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A simple engineered platform reveals different modes of tumor-microenvironmental cell interaction

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Abstract

How metastatic cancer lesions survive and grow in secondary locations is not fully understood. There is a growing appreciation for the importance of tumor components, i.e. microenvironmental cells, in this process. Here, we used a simple microfabricated dual cell culture platform with a 500 μm gap to assess interactions between two different metastatic melanoma cell lines (1205Lu isolated from a lung lesion established through a mouse xenograft; and WM852 derived from a stage III metastatic lesion of skin) and microenvironmental cells derived from either skin (fibroblasts), lung (epithelial cells) or liver (hepatocytes). We observed differential bi-directional migration between microenvironmental cells and melanoma, depending on the melanoma cell line. Lung epithelial cells and skin fibroblasts, but not hepatocytes, stimulated higher 1205Lu migration than without microenvironmental cells; in the opposite direction, 1205Lu cells induced hepatocytes to migrate, but had no effect on skin fibroblasts and slightly inhibited lung epithelial cells. In contrast, none of the microenvironments had a significant effect on WM852; in this case, skin fibroblasts and hepatocytes—but not lung epithelial cells—exhibited directed migration toward WM852. These observations reveal significant effects a given microenvironmental cell line has on the two different melanoma lines, as well as how melanoma affects different microenvironmental cell lines. Our simple platform thus has potential to provide complex insights into different strategies used by cancerous cells to survive in and colonize metastatic sites.

Keywords

cancer; microenvironment; micropatterning; co-culture; metastasis; *in vitro* model

Introduction

Cancer is the second leading cause of death in the United States; notably, cancer metastasis accounts for approximately 90% of cancer-related deaths [1]. Although early detection and improved targeted therapies have improved patient survival rates for most primary cancers [2], the survival rate for metastatic cancers remains very poor. For cancer cells to successfully metastasize, they must intravasate into the blood/lymph circulation, survive in the vasculature, extravasate out of the circulation, and colonize a new organ. Studies with various cancer models have led to numerous groundbreaking findings that explain how cancer progresses from a neoplasm to a deadly disease [3]. Among these findings are driver mutations and oncogenes [4] that unleash cancer cell proliferation, angiogenic switches [5] that enable tumors to increase in size, and cancer stem cells [6] that fuel cancer recurrence following treatment.

Although studies have been fruitful in defining critical pathways associated with tumor development and progression, researchers are recognizing that microenvironmental cells—non-cancerous cells integrated in the tumor—also contribute to the survival and growth of metastatic tumors. Cells within the tumor microenvironment may include endothelial cells [7], fibroblasts [7, 8], and immune cells [7], along with tissue-specific parenchymal cells. Cancer cells that extravasate out of circulation must adapt to a very different microenvironment from that of the primary tumor. Indeed, surviving and growing in a new hostile microenvironment is undoubtedly an important and potentially rate-limiting step in the progression from a lone cancer cell to macrometastases [9]. Proposed by Stephen Paget in 1889, the ‘seed and soil’ hypothesis has become one of the prevailing hypotheses attempting to explain how cancer metastasizes to a secondary site. Specifically, Paget hypothesized that macrometastases develop where cells within the secondary site provide a suitable ‘soil’ for cancer survival. Subsequent studies have provided evidence to support this hypothesis. Nakagawa *et al* showed that cancer-associated fibroblasts produce more growth factors and molecules that govern cell–cell interactions with cancer cells and wound healing than normal skin fibroblasts, thus supporting colon cancer growth in liver [10]. Similarly, Tabaries *et al* found that hepatocytes provide an adhesion bed for breast cancer cells by expressing a high level of claudin-2, a tissue-specific tight junction component normally found in liver that turned out to be crucial for breast cancer cells to seed and colonize the liver [11].

These observations underscore the essential influence of microenvironmental cells on whether a primary cancer cell is able to form a secondary metastatic malignancy. Accordingly, researchers have been using well-established as well as new methods to study cancer-microenvironmental cell interactions *in vivo* and *in vitro*. Mouse models are among the most widely used *in vivo* models for cancer research, and although they provide a physiologically relevant microenvironment for cancer cells, it is not feasible to precisely control microenvironmental cells in live mice. Additionally, the complex microenvironmental composition in mice makes it challenging to determine causal factors in cancer-microenvironmental cell interactions. Furthermore, although human cancer cells can be embedded in genetically modified mice, the microenvironmental cell is still of mouse origin, which may alter the relevance of such systems to human disease.

