Fibroblasts enhance migration of human lung cancer cells in a paper-based co-culture system

Gulden Camci-Unal, David Newsome, Brenda K. Eustace, George M. Whitesides*

Dr. G. Camci-Unal, Prof. G. M. Whitesides
Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA
E-mail: gwhitesides@gmwgroup.harvard.edu
D. Newsome, Dr. B. K. Eustace,
Vertex Pharmaceuticals Incorporated, 50 Northern Avenue Boston, MA 02210, USA
Prof. G. M. Whitesides
Wyss Institute for Biologically Inspired Engineering, Harvard University, 60 Oxford Street, Cambridge, MA 02138, USA
E-mail: gwhitesides@gmwgroup.harvard.edu

Keywords: tumor cells, fibroblasts, migration, coculture

Most studies of the cell biology of cancer have used monolayers of two-dimensional (2D) mono-cultures. Three-dimensional (3D) *in vitro* cell culture platforms that model the dynamic interactions between stromal and tumor cells are promising systems to develop physiologically relevant cancer models. In this work, we used a multi-layered paper-based platform to investigate the interactions between human lung tumor cells and fibroblasts that were isolated from primary patient tumor samples. We found that the human lung tumor cells migrated towards the layers where the fibroblasts were located in the stacked configurations, and suggest that this behavior could, in part, be due to the activity of the transforming growth factor-beta (TGF-beta) pathway. Blocking TGF-beta resulted in a decrease in the migration of the tumor cells. The efficacy of drugs that inhibit TGF-beta or other metastatic pathways, therefore, can now be more reliably evaluated in a simple assay using our paper-based platform rather than mono-cultures or transwell systems.
The microenvironment of tumors includes extracellular matrix proteins such as collagen, fibronectin, and laminin, and cell types including fibroblasts, endothelial cells, and immune cells.\[^{1-3}\] These components of the tumor stroma contribute to the development of cancer by influencing the growth, progression, and metastasis of small tumors.\[^{2,4,5}\]

Many studies of the cell biology of cancer rely on monolayer (2D) mono-cultures.\[^{6-10}\] This type of \textit{in vitro} culture does not recapitulate the complex environmental signals, interactions, and processes that characterize a tumor and its environment.\[^{11}\] There is, thus, a need for physiologically relevant cancer models that include multiple cell types and that model the dynamic interactions between stromal and tumor cells. While there are many three-dimensional (3D) systems available to study co-cultures, these are limited to a small number of configurations, use complex instrumentation, or require multi-step procedures.\[^{3,12,13}\] In addition, most of these systems cannot enable generation of physiologically relevant structures that resemble tumors because they cannot mimic the limitations (e.g. mass transport) to the supply of O\(_2\), nutrients, and signalling factors.\[^{3,10,14}\]

The objective of our work is to develop experimentally and conceptually simple, flexible scaffolds and systems with which to study the behavior of tumor cells in 3D co-cultures. We hope these systems will better recapitulate \textit{in vivo} tumor biology than more familiar monolayer (2D) culture of tumor cells.

We used a multi-layered, paper-based platform (which we call “cells-in-gels-in-paper” or CiGiP) to co-culture primary human lung tumor cell lines and tumor fibroblasts that were isolated from surgical tumor resections. We co-cultured these human lung cancer cells and fibroblasts in Matrigel within the stacks of 100-µm thick-paper scaffolds (Figure 1). We varied the locations of the two cell types in order to study patterns of cell migration.

We found that the human lung-cancer cells migrated towards an adjacent layer containing fibroblasts when it was included in the stacked layers of gel (and cells) supported in paper. The migration of the tumor cells increased upon co-culturing them with fibroblasts, an
observation consistent with examples from the literature.\cite{15} We suggest that this behavior could, in part, be due to the activity of the transforming growth factor-beta (TGF-beta) pathway. Literature examples have previously demonstrated that the TGF-beta pathway influences the migration and metastatic behavior of cancer cells.\cite{16,17} We therefore inhibited TGF-beta, and monitored the resulting effects on the migration of cells. Blocking TGF-beta decreased the migration of tumor cells toward fibroblast-containing layers.

Epithelial-to-Mesenchymal Transition (EMT) is a process by which the tumor epithelial cells lose their polarity and gain migratory properties.\cite{18} The epithelial cells acquire mesenchymal features when the cells undergo EMT.\cite{19} To determine the status of the lung tumor cells, we assessed their EMT behavior. We found that the inhibition of TGF-beta decreased EMT for the tumor cells (VXN2).

