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Establishment and Quantitative Imaging of a 3D Lung Organotypic Model of Mammary Tumor Outgrowth

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Abstract

The lung is the second most common site of metastatic spread in breast cancer and experimental evidence has been provided in many systems for the importance of an organ-specific microenvironment in the development of metastasis. To better understand the interaction between tumor and host cells in this important secondary site, we have developed a 3D *in vitro* organotypic model of breast tumor metastatic growth in the lung. In our model, cells isolated from mouse lungs are placed in a collagen sponge to serve as a scaffold and co-cultured with a green fluorescent protein (GFP)-labeled polyoma virus middle T antigen (PyVT) mammary tumor cell line. Analysis of the co-culture system was performed using flow cytometry to determine the relative constitution of the co-cultures over time. This analysis determined that the cultures consisted of viable lung and breast cancer cells over a 5 day period. Confocal microscopy was then used to perform live cell imaging of the co-cultures over time. Our studies determined that host lung cells influence the ability of tumor cells to grow, as the presence of lung parenchyma positively affected the proliferation of the mammary tumor cells in culture. In summary, we have developed a novel *in vitro* model of breast tumor cells in a common metastatic site that can be used to study tumor/host interactions in an important microenvironment.

Keywords

Breast cancer; metastasis; microenvironment; organotypic co-culture; host-tumor interactions

Introduction

Mortality from breast cancer occurs once the tumor spreads from the primary site to secondary metastatic sites, including the lung. The lung microenvironment, in particular, has been shown to be very important for metastasis and outgrowth of breast cancer cells through the interaction of the tumor cells and biological factors present in the lung parenchyma, which encompasses all cells comprising the tissue of the lung [1-4]. Animal models of breast metastasis to lung have given important information on factors that influence both the size

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and number of lung metastases, but their inherent complexity make it difficult to dissect cellular mechanisms important in this process. In light of these results, the development of an appropriate organotypic culture system consisting of both breast tumor cells and lung parenchyma can be regarded as an important research tool in order to study the mechanisms involved in breast tumor growth in the lung.

Two dimensional (2D) monolayer culture systems have been used for many years by cancer researchers to study *in vitro* processes. These systems, however, fail to fully recapitulate the morphology and function of cells in a 3-dimensional (3D) *in vivo* setting. Breast epithelial cells, in particular, need a 3D context in order to have the appropriate structural and functional cues for their growth and development [5-7]. Studies of the microenvironment have also shown that tumor cells require the presence of relevant host cells, including epithelial cells, endothelial cells, fibroblasts, and immune cells in order to successfully recreate a suitable *in vitro* co-culture model [8].

Several redundant 3D models have been employed to study breast cancer, including spontaneous cell aggregation, liquid overlay, spinner flasks, pre-fabricated engineered scaffolds, and scaffold based cultures such as those grown on collagen gels [9]. Each of these techniques has both advantages and disadvantages, including cost and difficulty in culturing certain cell types. In addition, many of these techniques are not suitable for repeated analysis of the same culture over time, and the architecture of the 3D matrix itself does not make it amenable to imaging by microscopy.

For this study we have developed a novel 3D *in vitro* organotypic model of breast tumor growth in the lung consisting of GFP-labeled mammary tumor cells and whole lung isolates that are cultured in a collagen sponge (Gelfoam), which serves as a scaffold. Flow cytometry analysis of the cultures was performed to characterize the percentage of viable cells present in the cultures over time. Additionally, the cultures were imaged at time points over 5 days using confocal microscopy in order to determine if the lung cells had a positive effect on the growth of the breast cancer cells in culture. This novel model should provide new opportunities for live cell imaging and studies of tumor-host interactions in a relevant microenvironmental setting.