Recreating cancer-microenvironmental cell interactions *in vitro* can overcome the complications from studying microenvironmental effects *in vivo*, as specific human cell lines can be used to represent both cancerous and microenvironmental cells in a controlled setting. Boyden chambers or Transwell systems use two physically distinct compartments connected by a porous membrane to study cell paracrine effects, allowing secreted factors from microenvironmental cells to diffuse to cancer cells and vice versa. In these systems, cancer cell migration across the membrane can easily be measured to quantify cancer cell response to soluble factors secreted by microenvironmental cells. For instance, Rhodes *et al* used the Transwell system to show that human mesenchymal stem cells stimulate migration of MCF-7 breast cancer cells [12]. However, interactions between the two cell types within the Transwell are exclusively of soluble form. Also in this type of study, because the two cell types are grown on two different substrates (i.e., polystyrene for the bottom well and polycarbonate or polyester for the membrane), additional variables, such as substrate stiffness and chemical composition, must be considered during data interpretation.

Recent advances in microfabrication and biomaterials enable more controlled studies to be carried out. Microfabricated stencils and stamps allow researchers to deposit different types of cells and extracellular matrices (ECMs) according to pre-defined patterns and can thus establish cell–cell interactions to a resolution of 100 μm . For example, Dickinson *et al* created a series of finely controlled cancer-endothelial interactions with microcontact printing, taking advantage of preferential adhesion of endothelial colony-forming cells to fibronectin and of breast cancer cells to hyaluronic acid [13]. More recently, Shen *et al* used high resolution analysis of similar micropatterned tumor-microenvironmental co-culture experiments to demonstrate that the proximity of microenvironmental cells can have a strong influence on the growth rate, gene expression profile, and drug response of a given cancer cell [14].

In this study, we used a simple engineered platform to investigate cancer-microenvironmental cell interactions between melanoma cells and lung epithelial cells, hepatocytes, or skin fibroblasts. Melanoma is a very aggressive form of skin cancer, for which skin, liver, and lung are common target organs for metastasis [15]. We investigated the microenvironmental interactions of two different melanoma cell lines, 1205Lu and WM852. 1205Lu is a metastatic melanoma cell line experimentally developed from a cell line (WM793) established from a vertical growth phase (VGP) melanoma lesion. After the lesion is trypsinized, the resulting cell suspension is subcutaneously injected into a mouse, and the 1205Lu line is generated from a subsequent spontaneous lung metastasis. It is a highly invasive and highly migratory cell line [16, 17]. WM852 is a less aggressive metastatic melanoma cell line originally isolated from a VGP stage III (as classified by American Joint Committee on Cancer) metastatic skin lesion excised from a patient [18]. To establish cancer-microenvironmental cell interactions, we used a microfabricated polydimethylsiloxane (PDMS) stencil to pattern cancer and microenvironmental cells with a 500 μm gap on tissue culture polystyrene. After patterning, both cancer and microenvironmental cells can freely migrate into the gap, and the short distance between cells allows paracrine communication. Despite its simplicity, the engineered platform revealed important differences in the interaction of the two metastatic melanoma cell lines

with microenvironmental cells. Significantly, our platform identified phenotypic differences between the two melanoma lines that may potentially reflect the state of tumor progression.

Materials and methods

Microfabricated stencil

The stencils for cell patterning (figure 1) were fabricated using soft lithography. Silicon wafers (University Wafers, Boston, MA) were cleaned with piranha solution before use. SU-8 2150 photoresist (Microchem, Westborough, MA) was spin-coated onto the wafer to achieve a thickness of approximately 0.7 μm . After soft baking, the wafer was exposed to ultraviolet light (UV) through a photomask (CAD/Art Service, Bandon, OR) with predefined features. The wafer was then hard-baked and developed. Polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) was mixed at a 10:1 ratio (PDMS base to curing agent), degassed, and poured over the patterned wafer. A Teflon sheet was laid over the PDMS, and a piece of glass with a 1 kg weight placed on top of the Teflon sheet was used to apply even pressure during overnight curing at 80 °C to ensure PDMS stencils had the same thickness as the features on the wafer. Cured PDMS stencils were peeled off the wafer and trimmed to fit a 12-well plate. Before use, the PDMS stencils were exposed to UV for 1 h and gently pressed down into a 12-well plate to ensure a tight seal between the stencil and the bottom of the plate.