In this report, we demonstrate the usefulness of stacked paper scaffolds for studying the migration patterns of tumor cells in a co-culture setting with tumor fibroblasts. The CiGiP approach provides a versatile tool for exploration of the areas of fundamental cell biology and development of new therapeutics. The multi-layered paper constructs enable combining distinct types of cells in different patterns and configurations as well as high-throughput preparation and analysis of the samples in parallel. Stacking the layers of paper impregnated with cells in gel matrices easily assembles them into a 3D construct with control over the distribution of the cells. The modular paper constructs permits us to generate in vitro tissue-like structures through straightforward fabrication. This paper-based cell culture platform is simple, tunable, cost-efficient, requires only small amounts of samples, (i.e. cells, materials for gels, medium), and allows co-culturing different types of cells. Using the CiGiP strategy allowed us to eliminate the cutting and sectioning steps in sample analysis and generate physiologically relevant constructs of tumors in 3D.

This work demonstrates that the migration of tumor cells is influenced by the TGF-beta pathway. The efficacy of drugs that inhibit TGF-beta or other metastatic pathways, therefore,
can now be evaluated more reliably, using a simple assay based on CiGiP, rather than using mono-cultures or trans-well systems.

VXN2 cells were grown in both mono- and co-culture configurations in the paper scaffolds within multi-layered stacks. The cell-containing paper scaffolds were cultured for three days. We removed the samples from the holders, de-stacked the layers, and analyzed them to determine the percent (%) of VXN2 cells that are present in each layer of the scaffolds (Figure 2a).

We analyzed the experimental data using GraphPad Prism (Version 4.02, La Jolla, CA) software. The statistical differences between groups were determined by one-way ANOVA tests using Bonferroni comparisons. We considered the $p$-values that are smaller than 0.05 as statistically significant. The $p$-values were defined by GraphPad Prism as follows: $^* p < 0.05$, $^{**} p < 0.01$, and $^{***} p < 0.001$.

Figure 2b-d demonstrates the results from the mono-cultures of human lung tumor cells in different layers of the paper stacks. In Figure 2b, VXN2 cells were cultured in Layer 1 (L1). In this configuration, the tumor cells did not migrate significantly beyond L4. Less than 2% of the cells were able to migrate to the bottom of the stack (L7). In Figure 2c, VXN2 cells were cultured in the middle layer (L4). The cells migrated to all of the layers within the stack. Migration of VXN2 cells to L3 was greater than to L5; we speculate that this difference reflects the higher availability of oxygen and nutrients in L3 than in L5. In Figure 2d, VXN2 cells were cultured in the bottom layer (L7). The tumor cells migrated all the way to L1, although from L5 to L1 migration was < 2%.

VXN2-TF co-cultures were designed to demonstrate the effect of fibroblasts on the migration of tumor cells within the stacked layers of paper. The results indicated that a subset of VXN2 cells migrated toward the layers that were occupied by the TF cells. Figure 3 represents the results from the experiments in which VXN2 cells and TF cells were co-cultured at different positions in the paper stacks. In Figure 3a, VXN2 cells were cultured in
L7 and TF cells, or no cells, were cultured in L1 and L2. There were higher numbers of VXN2 cells migrating to the layers containing TF cells compared to the same layers without TF cell culture.

VXN2 cells were cultured in the middle of the stack and TF cells were placed either in the top two or bottom two layers of the stack to study the migration behavior of VXN2s, in Figure 3b. We found that the VXN2 cells migrated toward the layers where the TFs were cultured, similar to the previous configuration in Figure 3a. We detected an increase in the number of migrating VXN2 cells as a result of seeding TFs in either the top or bottom layers. This observation suggests that the direction of migration has little bias resulting from position in the stack. Figure 3c summarizes the results from a co-culture in which VXN2 cells were initially in L1, and the TFs were in L3, L4, and L5. In this experiment, the VXN2 cells migrated toward the layers that were occupied by the TF cells. In Figure 3d, VXN2 and TF cells were cultured in L1 and L7, respectively. The number of VXN2 cells migrating toward L7 containing the TF cells was greater than those without the TF cells.

One reason for the increased migration of tumor cells in the presence of TFs might reflect the activity of the TGF-beta pathway. To test whether the VXN2 and TF cells secrete TGF-beta, we performed an ELISA assay (Figure S1). The results indicated that TGF-beta was secreted by VXN2s, TFs, and co-cultured VXN2-TF cells in the paper stacks.