Materials and Methods

Cell culture and generation of GFP –tagged mammary tumor cell lines

The R221A cell line was isolated from a spontaneous mammary tumor in an MMTV-PyVT transgenic mouse in the FVB/N background [10-11]. The R221A cell line was genetically manipulated to stably express GFP in the pMSCV retroviral vector (obtained from Dr. Shimian Qu, Vanderbilt University). Phoenix packaging cells (a kind gift from Dr. Albert Reynolds, Vanderbilt University) were transfected with the retroviral vector. Viral titer was collected and used to infect the R221A cells. Selection media (DMEM containing 10% FCS and 10 µg/ml puromycin (Sigma, St. Louis, MO) was added to cells after transfection and used for culturing the R221A-GFP cells. Cells were sorted by flow cytometry for GFP expression, and a population expressing a median GFP level was chosen for the experiments.

Animal Studies

All studies were conducted following review and approval by the Vanderbilt Institutional Animal Care and Use Committee. Both FVB/N mice and C57Bl/6-GFP mice (a transgenic mouse line with an enhanced GFP (EGFP) cDNA under the control of a chicken beta actin promoter and a cytomegalovirus enhancer) were purchased from the Jackson Laboratory for studies. For co-cultures, 6–8 week old mice were euthanized and the lungs removed, washed

in Hank's buffered sodium solution (Invitrogen, Carlsbad, CA), then digested overnight at 37°C in a mixture of Hank's Balanced Salt Solution (HBBS) containing gentamycin (Invitrogen, Carlsbad, CA) at 50µg/ml, fungizone (Invitrogen, Carlsbad, CA) at .25µg/ml, dispase (Invitrogen, Carlsbad, CA) at 2.5u/ml, and collagenase 1A (Sigma, St. Louis, MO) at 150µg/ml to form a single cell suspension. For the experimental metastasis assay, 1×10^6 R221A-GFP cells in a total volume of 100µl of phosphate buffered saline (PBS) were injected into the lateral tail vein of 6–8 week female FVB mice. After 14 days, the mice were sacrificed and the lungs were fixed in Bouin's fixative solution (Ricca Chemical, Arlington, TX) for visualization of surface metastases. Next, lungs were dehydrated through ethanols, embedded in paraffin, and sectioned for immunohistochemical analyses. For orthotopic injections 1×10^6 R221A-GFP cells in a total volume of 20µl PBS were injected into the fat pad of 6-week old FVB/N female mice (The Jackson Laboratory). After 4 weeks, the glands were removed and fixed in 10% formalin, dehydrated through ethanols, embedded in paraffin, and sectioned for immunohistochemical analyses. For the *in vivo* image of R221A-GFP cells, the mammary fat pad was excised, rinsed briefly in PBS, placed onto a microscope slide, then immediately imaged on a wide-field microscope using a GFP filter.

Organotypic Co-Cultures

Gelfoam® (Pharmacia, Kalamazoo, MI) was aseptically cut into 2cm² squares and placed into 35mm culture dishes (MatTek, Ashland, MA) to serve as a scaffold for the cultures. Small needles were placed at two points in the sponge to serve as positional markers for subsequent imaging experiments. 1.5×10^6 R221A-GFP cells in PBS in a volume of 20 µl was inoculated onto the top of the scaffold. The digested lung mixture was washed in PBS, and red blood cells were lysed in a solution of PharmLyse (BD Biosciences, San Jose, CA) in PBS. The lung cells were then washed once more in PBS, and 2×10^6 cells in PBS in a volume of 20µl was also inoculated onto the top of the scaffold. The cultures were placed at 37°C to allow the cells to soak into the scaffolds. One ml culture media (phenol red-free DMEM (Invitrogen, Carlsbad, CA) with 15% FCS supplemented with fungizone and gentamycin (Invitrogen, Carlsbad, CA) was then added to the cultures, and they were placed back into a 37°C incubator. Culture media was changed daily throughout the experiments. Before cultures were imaged using confocal microscopy, they were inverted such that the surface of the scaffold where the cells were seeded was flush against the glass surface of the dish.

Immunohistochemical and Cytochemical Analysis

Co-cultures and tissues were fixed in 10% formalin before embedding in paraffin. For GFP localization, anti-GFP (living colors A.v. GFP) (Clontech, Mountain View, CA) was used at a 1:600 dilution with 10mM citrate antigen retrieval. Hemotoxylin and eosin (H&E) staining were performed on sections using standard protocols.