Cell culture

1205Lu and WM852 metastatic melanoma cell lines expressing green fluorescent protein (GFP) were a generous gift from Dr Meenhard Herlyn (Wistar Institute, Philadelphia, PA). We selected different microenvironmental cells that best represented the primary parenchymal cells seen in metastatic tissues. Lung epithelial cells line the surface of the respiratory tree, making them good candidates for modeling lung tissue. Hepatocytes are the major constituent of the liver, accounting for 67% of all cells in liver [19]. Skin fibroblasts are the major type of cells in the dermis, where melanoma skin metastasis usually occurs. BEAS-2B (human bronchial epithelial) cells and BJ fibroblasts (foreskin) were purchased from ATCC (Manassas, VA). Immortalized human fetal hepatocytes were a generous gift from Dr Mark Zern from University of California, Davis. All cells were cultured in high glucose Dulbecco's Modified Eagle Medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; GE Healthcare, Logan, UT), 5 $\mu\text{g L}^{-1}$ insulin (Sigma-Aldrich, St. Louis, MO), 2.4 mg L^{-1} hydrocortisone (Sigma-Aldrich), and 2 mM L-glutamine (Life Technologies) at 37 °C and 5% CO_2 . We found that we needed to supplement basal media with hydrocortisone and insulin to maintain liver cells—the most difficult cell type to maintain. Fortunately, we found that this media was able to support all the other cell types, and this became the common media used for all experiments.

Cancer-microenvironmental cell interaction assay

Two days prior to conducting the assay, all microenvironmental cells were treated with mitomycin C-supplemented culture media for 24 h, with mitomycin C concentrations for lung, liver, and skin cells at 0.5, 1, and 5 $\mu\text{g ml}^{-1}$, respectively, to arrest their proliferation (mitomycin C titration data not shown). Microenvironmental cell proliferation was arrested

to mimic the naturally low proliferative state of these cells *in vivo* [20, 21]. After treatment, cells were washed with phosphate buffered saline (PBS) and kept in culture media for another 24 h. The wells of the PDMS stencils were filled flush to the air–liquid interface with 26 μl of fibronectin ($10 \mu\text{g ml}^{-1}$) and incubated at 37 °C and 5% CO₂ for 1 h to facilitate cell attachment. The volume was critical to minimize meniscus formation, which resulted in uneven seeding. The wells were then rinsed twice with PBS. All microenvironmental cells were stained with CellTracker Orange (Life Technologies, Carlsbad, CA) according to manufacturer's protocol before trypsinizing. Cells were seeded into the wells of the PDMS stencil at capacity (26 μl) and incubated at 37 °C and 5% CO₂ for 3 h to allow for cell attachment. Hepatocytes, lung epithelial cells, and foreskin fibroblasts were seeded at a density of 1.2×10^6 cells/ml to form a confluent monolayer upon cell attachment. 1205Lu and WM852 were seeded in full culture medium at 0.6×10^6 cells/ml, which ensured that they did not become overconfluent as they continued to proliferate over the course of the experiment. The stencils were then peeled off, and each well was rinsed with warm PBS and filled with culture media supplemented with 1% FBS. Immediately after, the plate was imaged using an Axiovert S100 microscope (Zeiss, Germany) equipped with MAC2002 motorized stage (Ludl Electronic Products, Hawthorne, NY). A motorized stage was used to take images at the specified coordinates of the 500 μm gaps of each cell combination, once at 0 h and again at 72 h. The plate was maintained at 37° C and 5% CO₂ for the 72 h period between imaging.

Data analysis and significance testing

Images for each fluorescent marker (GFP and Cell-Tracker Orange) were merged using ImageJ, and the merged pictures at 0 h and 72 h are stacked together. A box with 500 μm width was drawn on the 0 h picture to fit the gap, and the position of the box was transferred to the 72 h picture to mark the cell migratory front. The areas covered by both cancerous and microenvironmental cells in the gap were obtained. The number of cancer cells within the 500 μm gap was counted manually.

