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Review

Building on the foundation of daring hypotheses: Using the MKK4 metastasis suppressor to develop models of dormancy and metastatic colonization

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ABSTRACT

The identification of a novel metastasis suppressor function for the MAP Kinase Kinase 4 protein established a role for the stress-activated kinases in regulating the growth of disseminated cancer cells. In this review, we describe MKK4's biological mechanism of action and how this information is being used to guide the development of new models to study cancer cell dormancy and metastatic colonization. Specifically, we describe the novel application of microvolume structures, which can be modified to represent characteristics similar to those that cancer cells experience at metastatic sites. Although MKK4 is currently one of many known metastasis suppressors, this field of research started with a single daring hypothesis, which revolutionized our understanding of metastasis, and opened up new areas of exploration for basic research. The combination of our increasing knowledge of metastasis suppressors and such novel technologies provide hope for possible clinical interventions to prevent suffering from the burden of metastatic disease.

1. Introduction

At the time of diagnosis, many cancer patients already have disseminated cancer cells within secondary tissues far-removed from the primary tumor [1–3]. This points to the importance of understanding how cancer cells, which may persist at sites for long periods of time, ultimately form clinically-significant metastases. Many researchers are focusing on the detection and biological significance of such disseminated cells in the clinical setting. Given the fragmentary data on the process of secondary organ colonization, many investigators are relegated to piecing together information from various sources to develop working models of how dormant cancer cells initiate growth and go onto form detectable metastases. In recent years, discoveries from the study of metastasis suppressors (i.e. genes, proteins or non-coding RNAs which specifically suppress metastasis formation) have yielded new insights into molecular and cellular mechanisms controlling the process of metastatic colonization [4–6].

Given the tremendous progress that has been made in the field of metastasis suppressor research it is humbling to consider that the birth of this area of scientific endeavor hinged on a *single daring hypothesis* put forth by Dr. Patricia Steeg, who was then a postdoctoral fellow at the National Cancer Institute [6]. Since tumorigenesis and metastasis are separable processes, she hypothesized that one could identify genes that specifically suppress metastasis formation without affecting tumor growth. Once identified such *metastasis suppressor genes* could be used to tease out processes that are specifically involved in metastasis formation [7]. Through tenacious efforts, she identified and published the first metastasis suppressor *nm23* and showed that consistent molecular events underlying metastasis *could be identified* [8]. Not only did this open the door to the molecular studies of metastasis that are commonplace today, ultimately it forced investigators to reconsider fundamental tenants of metastasis biology. For example, when efforts to find metastasis suppressors were initiated, it was expected that their utility would be in predicting disease outcome. This is because escape of cells from the primary tumor was considered to be the rate-limiting step in metastasis formation. It logically followed that metastasis suppressors would block escape of cells from the primary tumor. However, robust in vivo studies showed

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that metastasis suppressors can also control *the growth of cancer cells at secondary sites* [9–12]. Interestingly, other investigators, working on completely different questions, also identified metastatic colonization as a rate-limiting step in metastasis formation [13,14].

As evidenced by this mini-review series, there is now a rich and vibrant literature on metastasis suppressors. As such, there are many excellent scholarly reviews and publications that provide detailed descriptions of molecular, biochemical, and cellular effects and functions of specific metastasis suppressors [6,15,16]. Thus, we will take a somewhat different approach in our discussion of the identification of a novel metastasis suppressor function for the stress-activated protein kinase c-Jun NH2-terminal kinase (JNK) kinase 1/mitogen-activated protein kinase (MAPK) kinase 4 (hereafter referred to as MKK4). The following sections will present a concise overview of the discovery of MKK4's metastasis suppressor activity, studies to discern its biological mechanism of action, and new engineering approaches that may be used to develop models of cancer cell dormancy and metastatic colonization.

2. MKK4 – A stress-activated signaling kinase that is involved in growth of cancer cells at secondary sites

Our laboratory identified MKK4 as a prostate cancer metastasis suppressor in 1999 [17] and subsequently as an ovarian cancer metastasis suppressor in 2002 [18]. MKK4 is a MAP kinase within the classically defined stress-activated protein kinase (SAPK) cascade. To date, three MAP kinase modules have been well characterized: extracellular signal-regulated protein kinase (ERK), c-Jun NH2-terminal protein kinase (JNK), and p38 [19]. The JNK and p38 pathways are generally activated by stress stimuli such as cytokines, pH changes, and ionizing radiation [19]. MKK4 is a dual-specificity kinase, which in response to extracellular stimuli, can become activated and in turn can phosphorylate and activate the JNK and p38 MAPKs. This distinguishes MKK4 from MKK7, which can only phosphorylate JNK. It also differs from MKK3 and MKK6, which can only phosphorylate p38. The signaling events and biological outcomes of activation of MKK4 within the SAPK cascade are the subject of several excellent reviews [20–24]. Thus we will focus on our efforts to determine the mechanism by which MKK4 impairs metastasis formation.