Flow Cytometric Evaluation of the Co-Cultures

Cells were released from the Gelfoam scaffold by digestion for 30 minutes at 37°C in a mixture of Dispase (Invitrogen, Carlsbad, CA) at 7.5u/ml in PBS. Cells were resuspended in 1ml cold PBS for sorting and incubated for 30 minutes on ice with Hoechst (Invitrogen, Carlsbad, CA) at 50µg/ml for analysis of cell viability. The stained cells were analyzed on a Becton Dickinson LSRII operated through the Vanderbilt Medical Center Flow Cytometry Shared Resource.

Confocal Microscopy

Microscopy was performed with a Zeiss LSM 510 Meta inverted confocal microscope with a motorized stage in the Vanderbilt Cell Imaging Shared Resource. A 10×/0.30 Plan Neofluar lens was used for image acquisition. GFP was excited with a 488nm laser, and a 530–550 bandpass filter was used to detect GFP-expressing cells. Cultures containing cells and positional markers were placed onto the microscope, and five different z-stacks of each culture were taken at 20µm intervals (for a total of 60 µm in depth). The position of each stack was recorded using the positional markers in the culture with a motorized stage. At 4 time points over a 5 day period, multiple z-stacks taken from the same location in each culture were re-imaged to provide data on the growth of the R221A-GFP cells in the culture. MetaMorph™ imaging software (Molecular Devices, Downingtown, PA, USA) was used to analyze the images, and total integrated intensity of GFP expression was used as a marker of growth of the R221A-GFP tumor cells in the culture.

Statistical Analysis

Comparisons of the difference in growth rates between the two sets of samples from the confocal studies were analyzed for significance at the 95% confidence level using Student's *t* test with GraphPad Prism 4 software (GraphPad Software, San Diego, CA).

Results

Establishment and Characterization of the R221A-GFP Cell Line

The R221A cell line was previously isolated from a mammary tumor in the fat pad of a MMTV-PyVT transgenic mouse in the FVB/n background [10]. The cell line was transduced with a retroviral vector to label the cells with GFP to allow for confocal microscopy. After flow cytometry sorting of the cells for a median level of expression of GFP, the cell line was characterized both *in vitro* and *in vivo* to ensure that expression of GFP did not alter its morphology or tumorigenicity. R221A-GFP cells were injected into an orthotopic site (the fat pad) and via the tail vein into a metastatic site (the lung), to examine tumorigenicity at both sites (Fig 1, a,c). After 14 days for the tail vein injections, and 4 weeks for the fat pad injections, the organs were excised, processed, paraffin-embedded, and sectioned into 5µm sections for histological analysis. Immunohistochemistry for GFP determined that tumors growing in both the lung (Fig 1b) and the fat pad (Fig 1d) retained expression of GFP *in vivo*, showing the cell line maintained GFP expression throughout tumor formation. To examine the expression of GFP *in vitro*, R221A-GFP cells were plated into culture dishes and imaged using fluorescence microscopy 24 hours later, revealing strong GFP expression in the cells (Fig 1e). Also, GFP expression could be seen in a freshly excised fat pad containing a mammary tumor (Fig 1f).

Development of the 3D Lung Organotypic Co-Culture Model

We chose to utilize Gelfoam as the 3-dimensional scaffold for our orthotopic cultures because it had been successfully used previously as a scaffold for a model of fetal rat lung cells [12]. Also, its porous structure made it amenable for microscopic imaging and histological analysis. To construct the cultures, lungs were taken from 6–8 week old FVB/N mice and completely dissociated overnight at 30°C in digestion solution. After lung cells were dissociated and red blood cells were lysed, the cells were counted and approximately 2×10^6 of the mixed cell population in a volume of 20µl were applied to the top of a 2mm² piece of scaffold. Along with the lung cells, 1.5×10^6 R221A-GFP cells, also in a volume of 20µl, were applied to the top of the scaffold to form a mixture of lung and breast cells. Once the cell mixture has soaked into the sponge, 1ml of phenol-red free media was added for maintenance of the cultures (Fig 2a). Culture media was changed daily throughout the

experiment. Confocal imaging was performed and clearly showed the GFP-labeled breast cells within the matrix (Fig 2b). We observed that the cells preferentially grew along the ridges of the scaffold, which were visible on a brightfield image (Fig 2b).