We quantitated the alignment of microenvironmental cells relative to the cancer patterning front within the gap using a two-dimensional fast Fourier transform (2D FFT) method [22]. Briefly, 2D FFT was performed on microenvironmental cells within the gap to convert spatial information about cell orientation into frequency information. The resulting FFT yielded oval spectra for angles from 0° to 360°. Radial sums over this oval were calculated using the ImageJ (NIH) 'Oval profile' plug-in with a sampling frequency of 12°, which yielded a π -periodic plot of the overall orientation angle of the cells. Data from 0°–180° was added to that from 180°–360° as 2D FFT is symmetric about 180°. All angles were shifted by 90° so that 180° represents an orientation that is perpendicular to the cancer cell patterning front. The data was then normalized such that the total sum was unity. Cells with random orientation would have a uniform angle distribution, with a value of 0.0667.

The overlap between cancer and microenvironmental cells was defined by the horizontal distance measured between the furthest migrating cells into the opposing population of cells at 72 h.

Each cell combination was performed in triplicate. Analysis of variance (ANOVA), followed by the Tukey–Kramer multiple comparison post hoc testing, was used to determine if the area covered by cancerous and microenvironmental cells within the gap was significant and if the difference in cell orientation of microenvironmental cells was significant, t-tests were used to determine if the cancer-microenvironmental cell overlap was significant. A p -value <0.05 was considered to be significant. Statistics were analyzed using PRISM (Graphpad, La Jolla, CA).

Results

Establishing cancer-microenvironmental cell interaction

A microfabricated PDMS stencil (figure 1) was used to segregate cancer from microenvironmental cells on the surface of tissue culture plastic. As shown in figure 2, the stencil leaves straight and clear boundaries with a 500 μm gap between cancer cells (left side) and microenvironmental cells (right side). The 500 μm gap size was chosen based on diffusion distance of chemokines, migration speed of cancer and microenvironmental cells, and ease of maintaining a good seal between PDMS and tissue culture plastic. Melanoma cells have been reported to migrate as fast as 20 $\mu\text{m h}^{-1}$ [23], and normal tissue cells have migration speeds on the same order of magnitude [24, 25], suggesting closure of the 500 μm gap can be achieved within 72 h. As the effective intercellular communication distance has been estimated to be around 250 μm for individual cells [26], and the separation between cell types decreases as cells cover the gap, it is reasonable to assume that cancer-microenvironmental cell chemokine communication occurs at this separation distance. Unfortunately gaps narrower than 500 μm were not possible to test in this system because the PDMS stencils were not able to seal against the tissue culture plastic surface, leading to mixing of the two cell types during seeding.

Influence of microenvironmental cells on cancer cells

1205Lu is a highly invasive and highly migratory metastatic melanoma cell line originally derived from a stage IV metastatic lesion [16, 17]. To examine the interaction of 1205Lu cells with different types of microenvironmental cells, they were cultured opposite lung epithelial cells, hepatocytes, or skin fibroblasts, and images were taken of the gap and the areas on either side of the gap after 72 h. A higher percentage of 1205Lu melanoma cells occupied the gap when co-cultured with lung epithelial cells or skin fibroblasts compared to when co-cultured with hepatocytes (figures 3 and 4). When co-cultured with lung and skin, 1205Lu melanoma cells moved a significantly greater distance ($p = 0.0058$) beyond their original patterning boundary than in the absence of opposing microenvironmental cells (figure 4(A)). A number of 1205Lu cells were also observed beyond the initial boundaries of these microenvironmental cells, confirming the high degree of invasiveness of this cell line. In contrast, hepatocytes had no apparent effect on 1205Lu cell movement. Coverage area and cell densities were not significantly different than values observed in the absence of microenvironmental cells (figures 4(A) and (E)), and this is reflected in a roughly equal distribution of 1205Lu cells and hepatocytes within the gap (figure 3(A)). 1205Lu coverage was decreased with 1205Lu cells on the opposing side. Since 1205Lu cells covered more

than half of the gap area when alone, patterning them on opposing side impeded its coverage when cell–cell contact occurred. (Supplementary figure 3.)

