2011; Visvader and Lindeman, 2008). Very little is currently understood about the mechanisms that drug-resistant quiescent TICs use to exit and re-enter the cell cycle, which has prevented significant advances in developing drugs to specifically target these pathogenic cells. The discovery, characterization, and quantification of drug-resistant quiescent TICs is absolutely required for developing therapies to treat metastatic disease and prognostic biomarkers.

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✉ Lisa A. DeLouise
lisa_delouise@urmc.rochester.edu

[1] Department of Biomedical Engineering, University of Rochester, Rochester, NY, USA

[2] Department of Dermatology, University of Rochester Medical Center, Rochester, NY, USA

In this work we used a high throughput cell screening technology based on microbubble (MB) well arrays to discover drug resistant cells in a cutaneous squamous cell carcinoma (SCC) cell line derived from a human tumor that was proven to be tumorigenic in mice (Mantel et al., 2012). SCC is the second most common form of skin cancer (Miller et al., 2007; Miller et al., 2010). It has a low mortality rate (~1%) however, in certain populations such as organ transplant recipients, the rates of metastatic disease and death are much higher (Mullen et al., 2006). Chemotherapeutic drugs commonly used to treat metastatic SCC include cisplatin (Shao et al., 2014), carboplatin (Cranmer et al., 2010) and 5-fluorouracil (Tallon and Turnbull, 2013) which all target actively proliferating cells. MB well arrays are **(Fig. 1)** formed in polydimethylsiloxane (PDMS) using the gas expansion molding process (Giang et al., 2014; Giang et al., 2007; Giang et al., 2008). PDMS is a clear polymer with an elastic modulus (2 MPa) that is more similar to soft tissue (e.g. skin =0.85 MPa) than the standard hard tissue culture polystyrene plate (2500 MPa) (Agache et al., 1980; Lee et al., 2004; Sun et al., 2012). PDMS is also hydrophobic which favors nonadherent cell growth (Aoun et al., 2014; Ratnayaka et al., 2013; Yeon et al., 2013). Normal cells find it difficult to adhere to and deposit matrix on hydrophobic substrates and consequently they die. TICs possess a privileged capacity to condition their microenvironment and proliferate in an anchorage independent fashion (Dick, 2009; Lin and Chang, 2008; Mueller-Klieser, 1997; Pastrana et al., 2011) and therefore may be enriched for by culturing in MB wells. The unique spherical architecture of the MB well also allows for the concentration of cell secreted factors (Bobo et al., 2014). This combined with the small MB well volume (0.5 to 10 nL), allows cells to rapidly condition their microenvironment. This promotes single cell survival and clonal proliferation which makes MB arrays an ideal platform to sort TICs, to study stem cell fate and drug resistance. The ability of a single cell to self-replicate is considered to be key stem cell (embryonic, somatic, or cancer) property (Reya et al., 2001). Studies report that when cancer cells are proliferated as nonadherent aggregates or spheroids their clonogenic potential increases as does their expression of stem cell markers, their migratory/invasive characteristics, and their resistance to chemotherapeutics (Bissell and Barcellos-Hoff, 1987; Chandrasekaran and DeLouise, 2011; Chen et al., 2011; dit Faute et al., 2002). MB well arrays are a proven high throughput single cell screening technology (Bobo et al., 2014; Giang et al., 2014; Jones et al., 2013) that have been exploited in various aspects of cancer research (Agastin et al., 2011; Chandrasekaran and DeLouise, 2011; Chandrasekaran et al., 2012; Chandrasekaran et al., 2011; Chandrasekaran et al., 2016; Chandrasekaran et al., 2014). For example, the MB microenvironment has been exploited to study the epithelial to mesenchymal transition (Chandrasekaran et al., 2011) which is an important process in cancer metastasis (Beuran et al., 2015; Cao et al., 2015; Huang et al., 2015; Katoh, 2005). MB arrays have been used to quantify the clonogenic potential of cancer stem cells (Chandrasekaran and DeLouise, 2011), to test the efficacy of therapeutic approaches to kill cancer cells (Chandrasekaran et al., 2014) and to screen for the metastatic potential of melanoma cells (Chandrasekaran et al., 2016). In this study, we demonstrate that MB array technology can be used to dissect the heterogeneity of the tumorigenic SCC cell line at the single cell level to discover morphologically distinct cell colonies and rare drug resistant cells.