The versatility of the culture system allows for fixation in 10% formalin, processing, and subsequent sectioning for histological analysis. H&E staining of the cultures showed the presence of cell aggregates within the culture that consisted of both large tumor cells with irregular nuclei and small lung cells, demonstrating that the R221A-GFP tumor cells and lung cells had direct contact with each other within the culture system (Fig 2c).

Immunohistochemistry for GFP expression was also performed on culture sections which showed GFP-positive R221A cells intermingled with unstained lung cells (Fig 2d).

Flow Cytometric Analysis of the Organotypic Co-culture System

We next wanted to examine the composition of the co-culture over time to determine the viability of both the breast and lung cells during the time frame in which the confocal imaging would be performed. To do this we constructed co-cultures in a similar fashion as those cultures used for imaging; however, in this case we used unlabeled R221A breast cancer cells along with lung cells isolated from a mouse expressing GFP from a chicken beta-actin promoter in all tissues in order to provide a marker for sorting the lung cells. We determined that we were able to reproducibly separate the breast cells from the lung cells due to their large size difference (Fig 3a-b). We chose in this experiment to use labeled lung cells because of their small sizes, and the fact that they tended to sort at approximately the same size as leftover cellular and Gelfoam debris that remained after isolating the cells from the culture. The use of GFP as a marker of this population thus ensured that we examined actual GFP-expressing lung cells, not extraneous material (Fig 3b).

Cultures were examined on days 1, 3, and 5. After cells were isolated from the cultures at the specified timepoints, they were stained with Hoechst to measure cell viability of the two populations. Flow cytometry analysis determined that viability of the two cell populations remained relatively constant at approximately 95% for the breast cells and 91% for the lung cells on days 1, 3, and 5, respectively (Fig 3c-e, Table 1). It is important to note that flow cytometry determined that the cultures contained significant numbers of viable breast and lung cells even out to five days, suggesting our co-cultures could be analyzed using confocal microscopy at least out to that time point.

Imaging and Analysis of the Organotypic Co-Culture Model

In order to study a cellular process over time, most *in vitro* culture systems set up multiple replicates that are processed at different timepoints. The disadvantage to this technique is that even though the cultures were constructed in the same manner, there would be experimental variation in the results because each timepoint constituted a different culture. We wanted to develop a co-culture system that would not only be able to be sectioned and histologically studied at specific time points, but also one in which live cell imaging could be used to monitor the growth of the same culture over time. One of the advantages of our co-culture system is that it uses a scaffold that can be imaged using microscopy. The R221A cells can be monitored using confocal microscopy using GFP expression. Total GFP signal can then be utilized as a marker of growth of the cells over time in the culture. Each culture contained two positional markers, in the form of fine needles, that served as markers of 'x' and 'y' positions respectively (Fig 4a). The bottom of the plate marked the 'z' position. By using a microscope with a motorized stage, it was possible to mark stack positions for imaging on day 1, and using the positional markers, to return to the same spot in the culture for subsequent imaging over time. For each culture 5 different z-stacks were taken at 20µm intervals for a total of 60µm for each stack (Fig 4a).