After confirming that the cells secrete TGF-beta, we used 25 µg/mL of anti-TGF-beta antibody (R&D Systems, AB-100-NA) to block the effects of TGF-beta in the co-culture experiments. Figure 4 displays the resulting plot. The VXN2 cells were cultured at the top layer (L1) and fibroblasts were placed at the bottom layer (L7). Blocking TGF-beta resulted in a significant decrease in the number of VXN2 cells that migrated to L7. In order to test whether the activity of the TGF-beta antibody is specific to the antibody, we carried out a control experiment using antibodies against IgG. As expected, this control antibody did not influence the number of migrating VXN2 cells in the direction of the layers containing TFs.
The migration pattern of the standard co-cultures without the addition of an antibody was similar to that of the cultures with the control antibody.

To assess the metastatic potential of the VXN2 cells, we immunostained for the markers of EMT, E-Cadherin and vimentin. Figure 5 shows the confocal microscopy images. The mesenchymal status of the VXN2 cells is demonstrated through the expression of vimentin in the co-cultures (Figure 5a). This observation is compatible with previous findings.[20] Tumor fibroblasts can stimulate the EMT and invasion of tumor cells through secretion of various cytokines. The cytokine TGF-beta, when secreted by tumor fibroblasts, can induce EMT of bladder tumor cells.[20] We used a TGF-beta neutralizing antibody and found that the inhibition of TGF-beta resulted in a decrease in the expression of vimentin in the VXN2 cells (Figure 5b).

Our results are consistent with literature examples. For instance, one study reported that lung colonizing potential of melanoma cells increased when they were co-cultured with fibroblasts,[21] plausibly as a result of signalling factors that were released from the fibroblasts. Similarly, another study reported that the fibroblasts increased migration and invasion of colon cancer cells.[22] Another research group found that TGF-beta from fibroblasts increased the malignancy of metastatic breast cancer cells.[23]

The primary goal of this paper was to develop a simple paper-based system to study co-cultures of tumor cells and fibroblasts. We used CiGiP for fabrication of multi-layered stacked scaffolds to explore the migratory behavior of tumor cells in 3D. This approach may better recapitulate the dynamic interactions between tumor cells and fibroblasts compared to 2D cultures. We have established that tumor cells migrated more when they are co-cultured with fibroblasts than when they existed as mono-cultures of tumor cells. We suggested that this finding could be partially relevant to the TGF-beta pathway, and found as a test of the hypothesis that when we inhibited TGF-beta, the migration of tumor cells decreased. TGF-beta, therefore, could be a factor participating in the migration process. In addition to
migration, EMT is indicative of the metastatic potential of the tumor cells. We tested the VXN2 cells for EMT as a means to understand the metastatic potential of tumor cells in the presence of fibroblasts. We found that EMT decreased when TGF-beta was inhibited using an anti-TGF-beta antibody. This result is expected since TGF-beta inhibits migration as well as EMT and metastasis. We emphasize that we do not claim that the increase in the migration of tumor cells is purely or even primarily caused by TGF-beta. We suggest that our finding could be, in part, due to the TGF-beta pathway. The exact mechanisms of tumor cell migration in the presence of fibroblasts can be studied in detail in the future. The types of experiments that can be performed with the stacked multi-layered paper include i) investigation of the interplay between different types of stromal cells (e.g. immune, endothelial, connective tissue) and tumor cells, ii) decoupling the effects of oxygen from soluble factors of fibroblasts, and iii) exploration of the influence of TGF-beta from different types of cells on the migratory behavior of tumors. The assessment of anti-migration therapeutics could be another useful application of the stacked paper-based platform. The ability to prepare and analyze samples in a high-throughput fashion makes the stacked paper platform broadly useful for generation of physiologically relevant tumor models and development of new drugs. Paper can also be chemically modified to introduce bioactive functional groups that interact with cells in specific ways offering new opportunities for future studies.

In summary, we have developed a paper-based co-culture system for human lung tumor cells and tumor fibroblasts that allowed us to study the migration of tumor cells. The tumor cells migrated towards the fibroblasts within the stacked systems. Blocking TGF-beta decreased this migration. We also determined the EMT for the lung tumor cells to estimate their metastatic potential. The inhibition of TGF-beta decreased the fraction of tumor cells undergoing the transition to the mesenchymal state. Additional work will be required to
determine the mechanisms of activity of TGF-beta pathway in this system; this work is beyond the scope of the current paper.