2 Materials and methods

2.1 Culture of SCC cell line

The SCC cell line was cultured in keratinocyte growth media supplemented with EGF (0.2 ng/mL) and bovine pituitary extract (25 μg/mL) at 37 °C and 5% CO_2. Media was changed every 3 days. Unlike normal keratinocytes, the tumorigenic SCC cell line does not proliferate more slowly with each passage nor so they senesce. SCC cells cannot be pushed to make filaggrin, a terminal keratinocyte differentiation marker, and they generate SCC-like tumors when subcutaneously injected into immunodeficient mice (Mantel et al., 2012).

2.2 Prepartion of primary SCC cells for MB array seeding

Primary SCC cells were generated from a human tumor as previously described (Mantel et al., 2012). Briefly, a deidentified SCC tumor was received within 1 h post resection and celluarized using a standard protocol. IRB approval (RSRB00039696) exists to bank SCC tumors removed as standard of care as well as adjacent normal skin for use in de-identified studies. The tumor was disinfected in 70% ethanol for 10 min, then minced, treated with collagenase I (1000 U/ml 1.5 h, 37 °C), then 0.25% trypsin-EDTA (15 min, 37 °C) after which an equal volume of FBS was added. Isolating cells from skin and especially skin tumor tissue is difficult and this process is very effective at digesting the matrix to release cells. The dissociated sample was filtered (100 μm), washed 1× in PBS, resuspended in keratinocyte growth media (KGM, Gibco) culture media. The cell concentration was determined (Biorad TC10) and the cells were seeded directly in MB well arrays (5×10^4 cells/cm^2) and cultured in KGM supplemented with EGF (0.2 ng/mL) and bovine pituitary extract (25 μg/mL) at 37 °C and 5% CO_2. Media was changed every 3 days.

2.3 PDMS microbubble well array preparation

MB wells are spherical compartments formed in polydimethylsiloxane (PDMS) using gas expansion molding (GEM)

technique (**Fig. 1**). PDMS is an optically clear, hydrophobic, gas permeable, flexible, and biocompatible polymer that is commonly used as a substrate for cell culture. In the MB well there is room for cell-cell contact and the elastic modulus of PDMS is similar to soft tissue (Agache et al., 1980; Lee et al., 2004). A mixture of 10:1 ratio of base to curing agent (wt%) of PDMS (Dow Corning Sylgard 184) was poured over a silicon wafer template (Giang et al., 2014; Giang et al., 2008) and cured at 100 °C for 2 h. For this work we used 10 × 10 MB well arrays. Each MB well had a 100 μm diameter circular opening and ~8 nL volume. Each array chip was placed in a well of a 24-well plate (2 cm^2). The planar surface of the MB chips was blocked with 2% BSA for 45 min. Chips were washed one time with 1xPBS, followed by de-priming the MBs in a desk top vacuum chamber with a 1:1 mixture of PBS and ethanol to sterilize. The MBs chips were then incubated in PBS overnight before cell seeding.

2.4 MB well cell seeding and live staining

SCC cells were seeded into MB well arrays by gravity with a fixed cell seeding density. To achieve limiting dilution seeding (MB wells seeded with 0, 1, 2 or 3 cells per well) we seeded at 0.5×10^4 cells/cm^2. Increasing the seeding density to 2.0×10^4 cells/cm^2 increased the number of cells deposited to 4–15 cells per well. The chips were submerged in the cell stock solution for ~5 min and washed twice with media to remove cells that deposited onto the planar chip surface. Cells in the MB well array were counted 30 mins after seeding. Media was changed every 2 days. The limiting dilution seeding protocol (0.5×10^4 cells/cm^2) ensures a total seeding efficiency of 80.1% ± 6.2% with a single cell seeding efficiency of 31.3% ± 4.9% and ~170 cells seeded per chip (1.7 cells/well). Clonogenic potential is defined as the ability of a single cell to generate daughter cells(i.e. self-renew). Cells were cultured in MB well arrays for up to 7 days and clonogenic potential values were scored after staining 2 h with CalceinAM (2 μM) to quantify live cells. Images were taken under bright field and fluorescent filter, and were analyzed using ImageJ.