Use of a motorized stage allowed for the imaging of the exact same z-stack positions over time, as shown in Fig 4b, in which one plane of a z-stack is shown for both a culture containing breast and lung cells (Fig 4b-top panel) and a culture containing only breast cells (Fig 4b-lower panel). MetaMorph™ software was used to determine total integrated intensities of GFP expression of each culture over time. These measurements were then graphed to illustrate cell growth as total integrated intensity over time. In cultures containing both breast and lung cells total integrated intensity increased greatly over days 1–2, dropped off, then increased over time to day 5. In contrast, cultures containing only breast cancer cells had slower growth as measured by smaller changes in total integrated intensity over time (Fig 4c). These results are summarized in graph form in Fig 4c, and demonstrate that the tumor cells in those co-cultures containing both breast cancer and lung cells grew at a significantly enhanced rate between days 1–2 ($p=0.009$) than those cultures containing breast cancer cells alone. Importantly, flow cytometry had previously determined that the cultures contained viable breast cancer and lung cells at these time points. This data determined that the presence of the lung cells provided a more permissive microenvironment for the breast cancer cells and aided in their enhanced growth inside the culture system. In order to determine that the difference in growth rates between the culture groups was not the result of different numbers of breast cells being present at the start of the assay, additional cultures were analyzed using a plate reader to determine total fluorescence 2 hours after the cultures were constructed. These analyses revealed that there was no statistical difference between measured fluorescence units from cultures containing both breast cancer and lung cells compared to those containing only breast cancer cells (data not shown). Additionally, a standard curve of R221A-GFP cells in culture was constructed and analyzed which revealed that cell number highly correlated with GFP fluorescence levels. Taken together, these analyses determined that equivalent numbers of GFP-labeled breast cancer cells were present at the beginning of the assay and that GFP signal is a valid surrogate for cell number.

Discussion

We describe the characterization and use of a 3D murine organotypic co-culture model of breast cancer cell growth in the lung. A PyVT mammary tumor cell line was GFP-labeled and characterized using both an orthotopic site (the fat pad) and a metastatic site (the lung) for growth *in vivo*. The cells were co-cultured *in vitro* with isolated murine whole lung cell suspensions on a Gelfoam support. Flow cytometric analysis revealed that cell viability remained relatively constant in the cultures over a 5 day period with both cell populations having on average greater than 90% surviving cells. Confocal analysis provided evidence for the importance of a relevant microenvironment in the outgrowth of tumor cells at a secondary site, since R221A-GFP cells in culture accompanied by lung cells grew at a statistically enhanced rate relative to those cultured alone. The presence of lung cells in the co-culture thus aided the early growth of the R221A-GFP cells, possibly by secreting factors into the co-culture matrix that facilitated establishment and early proliferation.

Gelfoam was first used in an *in vitro* organotypic culture system by Liu et al. in which the Gelfoam collagen sponge served as a scaffold for the growth of fetal rat lung cells [12]. The authors used this model to measure the effect of mechanical stretch on the proliferation of the fetal lung cells present in the culture. Subsequent work by the same group used the culture system to examine the differentiation of the fetal lung cells in culture using both light microscopy and electron microscopy [13], and the impact of mechanical stretch on the stimulation of macrophage inflammatory protein-2 secretion from the fetal lung cells in culture [14]. Gelfoam provided an excellent substrate for these studies, allowing live cell imaging of the cultures in real time using confocal microscopy without autofluorescence in any of the channels tested, as well as the ability to perform histologic and flow cytometry

studies. The organotypic culture system permits real time live cell imaging of the same cultures, producing a more accurate representation of changes in the cultures over time than analyzing different cultures at sequential timepoints.

Using this co-culture system, we were able to determine that the presence of lung cells provided a more permissive microenvironment for the establishment and early growth of the mammary tumor cells. It is not clear if this effect required cell:cell contact, or was mediated by soluble factors. A recent study using human bone marrow stromal cells cultured with different human breast cancer cell lines, including MDA-MB-231 and MCF-7, while embedded in Matrigel basement membrane extract determined that the bone marrow cells could enhance the growth rates of some, but not all, cell lines in culture [15]. The co-culture system provides the opportunity for further, mechanistic studies to identify factors critical for mediating host:tumor cell interactions.