WM852 melanoma cells responded very differently to the three types of microenvironmental cells than 1205Lu. WM852 was isolated from a localized stage III metastatic lesion in the skin of the patient [18], and the cell migration results showed that it is not nearly as invasive as 1205Lu (figure 4). Although all three microenvironmental cell lines have a slight tendency to increase WM852 movement into the area between the patterning boundaries, this trend was not significantly different ($p = 0.3392$) than the movement observed in the absence of microenvironmental cells. Consistent with the absence of a cytokinetic effect, none of the microenvironmental cells had an effect on WM852 cell number. Accordingly, WM852 cells accounted for a much lower percentage of cells moving into the area between the patterning boundaries than for 1205Lu cells when incubated with a given type of microenvironmental cell. Culturing WM852 cells on opposing sides did not affect their coverage in the gap. Since WM852 cells alone covered less than half of the gap area, cell/cell contact did not occur when they were cultured on opposing sides. (Supplementary figure 3.)

We observed that migration of melanoma cells on the opposite side of the cancer pattern, the side that is not next to microenvironmental cells, was similar to that of melanoma cells alone. (Supplementary figure 4.)

We confirmed that the advance of melanoma cells into the gap was due to both proliferation and migration. Ki67 staining showed that 40%–60% of melanoma cells within the gap were proliferating. (Supplementary figure 1.)

We treated microenvironmental cells with mitomycin C to mimic their stationary feature *in vivo*. To confirm this was necessary for observing cancer-microenvironmental cell interactions, another set of experiments was done without mitomycin C treatment. Unleashing proliferation of microenvironmental cells changed their migration pattern as well as the subsequent influence on cancer cell migration. (Supplementary figure 2.) All microenvironmental cells proliferated and migrated into the gap significantly over the 72 h period. 1205Lu migration was retarded by all microenvironmental cells comparing to the non-mitomycin C treated conditions. Specifically, lung epithelium inhibited 1205Lu migration by covering the majority of the gap and as a result, 1205Lu barely migrated beyond their patterning boundary. On the other hand, WM852's migration was mostly unchanged. This could be due to the fact that their migration was minimal even when the microenvironmental cells were not treated with mitomycin C. Interestingly, when co-cultured with skin fibroblasts, WM852's migration was slightly stimulated ($p < 0.05$). This might indicate that the mitomycin C treatment changed the paracrine signal secretion of skin fibroblasts. Due to the significant advancement of microenvironmental cells into the gap, we focused our analysis on mitomycin C-treated conditions, where proliferation of microenvironmental cells did not dominate the gap area.

Influence of cancer cells on microenvironmental cell migration

It is evident from figure 2 that, just as microenvironmental cells differentially influence cancer cell migration, cancer cells also differentially influence microenvironmental cell migration. These data are quantified in figure 5. In the absence of cancer cells, all three microenvironmental cell lines migrated into an area beyond their initial patterning boundaries, with hepatocytes and lung epithelial cells moving further than skin fibroblasts. Addition of 1205Lu cells in co-culture significantly increased the area occupied by hepatocytes, and, surprisingly, significantly decreased the area occupied by lung epithelial cells. 1205Lu cells had no effect on the area occupied by skin fibroblasts. The advancing fronts of lung epithelial cells and skin fibroblasts were relatively straight. However, hepatocytes migrated further and displayed a less distinct front, resulting in greater interaction with tumor cells. Addition of WM852 cells into co-culture gave markedly different results than those observed with 1205Lu cells. WM852 cells induced a striking increase in migration distance observed for skin fibroblasts ($p < 0.05$); on the other hand, only a slight but non-significant increase in the migration distance of hepatocytes and no effects on migration of lung epithelial cells were observed. We observed that migration of microenvironmental cells on the opposite side of the microenvironmental cell pattern, the side that is not next to cancer cells, was similar to that of microenvironmental cells alone. (Supplementary figure 4.)