2.5 CD44 and CD133 cell marker analysis

The MB chip was transferred into a 24-well TCP containing 1 × PBS to rinse and then it was transferred into a well containing 10% formalin fixative with 0.1% Triton for 20 min at room temperature. The chip was rinsed twice with 1 × PBS, blocked with 2% BSA for 30 min at room temperature to prevent any non-specific adsorption of antibodies, followed by another wash with PBS. Primary antibody solutions were prepared using 1/500 dilution of each antibody in 2% BSA/PBS. The primary antibodies were Rabbit-Anti-human-PROM1/CD133 (Rockland Immunochemicals, Inc.# 600–401-P83) and Mouse-Anti-human-CD44 (BD Biosciences Clone #G44–26). The chips were transferred into 1 ml of primary antibody solutions and incubated overnight at 4 °C or for 1 h at room temperature. The chips were then washed twice with PBS before staining the secondary antibodies. The secondary antibody solutions were prepared using 1/500 dilution of each antibody in 2% BSA/PBS. The secondary antibodies were Goat-Anti-Rabbit IgG (Texas Red conjugated, abcam #ab6719,) and Goat-Anti-Mouse IgG1 (CF350 conjugated, Biotium, Inc. #CF350). After washing, the chips were transferred to the secondary antibody solution and were incubated for 1 h followed by two washes with 1xPBS. The chips were imaged using an Olympus IX70 inverted fluorescent microscope equipped with QImaging Retiga EXL camera.

2.6 Cisplatin drug resistance

Cisplatin (10 μM) was diluted in cell culture media. The chips were transferred to the cell culture media with cisplatin and incubated for 2 days after which chips were transferred into fresh media prior to a live/dead staining with CalceinAM (2 μM) /Propidium Iodide (1 μM).

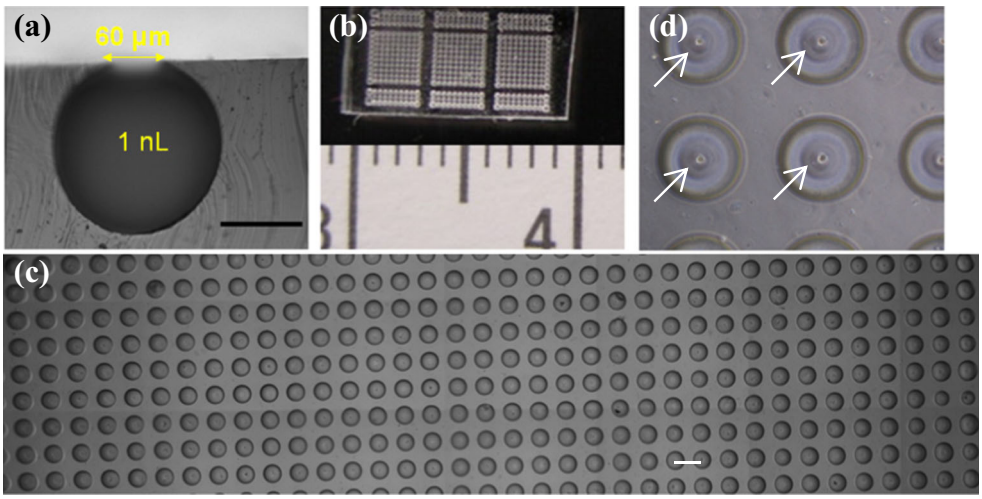

Fig. 1 Images of MB array technology. a Cross-section of a single MB well with 60 μm circular opening and ~160 μm diameter. Scale bar 80 μm. **b** A MB array chip (1 cm x 0.5 cm) with 480 wells. **c** Section of a large array with ~2K MB wells/cm^2. Scale bar 240 μm. **d** Close view of MB wells seeded with 1 cell per well (white arrows)