Ectopic expression of MKK4 decreased the number of spontaneous AT6.1 rat prostate cancer metastases by $\geqslant 90\%$ ($P < 0.0001$) without affecting primary tumor formation [12,17]. Spontaneous metastatic ability is defined as the ability of the cells to disseminate from the primary tumor and form overt metastases at the secondary site (lung). The metastasis suppressors identified prior to MKK4 (Nm23, Kai1, Brms1, and Kiss1) were all novel proteins. In contrast, MKK4's known functions in the SAPK pathway provided us with the huge advantage of information and tools that enabled mechanism-based studies. Based upon its known biochemical function, we hypothesized that MKK4's metastasis suppressor activity would be dependent on its kinase activity. Studies using a kinase-inactive mutant showed that this is indeed the case [10,12]. At this point much of our data fit the limited knowledge of in vivo effects of metastasis suppressors and how MKK4 should function. One curious observation, however, was the presence of microscopic lesions in the lungs of animals bearing tumors, which expressed ectopic MKK4 [17,25]. This suggested that MKK4 did not block the escape of cancer cells from the primary tumor, but rather impaired the growth of cells after they had lodged in the lung (the secondary site for metastasis formation in this model system). Subsequent quantitative studies indicated that MKK4 did not affect the number of cells lodging within the secondary site [12,26,27].

Our findings, which countered the prevailing view that escape from the primary tumor was the rate-limiting step in metastasis formation, emerged at a time when data from the laboratories of Dr. Ann Chambers, Dr. Dan Welch, Dr. Lilly Ossowski, and others also suggested "growth control" at the metastatic site could be a rate-limiting step for metastasis formation [14,28–32]. As we considered these apparently contradictory data, we recalled the wisdom of Dr. Donald S. Coffey, "If two good investigators disagree and a paradox seems to exist, both of their data are probably correct, and we just need a new explanation to encompass both observations" [33]. To this end, members of our team set forth to develop daring hypotheses to address such paradoxes and or critical gaps in knowledge. The first such hypothesis was proposed and tested by Dr. Donald Vander Griend. He proposed the *loneliness hypothesis*, which compared cancer cells leaving from a primary tumor to explorers colonizing distant lands. He suggested that as lone disseminated cells or cells within small aggregates, these cancer cells would "miss the environment of their homeland (primary tumor)" and respond to physical and chemical stresses in their microenvironment. With respect to MKK4, he speculated that early in the process of colonization, the stress experienced by the relatively small number of "lonely" cells that lodged in the potentially hostile environment of the lung would activate ectopic MKK4 (which lies within the SAPK cascade) resulting in impaired metastasis formation. To test this, in vitro kinase assays were used to measure the activity of HA-tagged MKK4 protein isolated from disseminated cancer cells within the lung. The full details of this study are presented in Ref. [12]. In sum, these studies showed that HA-MKK4 immunoprecipitated from cells within the lung could phosphorylate a purified JNK substrate, while protein similarly isolated from primary tumors could not [12] (Fig. 1). These data are consistent with findings that ectopically expressed MKK4 suppresses metastasis formation without affecting 1° tumor growth.

Despite the compelling data, the possibility remained that MKK4 could be activated during the process of dissemination from the primary tumor. Szmulewitz et al. designed a clever set of experiments to rule out this possibility [27]. First, using quantitative PCR they showed that in standard spontaneous metastasis assays using AT6.1 cells, there are approximately 1×10^4 cells per lung at early timepoints during metastatic colonization. Using this baseline information they showed that ectopically expressed MKK4 suppressed metastasis formation of three highly malignant rat prostate cancer cell lines (i.e. AT6.1, AT3.1 and Mat-Lu) in experimental metastasis assays (i.e. cells injected via tail vein). Indeed, the extent and duration of metastasis suppression and increased survival of animals in these experimental metastasis assays paralleled that seen in previous work using spontaneous assays [12]. As indicated in Fig. 1, whether cells are delivered to the secondary site by the primary tumor (spontaneous metastasis assay) or delivered directly (experimental metastasis assay) MKK4 becomes activated in cells lodged within the target organ and controls subsequent steps in the colonization process.