Influence of cancer cells on microenvironmental cell orientation

Close examination of photomicrographs such as those depicted in figure 2 revealed that WM852 cells induced skin fibroblasts to polarize and become elongated along an axis perpendicular to the original WM852 melanoma cell front, a finding consistent with directed cell migration. Figure 6 depicts results of the 2D FFT analysis for the directional distribution of each microenvironmental cell line in response to the two cancer lines. Consistent with what can be discerned visually, skin fibroblasts showed a striking tendency to orient around 180° ($p < 0.05$), which is perpendicular to the WM852 patterning front. Hepatocytes had a slightly lesser, but still strong tendency to orient towards WM852 cells, but only a weak (if any) orientation of lung epithelial cells was observed. In contrast, 1205Lu cells induced very little cellular reorientation, with the exception of a small effect on hepatocytes. Overall, the influence of the two cancer lines on the orientation of each type of microenvironmental cell was highly consistent with the corresponding influence on microenvironmental cell movement beyond the patterning boundary. Together, these results suggest that WM852 cells have a strong influence on the directed cell migration of skin fibroblasts, and that both WM852 and 1205Lu cells have a smaller directed migration effect on hepatocytes.

Cancer and microenvironmental cell overlap

As successful metastatic tumor growth requires close interactions between tumor and microenvironmental cells, we investigated this issue using results obtained from our engineered platform. Accordingly, photomicrographs exemplified by those in figure 2 were reanalyzed to determine the overlap distance between the cancer and microenvironmental cell fronts. This metric puts more weight on the fastest moving cell in the population without regard to collective cell migration. 1205Lu cells had a larger overlap distance than WM852

cells when co-cultured with hepatocytes and lung epithelial cells. WM852 cells had a slightly larger overlap area than 1205Lu cells when co-cultured with skin fibroblasts, but the effect was not significant (figure 7). The large overlap or 'level of interaction' of 1205Lu co-cultures can be mainly attributed to the high 'invasiveness' of 1205Lu cells. In contrast, the large overlap of WM852 with skin is mainly due to the 'receptiveness' of the two cell types to each other as both types of cells can be found within each other's original pattern.

Discussion

Conducting migration assays using our simple microfabricated stencil offers some distinct advantages over traditional scratch assays, including ability to co-culture experiments and cleaner pattern boundaries free of dead or peeling cells. These advantages make image analysis and data interpretation more straightforward. The 500 μm gap is small enough to allow paracrine signaling and to enable capture by microscope in one field of view. However, the gap is big enough so cells can migrate freely in response to any potential paracrine signals before cell/cell contact occurs. Our system is also superior to Transwell-type assays in that it allows cell-to-cell contact in addition to paracrine interactions between cancer and microenvironmental cells, thereby more closely approximating interactions *in vivo*. Although the gap is not coated by ECM at the time when the stencil is peeled off, the ECM contained in the common media will gradually adsorb to the surface, providing a consistent ECM coating for all cell combinations. Finally, the effects of microenvironmental cells on cancer cells as well as the effects of cancer cells on microenvironmental cells can be evaluated simultaneously.

This study has two underlying hypotheses. First, independent of microenvironmental influences, not all metastatic tumors are alike. Second, the microenvironmental tissue surrounding a tumor provides essentially an *in vivo* selection process, and any cell lines derived from such a tumor may be predisposed to interact more strongly with microenvironmental cell lines derived from the site of origin of the parent cell line. The 1205Lu and WM852 melanoma cell lines are appropriate for this comparative study because (1) although they are both metastatic melanoma cell lines, they are derived from tumors taken at different stages of cancer development (distant metastasis in a mouse xenograft system versus primary metastatic lesion from a patient, respectively), and (2) the parent tumors are derived from different metastatic sites (lung and skin, respectively). By showing differences in the inherent mobility of the two cancer lines and their corresponding response patterns to microenvironmental cells derived from three different tissues, our results support both hypotheses.

Our mobility assay showed that 1205Lu cells migrate significantly further than WM 852 cells in the absence of microenvironmental cells, achieving a migratory front of at least 250 μm into the 500 μm gap. It is widely accepted that high cell motility correlates with high metastatic potential [27]. It should be noted, however, that 1205Lu and WM852 are isolated from different patients and subject to different isolation protocols; hence any firm conclusions regarding the relationship between cancer stages are impossible. Lung epithelial cells and skin fibroblasts both promoted an even greater 1205Lu migration beyond their patterning boundaries, and with lung epithelial cells, this increased motility is associated