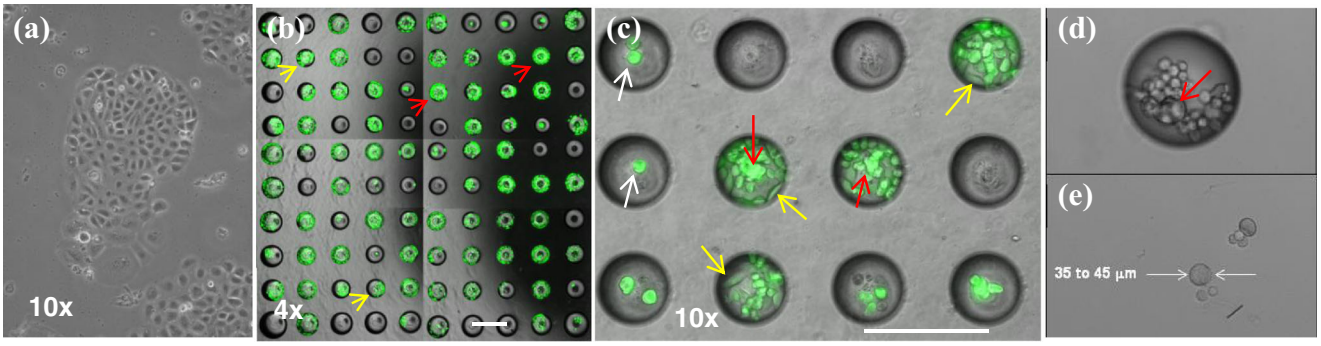

Fig. 2 Representative images of SCC cells growing (**a**) on 2D tissue culture plate and (b-d) in MB wells cultured for 7 days. Calcein-AM staining (green) indicates live cells. SCC cells adopt a spread morphology on planar TCP and are indistinguishable from normal keratinocytes. The SCC cell colonies that form in MB wells starting from 1 or 2 cells exhibit distinct morphologic and proliferation characteristics. (**b**) Some wells exhibit high proliferation (red arrows) where the MB well is nearly full of cells or low proliferation where MB wells are only half full (yellow arrows). Scale Bar is 400 μm. (**c**) Morphologically we observe formation of isolated large spheres (white arrow), spheres in mixed populations (red arrow) and spread cells (yellow arrow). Scale Bar is 400 μm. (**d**) Magnified view of low proliferation MB well treated with trypsin to loosen cells for manual plucking with Eppendorf micromanipulation tools. Large sphere is evident, red arrow. (**e**) After plucking the cells were dispensed onto a TCP where large sphere cells are evident and measure ~40 μm diameter

2.7 Cell recovery from MBs

Following several days in culture the SCC MB chips were transferred into a 24-well TCP containing serum free DMEM media and rinsed two times in 1xPBS, then incubated for 10 min in the serum free media. The MBs chips were then submerged into 0.25% trypsin-EDTA solution (5–10 min at 37 °C) to loosen cells prior to aspiration. The chips were then transferred to 35 mm TCP with DMEM and 10% FBS to quench the trypsin. Micromanipulation tools (Eppendorf CellTram and InjectMan® NI 2 Micromanipulator) were used to recover cells from individual MB wells as previously described (Bobo et al., 2014). Recovered cells were plated initially into 96-well TCP, and later expanded into larger wells.