It has been well established that important microenvironmental cues that cells require for growth are absent in typical two-dimensional culture systems [16]. Several groups have shown that breast tumor cells, in particular, are very sensitive to changes in their environment and behave more like their *in vivo* counterparts when they are placed inside a three-dimensional matrix in which to grow [5,7,17-19]. Another benefit of three dimensional cultures is that they typically consist of cells with several different phenotypes, including those both proliferating and non-proliferating, and also those that may have become necrotic. This situation is very similar to that which is found inside human tumor masses *in vivo* [9]. 3D *in vitro* systems consisting of well characterized breast tumor cell lines such as MDA-MB-231 have also been shown to recapitulate the drug sensitivities of tumor cells grown *in vivo* [20]. Taken together, these results have shown the importance of studies conducted in a three-dimensional culture system which more faithfully recapitulates the *in vivo* setting of tumorigenesis or tumor growth and progression.

The lung is a very common site of breast cancer metastasis; 39.5% of women diagnosed with metastatic disease having evidence of disease in the lung [21]. The testing of new pharmacological therapies for use in the setting of metastatic disease is a relevant *in vitro* setting is of crucial importance for the testing and development of promising agents for use *in vivo*. We suggest that our *in vitro* model of breast tumor cells in a common metastatic site will be useful for short-term testing of novel anti-metastatic therapies and imaging of host-tumor interactions in an appropriate microenvironmental context.

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Abbreviations

GFP	Green Fluorescent Protein
PyVT	Polyoma Virus Middle T Antigen
PBS	Phosphate Buffered Saline
H&E	Hematoxylin and Eosin
FCS	Fetal Calf Serum
2D	Two-dimensional
3D	Three-dimensional

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Figure 1.

Characterization of the R221A-GFP cells. R221A mammary tumor cells were isolated from the fat pads of PyVT mice and infected with a retroviral GFP vector to make the cells visible for imaging studies. To determine if the GFP-labeled cells maintained their tumorigenic potential, they were injected into mice at both a metastatic site (lung) (a) and an orthotopic site (the fat pad) (c), where they formed tumors. Immunohistochemistry for GFP expression, in which the brown precipitate indicates positivity, is also shown for the lung (b) and the fat pad (d). Also shown are fluorescent images showing GFP expression of the cells both in culture (e), and in a resected fat pad containing a tumor (f). (b) and (d) taken at 20x mag. Scale bar in E = 100 μ m.

Figure 2.

Setup of the co-culture assay. After the lung cells are completely dissociated, they are placed on top of the Gelfoam sponge with the R221A-GFP cells in a tissue culture dish containing media (a). Shown is a confocal image of a GFP and brightfield overlay that displays both the sponge matrix (white arrow) and the R221A-GFP cells (black arrow) within the sponge matrix (b). Panels (c) and (d) show histological sections of the cultures with both hematoxylin and eosin staining (c) and immunohistochemistry for GFP expression, in which the brown precipitate indicates positivity (d). Scale bar in (b) = 50 μ m, in (c-d) = 100 μ m.

Figure 3.

Flow cytometry analysis. Cultures containing R221A mammary tumor cells and GFP-labeled lung cells were analyzed by flow cytometry at day 1 (c), day 3 (d), and day 5 (e) timepoints to determine the percentage of viable lung and breast cells present in the cultures over time (shown in parentheses). Cultures containing R221A cells only (a) and GFP-labeled lung cells only (b) were used as gating controls for the analysis.

Figure 4.

Confocal imaging and analysis. Confocal microscopy is performed on the co-cultures by taking Z-stacks at multiple locations, and at multiple planes in each culture. Using positional markers, the same locations on each culture are imaged at each time point (a).

Photomicrographs are shown in (b) of one plane of one z-stack over time for 5 days of a culture containing breast and lung cells (upper panel) and a culture containing only breast cells (lower panel). GFP expression is converted into numerical values for analysis using MetaMorph© software to determine total integrated intensity of GFP for each sponge over time. These numbers are shown below the photomicrographs in (b) for the one plane shown, and study results are summarized in graphical form in (c). *p=0.009.