with an increase in number of cells migrating into the gap, which may be expected to result from directed cell migration. Although these observations would be consistent with the hypothesis that selecting a spontaneous lung metastasis to create the 1205Lu line selects for cancer cells that respond positively to paracrine communication from lung epithelial cells, far more comprehensive testing with different cell lines derived from lung and other tissues is required to obtain a definitive conclusion. What we can conclude, however, is that the 1205Lu cell line clearly responds differently to different microenvironmental cell lines. In contrast, WM852 cells are less migratory, and their mobility seems unaffected by any of the microenvironmental cell lines tested in our study. WM852 cell also require a longer time to attach to tissue culture plastic than 1205Lu. Interestingly, it appears as though there are two sub-populations of WM852 cells: a small population that migrates well into the microenvironmental-patterned area, and a much larger population that migrates very little. It is possible that the cells that migrate into microenvironmental-patterned areas are indicative of a sub-population of tumor cells obtained from the original tumor tissues versus an adaptive response of tumor cells that was acquired *in vitro*, which would be consistent with finding of a heterogeneity in DNA mutations and therefore within the same tumor [28].

Whereas the 1205Lu line is more motile and responsive to microenvironmental cell influences than the WM852 line, the WM852 line appears to have a greater influence on microenvironmental cells. When cultured next to WM852, all three microenvironmental cell lines became more migratory and occupied most of the 500 μm gap. In particular, skin fibroblasts and hepatocytes displayed strong directed migration towards WM852 cells (figures 2 and 6), including the appearance of an elongated phenotype where the elongational axis is directed toward the WM852 cells. Given that WM852 cells are derived from skin metastasis, the observation that they elicit the greatest directed migration effect on skin fibroblasts is not surprising. The most important result gleaned from the data in figure 6, however, lies in the comparison of the weakly migratory/high migration-promoting phenotype of WM852 cells with the highly migratory/weak migration-promoting phenotype 1205Lu of cells and in the implications this dichotomy has on the mechanisms by which cancer cells and microenvironmental cells interact to form a tumor. In essence, 1205Lu migration is stimulated by co-culture with certain types of microenvironmental cells, and in those situations they aggressively move toward and even into the microenvironmental cells. WM852 are less mobile, and their mobility was not significantly affected when co-cultured with the same types of microenvironmental cells, but they actively recruited microenvironmental cells in a cell-type (and potentially tissue-) selective manner, possibly through paracrine signals they secrete. If representative of the parent tumor behavior *in vivo*, these observations have potentially significant clinical ramifications. While both mechanisms would allow for the eventual development of tumor structure (the 3D arrangement of cancer cells and microenvironmental cells), the mechanism by which the tumor is constructed would be very different, and it would be reasonable to speculate that different therapeutic regimens would be required for treatment.

Future studies focused on identification of paracrine factors from microenvironmental cells that stimulate 1205Lu migration as well as those from WM852 that attract microenvironmental cells could prove useful in elucidating targetable chemokine signaling pathways for each class of tumor cells. For example, TGF β [29], interferon- α [30], and IL-6

[31] are potential growth factors secreted by fibroblasts that can modulate melanoma behaviors. Ultimately, 'brute force' high-throughput pharmacological testing could also be carried out using our simple assay to identify drugs with potential to block these paracrine factors.

Finally, it should be noted that the microenvironmental cells used in this study represent only a small fraction of organ-specific cells. Testing other organ-specific cells may help identify the key type of cells in each organ that interact with tumors. Cell lines are easy to obtain and use, but cells from patients with known cancer diagnosis and prognosis would be of more clinical value, and our simple microfabricated platform could easily be adapted to conduct this type of study. The current 2D platform could also potentially be modified into a 3D platform to better mimic the 3D microenvironment in living tissue [13, 32] and hence improve its predictive capabilities.