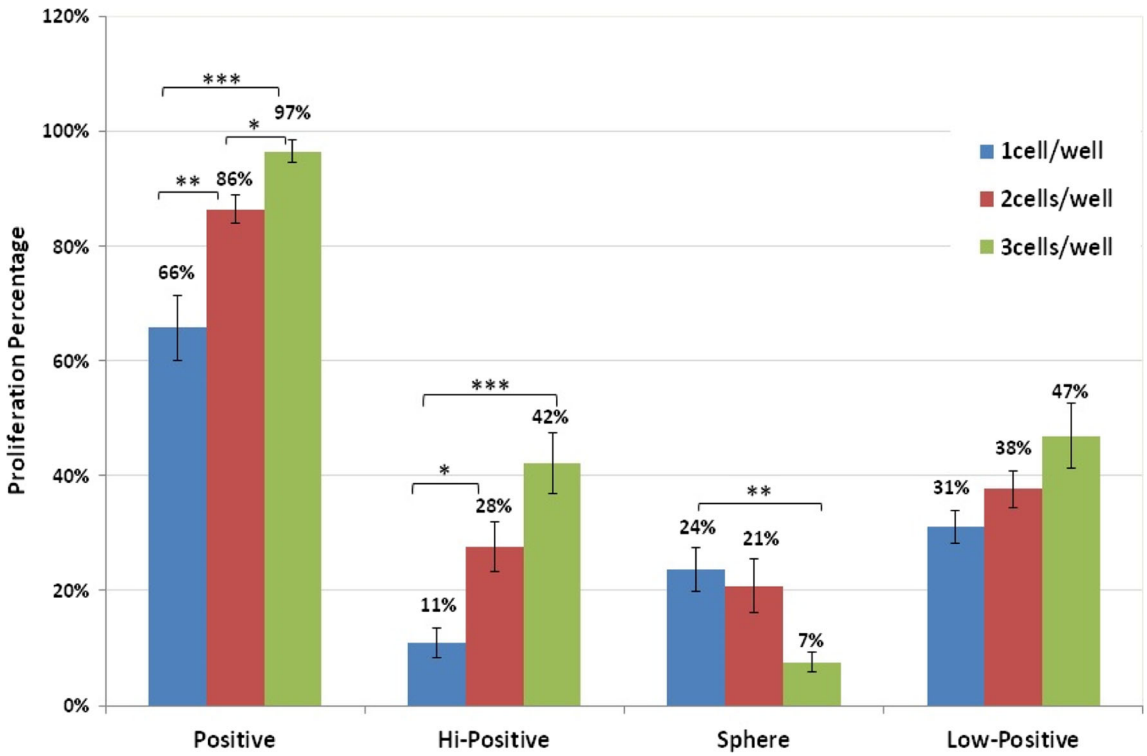

Fig. 3 Enumeration of overall clonogenic potential (positive), high positive, sphere and low positive formation as a function of initial MB well cell seeding. Results suggest that colony survival and sphere formation depends on the number of cells that start in the MB well. Proliferation percentage (mean ± Standard Error); Kruskal-Wallis test with post hoc Wilcoxon analysis. *, $p<0.01$; **, $p<0.005$; ***, $p<0.0005$. N=9 chips, ~100 MB wells per chip

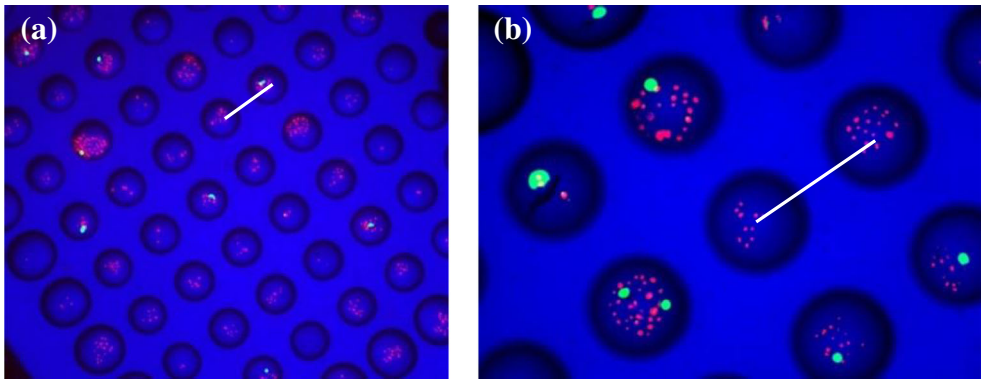

Fig. 4 MB well arrays were cultured with SCC cells for 5 days prior to 48 hr treatment with 10 μM cisplatin. (**a**) 4X and (**b**) 10X. Live calcein-AM (*green*) and dead propidium iodide (*red*) stain shows cisplatin killed the actively dividing cells whereas the spheres remain alive. Scale bars 400 μm

2.8 Statistical analysis

The JMP® statistical analysis software package was used to test for significance. We used the nonparametric Kruskal-Wallis one-way ANOVA analysis to test for significance within a group and a Wilcoxon post hoc analysis to determine which specific groups differed. The results are presented as mean ± Standard Error and results were considered significant when $p < 0.05$.