3. MKK4-mediate suppression is due to induction of a reversible cell cycle arrest of cells lodged at secondary sites

Various studies support a role for MKK4 dysregulation in clinical disease [18,19,34–43]. In ovarian cancer, the relationship between its expression and metastasis formation has been particularly informative. MKK4 protein levels were significantly decreased in metastases as compared to normal ovarian surface epithelium [18]. Profiling studies identified high MKK4 expression as a significant predictor of improved response to surgical cytoreduction [44]. In vivo functional studies used SKOV3ip.1 human ovarian cancer cells, which form metastatic deposits of a serous

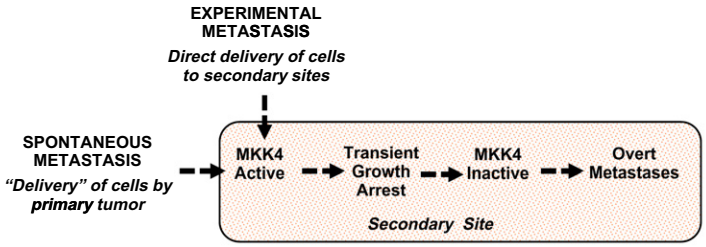

Fig. 1. Summary of MKK4's activity and biological effect during metastatic colonization.

papillary histology and produce highly reproducible numbers of metastases on the omentum, liver, and bowel [32]. SKOV3ip.1 cells have low endogenous levels of MKK4 but retain physiologic levels of other components of its signaling cascade [18]. Ectopic MKK4 decreased the number of SKOV3ip.1 metastases by 88% ($P < 0.0001$) and increased the animal lifespan by 70% (Wilcoxon, $P = 0.0045$) [18]. Its metastasis suppressor function is kinase-dependent and studies showed that selective activation of p38 reduced SKOV3ip.1 metastasis formation by 70% ($P = 0.0082$) [10]. These data further defined MKK4's metastasis suppressor activity and prompted the question – *What is the biological mechanism of MKK4-mediated metastasis suppression?*

In both spontaneous and experimental metastasis assays using prostate cancer cell lines, MKK4-mediated suppression is transient, with eventual outgrowth of MKK4-expressing cells [12,27]. Lotan et al. similarly found that found that mice injected with MKK4-expressing SKOV3ip.1 ovarian cancer cells eventually develop macroscopic metastases and succumb to their disease burden [26]. This raised an interesting question of whether bypass of metastasis suppression was due to selection of cells, which had permanent alterations in MKK4 or its signaling cascade, or alternatively, the population had undergone an adaptive process, which rendered MKK4 no longer active. To distinguish between these possibilities a mathematical analysis of the rate of accumulation of overt experimental metastases in the SKOV3ip.1 model, as well as the extent and duration of suppression by MKK4 was conducted. Biological and molecular assessments showed that outgrowth of MKK4-expressing SKOV3ip.1 cells was due to a population-wide adaptation and not permanent genetic alterations that impaired MKK4-signaling [26]. Further support for these findings was provided by the study of overt implants arising from HA-MKK4-expressing SKOV3ip.1 cells. Molecular analyses showed that these metastases continue to express HA-MKK4, indicating that no selection occurred. Additional studies showed that metastasis-derived cell lines retained biochemically functional MKK4 and were still suppressed in their ability to form overt experimental metastases when reinjected into naïve mice [26]. Subsequent work by Szmulewitz et al. showed similar results for MKK4-expressing AT6.1 prostate cancer cells. In further support of the loneliness hypothesis, using in vitro kinase assays they also showed that MKK4-purified from macroscopic metastases was not activated [27] (Fig. 1). Taken together, accumulated data from the prostate cancer and ovarian cancer model systems showed that ectopically expressed MKK4 did not affect the number of cells lodging at secondary sites, but rather caused a reversible impairment in the progressive growth of cells.