Conclusion

In this study, we report a simple microfabricated stencil to pattern different melanoma and microenvironmental cell types in close vicinity. Although bidirectional migratory effects were seen between microenvironmental cells and both melanoma lines, the nature of these effects differs. 1205Lu metastatic melanoma cells exhibited a highly migratory pattern and became even more migratory in the presence of specific microenvironmental cell types in co-culture experiments. The high motility of 1205Lu correlated with the fact that they develop into lung metastasis in mouse model. In the opposite direction, 1205Lu melanoma cells enhanced hepatocyte migration, had no effect on skin fibroblast migration, and slightly inhibited lung epithelial cell migration. Our findings also revealed that WM852 metastatic melanoma adapts an entirely different pattern that is much less motile than 1205Lu; it does, however, actively recruit skin fibroblasts and hepatocytes but does not affect the movement of lung epithelial cells. The highly directed migration of skin fibroblasts towards WM852 cells correlated well with the origin of WM852, a skin metastatic lesion from a human patient. Thus, our simple engineered system allows for unique observations of tumor-microenvironmental communication networks that are expected to provide critical insights into tumor-specific behaviors. Such communication networks appear to be associated with specific metastatic phenotypes and could be targeted through the development of novel therapeutic strategies for cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Microfabricated stencil to pattern cancer and microenvironmental cells. PDMS Stencil was laid onto tissue culture plastic and pressed gently to ensure a tight seal. Cancer and microenvironmental cells with appropriate density were added to either well. Cells were allowed to attach to tissue culture plastic for 3 h before the stencil was removed, leaving patterned cell islands behind. The gap between the two cell islands is 500 μm .

Figure 2.

Interaction between metastatic melanomas and different microenvironmental cells. (A) Melanoma cells (green), 1205Lu and WM852 were patterned next to lung epithelial cells, skin fibroblasts and hepatocytes with a 500 μm gap at 0 h. After 72 h, 1205Lu cells occupied the majority of the gap except when they were cultured with hepatocytes. On the other hand, microenvironmental cells occupied the majority of the gap when they were co-cultured with WM852. In addition, skin fibroblasts showed strong directed cell migration toward the cancer cells. (B) Magnified picture of 1205Lu with hepatocytes at 72 h and of WM852 with skin fibroblasts at 72 h.

Figure 3.

(A) 1205Lu cells constituted over 80% of the cells within the gap when cultured with lung epithelial cells and skin fibroblasts, but only about 40% when cultured with hepatocytes. 1205Lu distributions under lung and skin co-culture were significantly different from that under liver co-culture but not different from each other. (B) WM852 cells constituted less than 40% of the cells within the gap. For each type of melanoma cell, the distribution measurement was compared among three co-culture conditions, using one-way ANOVA followed by multiple comparison. ‘*’ indicates a $p < 0.05$.

Figure 4.

(A) and (B) 1205Lu cells covered more area within the gap when co-cultured with lung epithelium cells and skin fibroblasts than when cultured alone. WM852 cell migration was not significantly affected when co-cultured with microenvironmental cells. (C) and (D) Number of cancer cells present within the gap after 72 h. More 1205Lu cells migrated into the gap when co-cultured with microenvironmental cells than mono-culture. No difference was observed for WM852 cells. For each type of melanoma, measurement obtained through co-culture was compared to that obtained through cancer mono-culture, using one-way ANOVA followed by multiple comparison testing.

Figure 5.

Coverage of microenvironmental cells when co-cultured with melanoma cells. Hepatocytes covered more area in the gap, while lung epithelium covered less of the gap area when co-cultured with 1205Lu than alone. Skin fibroblasts covered more of the gap area when co-cultured with WM852 than alone. For each type of microenvironmental cell, coverage under the influence of 1205Lu and WM852 was compared with that when microenvironmental cells were cultured alone, using one-way ANOVA followed by multiple comparison testing.

Figure 6.

Microenvironmental cell orientation quantified by 2D FFT. (A) and (B) Orientation peaks of all WM852 and skin fibroblasts co-culture experiments were located around 180° , indicating that WM852 cells oriented toward cancer cells. Skin fibroblasts co-cultured with 1205Lu displayed no obvious peak orientation. (C) and (D) Hepatocytes showed alignment toward both WM852 and 1205Lu cells, with less pronounced alignment toward 1205Lu. (E) and (F) Lung epithelium showed no alignment toward either melanoma cell line as indicated by flat orientation distribution curves. Alignments for three independent experiments were plotted for each condition. The baseline for orientation observed in control experiments conducted in the absence of melanoma cells is 0.0667.

Figure 7.

The overlap between cancer and microenvironmental cells was defined as the horizontal distance between the furthest migrating cells of either cancer or microenvironmental population towards the other population. After 72 h, co-culture with 1205Lu showed more overlap than co-culture with WM852; however, WM852 and 1205Lu co-cultured with skin fibroblasts showed similar amounts of overlap. For each type of microenvironmental cell, t-test was performed to compare the influence of 1205Lu and WM852 on overlap.