3 Results/discussion

SCC cells were seeded into MB well arrays under limiting dilution conditions (wells initially seeded with 0, 1, 2 or 3 cells). SCC cells were observed to readily proliferated in MB wells. Colonies that grew from single cells after 5 to 7 days in culture exhibited unique morphologies not observed when the SCC cells were cultured on standard 2D tissue culture plastic (TCP). Representative images of SCC cells grown on TCP and in MB wells are shown in **Figs. 2a**, **b**, respectively. On TCP, the cells exhibit a spread morphology similar to primary keratinocytes (**Supplemental Fig. S1**). Colonies grown in MB wells were categorized as high proliferation (~100% MB full) or low proliferation (50% MB full) containing spread cells and spheres (**Fig. 2c**).

Spread cells (~15 μm diameter when trypsinized) were commonly observed in MB wells exhibiting high and low proliferation whereas spheres (~40 μm diameter when trypsinized) were more frequent in isolation but they also were present in low and high proliferating wells (**Figs. 2d**, **e**). Enumeration of the high and low positive wells, and sphere presence was performed by counting bright field (BF) images taken from 9 chips with ~100 MB wells per chip. Results (**Fig. 3**) reveal that the clonogenic potential (ability of cells to proliferate) depends on the number of cells initially seeded in the MB well. Approximately 68% of the wells initially seeded with a single cell were positive for proliferation (high or low). The clonogenic potential approached 100% when 3 cells were seeded per MB well. The remarkable ability for SCC cells to survive and proliferate in MB wells likely results from an enhanced capacity of the cancer cells to condition the nanoliter MB microenvironment as primary keratinocytes do not thrive in hydrophobic MB wells (not shown). We also observe that the tendency for spheres to form decreases with increasing cell seeding, however, our ability to distinguish the presence of spheres in mixed cell populations in the BF images may have underestimated their frequency.

3.1 Discovery of drug resistant spheres:

Cisplatin is a common chemotherapeutic drug used to treat various cancers and advanced SCC (Cranmer et al., 2010; Shao et al., 2014). MB well arrays were seeded with SCC cells that were allowed to proliferate for 5 days. After colonies developed as pictured in **Fig. 2b** the media was changed to include 10 μM cisplatin which we determined was sufficient to kill ~90% of SCC cells cultured on 2D TCP **(Supplemental Fig. S2)**. After 48 h the array was treated with a live/dead stain. The results (**Fig. 4a**) show that the cisplatin treatment killed the actively dividing cells whereas the sphere cells were drug resistant. It is plausible that the spheres exist in either a slow-cycling quiescent or senescent state; both of which can drive tumorigenesis (Li and Bhatia, 2011; Liu et al., 2013; Moore and Lyle, 2011). If the spheres are multicellular, drug

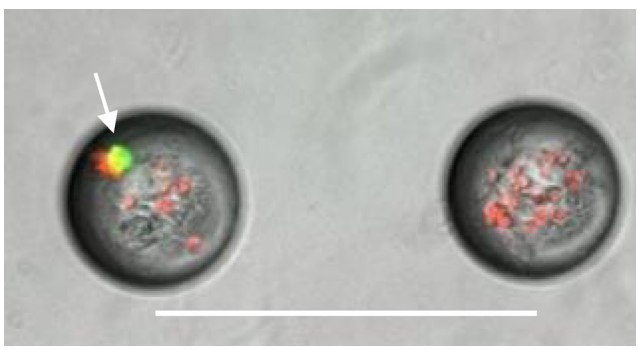

Fig. 5 Representative live (green) / dead (red) image of MB wells 7 days post seeding with cells derived from a primary SCC tumor without prior passage on TCP. Arrow shows a possible drug resistant sphere cell. Scale bar 400 μm