In most cases the formation, or growth, of overt metastases is the net result of both apoptosis and cell proliferation. Because of MKK4's role in the SAPK pathway and the association of this pathway with induction of apoptosis, conventional wisdom dictated that MKK4-mediated metastasis suppression was due to increased apoptosis of disseminated cancer cells. Based upon evaluation of the histopathology of the lesions, Lotan et al. put forth the daring hypothesis that MKK4 was actually affecting proliferation [26]. To test this, MKK4-expressing SKOV3ip.1 omental lesions and paired vector-only controls were assessed molecularly for apoptosis early in the time course of in vivo metastatic colonization. These data found only rare apoptotic cells in both groups. In contrast, analysis of proliferation revealed that BrdU incorporation was significantly decreased in MKK4-expressing cells as compared to vector controls (e.g. average of 6% versus 19% positive cells, $P < 0.0001$). Similar results were found using phospho-histone H3 staining. The decrease in both BrdU incorporation and phospho-histone H3-positive cells in MKK4-expressing microscopic lesions suggested that fewer cells were traversing S phase and subsequently M phase compared with controls. This finding, coupled with the fact that these cells eventually proliferate to form macroscopic implants, is consistent with a reversible cell cycle arrest (Fig. 1).

To further examine the possibility of a reversible cell cycle arrest, Lotan et al. evaluated the expression of cell cycle inhibitory proteins, potentially acting in the MKK4-expressing microscopic lesions. SKOV3 cells are known to be null for a variety of cell cycle inhibitors including p53 and p16 [26,45–47]; thus, protein expression of p21(Waf1/Cip1) was quantified in MKK4-expressing microscopic metastases. p16 immunostaining provided a negative control for this series of experiments. This approach revealed that p21 expression was increased nearly 10-fold in HA-MKK4-expressing microscopic lesions compared with vector-only controls (average of 9% versus 1%, $P < 0.0001$). Taken together with the decreased BrdU incorporation and phospho-histone H3 staining, these data were consistent with impaired cellular proliferation [26]. To further test this observation, Szmulewitz et al. dissociated lungs from mice with disseminated AT6.1 cells and used flow cytometry to analyze the cell cycle distribution of the disseminated cells. They showed that MKK4 over-expressing cells have a significant increase in G1-phase cells ($P = 0.024$), with a corresponding decrease in S-phase cells ($P = 0.036$), when compared to a vector-only expressing control [27]. Taken together, results from both the ovarian and prostate cancer model systems indicate that activation of MKK4 in disseminated cells causes reversible alterations in the cell cycle which are linked to impaired proliferation and inhibition of metastatic colonization.

4. Considering the role of the microenvironment in the tissue-specific activation of MKK4

The two key aspects of the loneliness hypothesis are the number of disseminated cancer cells and the environment of the secondary site. Our initial studies of MKK4 focused on the effect of its activation on cancer cells, but what is the specific microenvironment that the cells see? We reasoned that in order to fully understand how MKK4 functions as a metastasis suppressor, we needed to investigate the neighbors and the neighborhood in which disseminated cancer cells must survive and adapt to in order to establish themselves. To begin this effort, we chose to focus on the

ovarian cancer metastatic colonization model. In clinical ovarian cancer, as well as experimental models, the omentum is the primary site of metastasis formation [48,49]. The human omentum is a peritoneal fold composed of layers of mesothelial cells that envelope adipose tissue [50]. This highly vascularized organ is a storage site for lipids, a regulator of peritoneal fluid transit, and a reservoir for immune cells in the peritoneal cavity [50–52]. It extends from the stomach and blankets most abdominal organs, which may partly explain its susceptibility to colonization by ovarian and peritoneal cancer cells within ascites [53,54]. Despite its clinical importance, the omentum remains an understudied organ, and fundamental aspects of it structure, function, and composition have not been well-integrated into current models of ovarian cancer metastasis formation. This could be of crucial importance since dissemination of cells to secondary sites often occurs early in the process of tumor progression. Indeed Klein has proposed that metastases and primary tumors undergo parallel progression [1]. If this is true, it implies that disseminated cells evolve in response to the conditions of the microenvironment of the secondary site.

In their studies, Lotan et al. found that during metastatic colonization SKOV3ip.1 ovarian cancer cells were invariably found in association with immune aggregates within the omentum [26]. This observation prompted a rigorous evaluation of the literature. We learned that omenta from a wide variety of animals contain aggregates of immune cells which were first described by von Recklinghausen in 1863 [55] and termed milky spots by Ranvier in 1875 [55] (Fig. 2A). As indicated in Fig. 2B, the immune aggregates that define milky spots overlie dense vasculature and are surrounded by adipocytes. These structures are specialized to enable mobilization of immune cells for migration into the peritoneal cavity. Scanning electron micrographs indicate that the mesothelial layer over milky spots is discontinuous with stomata that may provide openings for transit of cells [56]. In agreement with our observations, two recent papers showed the preferential localization of cancer cells to milky spots on the omentum [53,57]. In our previous studies we have found that SKOV3ip.1 cells rapidly localize to milky spots and initially are interspersed throughout the immune cells of the milky spot (Fig. 2C). In later time points, cancer cells exist in discrete islands of cells surrounded by immune cells (Fig. 2D). The mechanisms and timing of this apparent transition