The paper-based cell culture system is simple, flexible, inexpensive, and convenient to use for studying dynamic interactions between physiologically relevant types of cells. We think that the CiGiP platform recapitulates in vivo tumor biology better than conventional 2D cultures. The paper-based scaffolds made it possible to generate physiologically relevant cancer models that include multiple cell types, and that model the dynamic interactions between tumor and stromal cells.

The paper-based tumor model also has several shortcomings. The lens paper is only 40 \(\mu\text{m}\) in the z-direction, therefore, may not be suitable for generation of constructs that would require significant mechanical strength. If an experiment utilizes the types of paper that are thicker than 200 \(\mu\text{m}\) in the z-direction, fluorescent imaging can be challenging due to scattering of light. In this work we co-cultured tumor cells and fibroblasts; we can, however, include other cells such as immune and endothelial cells in order to better mimic the native microenvironment of tumors.

We believe that understanding 3D co-cultures of cancer cells and fibroblasts will contribute to understanding the interactions of these cell types in tumors. The paper-based co-culture platform may be useful for screening the inhibitors of TGF-beta pathway and perhaps investigating other stromal interactions that might suggest targets for therapy.

CiGiP also has the potential for use in personalized medicine. For example, patient biopsies might be harvested and grown to sizes relevant to vascularization and necrosis in this system. Different chemotherapeutic agents, or doses of radiation, could then be tested to determine effective treatment strategies specifically designed for the patient. The paper-based co-culture approach simplifies the concept of patient-specific drug testing by miniaturizing the clinical trial in a dish, and by extending it in a new way into three dimensions.
Experimental Section

Preparation of paper scaffolds: We use paper as a scaffold to support thin layers of hydrogels containing cells; a number of prior applications of the method (CiGiP) are described elsewhere.\(^{[24-26]}\) For applications in cell culture, the paper should have sufficient mechanical strength to hold the gel matrix containing the cells. In addition, it should have pore sizes that are large enough to support cell growth, spreading, elongation, and migration. For this work, we initially explored lens paper. Lens paper has large pore sizes (20-200 µm), but because it is only 40 µm thick in the z-direction, it is challenging to handle a single layer of lens paper in cell culture applications. We have, therefore, fabricated hybrid scaffolds that comprise two layers of lens paper sandwiching one layer of poly(vinyl chloride) (PVC). The resulting scaffolds are thick enough to easily handle the cell cultures and have pores sufficiently large to allow migration of cells.

Co-cultures of human lung cancer cells and tumor fibroblasts in multi-layered paper stacks: VXN2 cells were initially mono-cultured at different layers in the paper stacks to explore their migratory behavior. As reported previously, these and other mono-cultures, do not adequately represent the environment that is important for growth of cells.\(^{[27-29]}\) Mono-cultures, additionally, do not exhibit \textit{in vivo} physiological behavior of cancer cells properly.\(^{[30]}\) To provide an improved mimic of natural tumors, VXN2 and TF cells were grown as co-cultures in multi-layered paper stacks with different configurations.

Inhibition of TGF-beta: To explore the influence of the activity of TGF-beta on migration of tumor cells, we used an antibody (R&D Systems, AB-100-NA) against it to block its effects. It is important to test a control antibody with the same isotype for validation of the results. We used an IgG antibody (R&D Systems, AB-105-C) and showed that the control antibody did not influence the migration of the tumor cells. We, therefore, concluded that the migration of tumor cells was decreased due to the addition of TGF-beta blocking antibodies.
Epithelial-to-Mesenchymal Transition (EMT): EMT is a phenomenon by which the epithelial cells become migratory and invasive by transitioning into the mesenchymal phenotype. According to literature reports, EMT is involved in initiating the events for the metastasis of tumor cells into blood vessels, lymph nodes, or other organs.\textsuperscript{[31]} The metastatic potential of a tumor is routinely analyzed by testing for the epithelial and mesenchymal phenotypes of the tumor cells.\textsuperscript{[32]} The mesenchymal state is high in vimentin and low in E-cadherin, whereas the epithelial state is high in E-cadherin and low in vimentin.\textsuperscript{[33]} We stained for E-cadherin and vimentin expression of VXN2 cells, and used the ratio of vimentin to E-Cadherin, which may be considered as one measure of the metastatic potential of the VXN2 cells.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was partially funded by a collaborative research agreement with Vertex Pharmaceuticals, Incorporated (Boston, MA), Bill & Melinda Gates Foundation Award #51308, and the Wyss Institute of Biologically Inspired Engineering. We acknowledge use of tissues procured by the National Disease Research Interchange (NDRI) with support from NIH grant 5 U42 RR006042.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))
References


Supporting Information

Fibroblasts enhance migration of human lung cancer cells in a paper-based co-culture system

Gulden Camci-Unal¹, David Newsome², Brenda K. Eustace², and George M. Whitesides¹,³*

¹ Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, MA 02138, USA.