resistance may also result from a lack of cisplatin bioavailability to innermost cells as recently reported (Cao et al., 2011; Liao et al., 2014). It is of interest to note that drug resistant spheres commonly exist in the presence of dividing cells **(Fig. 4b)**. This suggests that the sphere phenotype is not induced by a lack of nutrients required for cell proliferation. This also suggests that the drug resistant spheres are not influenced by paracrine factors secreted by cells co-existing in the MB well niche. From our data, we estimate that the frequency of sphere formation is low, ~3% of the cells initially seeded in the array, which is within the 1%–5% range that cancer stem cells have been reported to exist in SCC tumors (Oshimori et al., 2015). Although the frequency of TICs in human tumors can be very low (<<1%) (Ishizawa et al., 2010), the TIC frequency is less important than their capacity to evade therapy and to drive metastasis.

3.2 Expression of stem cell surface markers on spheres

For many cancers, including SCC, the cellular origin of TICs and the regulatory pathways essential for sustaining stem-like properties and quiescence are not well understood. High expression of stem cell markers, including aldehyde dehydrogenase 1 (ALDH1), CD34, CD133, and CD44 are characteristic markers of TICs isolated from cutaneous SCC and many other types of cancers (Adhikary et al., 2013; Collins et al., 2005; Malanchi et al., 2008; Margaritescu et al., 2011; Moore and Lyle, 2011; Nam-Cha et al., 2013; Nosrati et al., 2014; Schober and Fuchs, 2011; Wang et al., 2012). We conducted preliminary immunofluorescence studies *in situ* to test whether CD133 and CD44 are expressed on the drug resistant spheres **(Supplemental Fig. S3)**. Of 29 spheres counted, 15 were positive for either CD133 or CD44 and 13 were double positive.

3.3 Presence of sphere cells in primary a SCC tumor

Discovery of drug resistant sphere cells in a SCC cell line derived from human patient tumor is an important finding; however, their presence must also be validated in SCC tumors directly. The frequency of drug resistant cells in tumors is expected to be 1–3 orders of magnitude lower than that in a SCC cell line due to the heterogeneity of the tumor tissue which is comprised of cancer cells, normal keratinocytes, stromal cells and immune cells, all of which are retained in the tumor cellularization protocol. In a preliminary study, we cellularized a human SCC tumor **(Supplemental Fig. S4)** and directly seeded a MB well array at high density (4–15 cells per well) without prior passaging on TCP. After 7 days in culture a live/dead assay was performed. Results indicate that similar to primary keratinocytes (derived from human skin) the majority of cells died. However, despite use of small arrays (600 MB wells only) and sampling of only ~4000 cells, viable cells were detected and some exhibited a sphere morphology **(Fig. 5)**. Although cisplatin treatment was not used in this study we are encouraged that use of much larger arrays (>100 K MB wells) will provide the ability to screen many more cells that will enable the discovery of drug resistant cells directly from tumors that can then be enumerated, recovered and characterized.

4 Conclusions

We demonstrate here that the MB well array provides a high-throughput means to enumerate rare drug-resistant cancer cells from a heterogeneous population. The unique spherical MB architecture creates a niche that allows for the concentration of cell secreted factors and the hydrophobic PDMS helps to select for cells that can exhibit nonadherent growth. We used the MB well array to measure the clonogenic potential of SCC cells that depended on the number of cells initially seeded in the MB well. We followed the growth of morphologically distinct colonies and enumerated spheres that were resistant to cisplatin. Our preliminary studies suggest that the spheres express the commonly excepted stem cell markers, CD133 and CD44. Future studies are planned to recover and characterize the sphere gene expression profile to more fully understand this phenotype. We also plan to conduct *in vivo* studies in mice to functionally validate the tumorigenic potential of the drug resistant SCC spheres. In summary, we believe that MB array technology constitutes a new TIC discovery platform that can be used to expand our fundamental knowledge of cancer metastasis, identify novel biomarkers and patient specific treatment strategies.

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