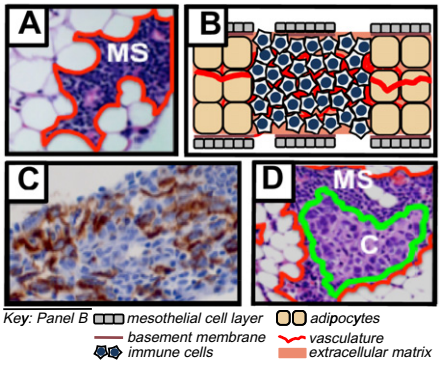

Fig. 2. Cancer cells colonize milky spots during metastatic colonization of the omentum. (A) H and E of omentum showing intense purple staining of immune aggregates referred to a milky spots (designated MS); (B) schematic of omentum showing characteristic features of a milky spot including immune cells, overlying stomata in the mesothelial layer, vasculature, and surrounding adipocytes; (C) immunohistochemical detection of cancer cells within the milky spot via cytokeratin staining (detected as discrete brown-staining cells) 6 h after intraperitoneal injection; (D) H and E staining of omentum showing the presence of discrete islands of cancer cells within milky spots within the 10 days after intraperitoneal injection. Panels (A) and (C) are reproduced from Khan et al. [55], while panel (D) is reproduced from Lotan et al. [26].

are currently under investigation. In our view, the omental metastatic colonization system seems to be amenable to studies of interactions between cancer cells and host cells during this complex process. Further, our mechanistic knowledge of how MKK4 disrupts metastatic colonization can be used to develop in vitro models that enable dissection of physical and biochemical signals important to survival and progressive growth of cells lodged at secondary sites (such as the omentum).

5. Developing models to study metastatic colonization

Identifying specific factors that regulate the biological fate of disseminated cells holds great promise for the development of adjuvant therapies to control minimal residual disease. A search of electronic databases shows that many investigators are focusing on this problem, yet much work remains to be done. For the most part, in vitro systems are designed to model cellular behaviors on a large scale. That is, large numbers of cancer cells interacting with large amounts of host tissue, cells, matrix, etc. [58–63]. Such strategies are based on the combination of the technologies generally available to cancer biologists and longstanding approaches to the study of biological problems. In some cases such approaches do yield data that can be translated back into animal models, although, based upon clinical and experimental findings often these systems do not mimic the physical constraints that lone disseminated cells (or small numbers of cells) likely experience as they colonize target tissues. This potentially has critical ramifications in understanding cancer progression within secondary tissues, as the behavior of cells can vary widely depending, for example, on their access to nutrients, diffusible signals, cell-adhesion opportunities, and other factors that depend on population size and the volume and geometry of microscopic niches. Recent technological advances are enabling the construction of microstructures that can recapitulate key attributes of microscopic niches in tissues, providing the capacity to tune the size, mass transport properties, chemistry, and elasticity of micrometer-scale enclosures capable of sequestering small numbers of cells. Our laboratories are now pursuing the daring hypothesis that such microstructures will offer unique opportunities to model the earliest steps of metastatic colonization and cancer-cell dormancy.

Connell et al. recently reported using such microstructures to study population-dependent changes in cellular behavior, probing the capacity of pathogenic bacteria to engage in quorum sensing and acquire biofilm-like antibiotic resistance [64]. Methods were developed for fabricating three-dimensional (3D) picoliter-scale microcavities out of photo-cross-linked bovine serum albumin (BSA) walls using a mask-based multiphoton lithography technique [64]. Microcavities could be fabricated with arbitrary size and shape, and typically were created with a funneled entry-way to help guide flagellated (i.e., swimming) cells from the bulk medium into the lumen of a cavity. By increasing the bath temperature from ambient to physiologic levels, the crosslinked protein walls expanded, thus causing entry-ways to pinch closed and retain cells within the trap. Once confined, cells could be tracked optically for periods of several hours. The protein-based walls enabled facile mass transport of nutrients, gases, and waste products, allowing trapped cells to divide at rates indistinguishable from those in bulk medium (a fundamental hallmark of physiologic normalcy). Initial results revealed that in populations of a few thousand cells, quorum sensing in *Pseudomonas aeruginosa* was modulated by population number and by the flow rate of external medium at constant bacterial densities. More surprisingly, trapped cells were observed to become resistant to gentamicin, a common aminoglycoside antibiotic, in populations as small as a few hundred cells. We believe these initial studies portend a much broader utility for protein-