² Vertex Pharmaceuticals Incorporated, 50 Northern Avenue Boston, MA 02210, USA.

³ Wyss Institute for Biologically Inspired Engineering, Harvard University, 60 Oxford Street, Cambridge, MA 02138, USA.

(*) Author to whom correspondence should be addressed:

gwhitesides@gmwgroup.harvard.edu

Keywords: tumor cells, fibroblasts, migration, coculture
Materials and methods

Whatman lens paper, 4,6-diamidino-2-phenylindole (DAPI), and Draq5 were supplied by Sigma-Aldrich (St Louis, MO). Antibodies to TGF-beta and IgG were obtained from R&D Systems (Minneapolis, MN). Antibodies for E-Cadherin and vimentin were bought from Cell Signaling Technology (Danvers, MA). Trypsin-EDTA, penicillin-streptomycin, fetal bovine serum (FBS), GlutaMax, Dulbecco’s minimal essential medium (DMEM) medium, and Dulbecco’s phosphate buffered saline (DPBS) were purchased from Invitrogen (Carlsbad, CA). The 16% (v/v) paraformaldehyde solution was purchased from Electron Microscopy Sciences (Hatfield, PA). All reagents were used as received without further purification.

Preparation of paper scaffolds

A 40 µm-thick polyvinylchloride (PVC) sheet was laser cut to create the holes for cell-seeding zones. The PVC sheet was placed between two layers of lens paper and hot-embossed at 450 °F for 30 sec. The resulting PVC-infused paper scaffolds were cut and sterilized by autoclaving. The scaffolds were kept sterile at room temperature until further use.

Cell cultures

The tumor cells (VXN2) and fibroblasts (TF) were isolated from the biopsies of human patients. The VXN2 cells (Vertex Pharmaceuticals Inc.) were isolated in vitro from a primary non-small-cell lung carcinoma (NSCLC) tumor sample, which was first expanded in mice. The TF cells (Vertex Pharmaceuticals Inc.) were isolated directly from a primary NSCLC sample. Both cell types were cultured on tissue-culture treated plastic in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) Glutamax, and 1% (v/v) penicillin/streptomycin. The cell cultures were maintained in a standard 37°C incubator equipped to provide 5% CO₂. We changed the medium every other day to provide a fresh environment for cells.

Co-cultures of human lung cancer cells and tumor fibroblasts in multi-layered paper stacks
In the co-culture experiments, we used VXN2 (passage number 10-19) and TF (passage number 3-12) cells. After harvesting the cells from tissue cultures flasks, we labeled the VXN2 cells by cell tracker green. We seeded them in the culture zones of the double lens papers and assembled them in different configurations. After culturing the paper stacks for three days, we separated the layers from the scaffolds and scanned them with high resolution Typhoon scanner. We quantified the amount of fluorescence for each cell-seeded zone by using the NIH Image J software. Subsequently, we generated the bar plots based on the percent (%) of VXN2 cells that are present in each layer. We performed the experiments in triplicates for each condition.

*Immunocytochemistry for Epithelial-to-Mesenchymal Transition (EMT)*

To assess the epithelial and mesenchymal phenotype of the tumor cells, they were tested for expression of E-Cadherin and Vimentin. The samples were fixed in a 4% (v/v) paraformaldehyde solution and permeabilized using 0.5% (v/v) Triton X-100. A 10% (v/v) solution of goat serum was used to block the non-specific protein binding. The samples were incubated with 1/100 diluted primary antibody mixture for E-Cadherin and vimentin overnight at 4°C. 1/100 diluted secondary antibodies were then used to incubate the samples at room temperature for 1 h. The samples were washed with PBS and imaged using a Zeiss LSM710 confocal microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY).

*Statistical analysis*

The experimental data was analyzed by GraphPad Prism (Version 4.02, La Jolla, CA). The statistical differences between groups were determined by one-way ANOVA tests with Bonferroni comparisons. *p*-values < 0.05 are considered statistically significant (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001).