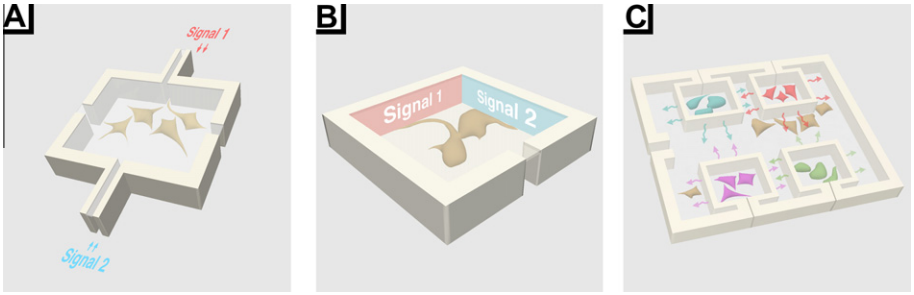

Fig. 3. Protein-based microcavities offer a versatile platform for characterizing the behavior of small cellular populations. Clusters of cells retained within microcavities can be exposed to defined chemotactic (A) and haptotactic (B) agents, providing a means to systematically evaluate cellular responses to spatially-defined, competing stimuli. Entry-ways in (A) and (B) are shown in an open state, but can be designed to close under certain environmental conditions [64]. In addition, this approach provides possibilities for evaluating the relative efficacy of diffusible signals generated by different populations of cells. For example, panel (C) shows four distinct small cellular populations, each of which release potentially different types and/or quantities of chemotactic agents. Because microcavity walls are porous to these signals, the relative ability of each population to effect clustering of a fifth cell type could be evaluated in a competitive format. For presentation purposes, microcavities in (A), (B), and (C) all are shown with thin, nearly transparent roofs.

based microstructures in studying the population-dependent behavior of many cell types, including metastatic cancer cells, in well-defined microenvironments. This notion is supported by recent reports which make strong connections between societal behaviors in bacteria and those of cancer cells [65–67]. One challenge in extending this method to non-swimming cells is microcavity loading. A number of alternate strategies may be useful for introducing mammalian cells into microcavities, including optical trapping, a method used successfully for loading immotile bacteria, microinjection, and reliance on random or guided (chemotactic or haptotactic) motility of adherent cells.

In addition to offering a potential means to isolate small numbers of cancer cells for study within microscopic cavities of defined size and shape, this technology could provide vital opportunities for manipulating attributes of protein walls to model features of tissue microenvironments. For example, movement of small molecules (such as fluorescently labeled antibiotics) across the BSA walls can be rapid, with microcavity concentrations reaching external levels within seconds (Fig. 3A) [64]. Diffusion rates of larger molecules, such as cytokines, will likely be smaller, although in preliminary studies a variety of molecules ranging up to tens of kilodaltons have been observed to efficiently permeate into photo-cross-linked BSA. As a result, it should be possible to direct key signaling molecules into the traps at defined times, allowing the impact of these species on small populations of cancer cells to be studied under well-defined conditions. In addition, it may be feasible to collect the media immediately surrounding a trap to analyze cytokines that have diffused from confined cancer cells and across trap walls. With regard to the ovarian cancer–milky spot interactions, such structures would be applied to testing the effect of specific candidate chemoattractants which may prompt the cancer cells to invade milky spot structures.

Another feature of such structures is the ability to modify the protein walls with biotinylated molecules of interest (Fig. 2B). Such functionalization can be accomplished by constructing walls from photo-cross-linked avidin, which has been shown to retain substantial biotin-binding capacity, or by photocrosslinking biotinylated BSA and decorating walls with avidin after they are fabricated [64]. Such modifications to the interior walls would allow one to observe the behavior of cells in response to specific chemical characteristics, depending on the design of the experiment. For example, if we consider the ovarian cancer metastatic colonization model, we could envision decorating the walls of the structure with candidate effector molecules and examine motility or proliferative responses of cancer cells placed within the structure. Given the composition of milky spots we suspect that such effectors could be macrophage, adipocyte, or endothelial cell derived factors. Moreover, use of mask-based multiphoton lithography enables intricate 3D structures to be rapidly designed and prototyped [68,69]. This allows experimenters, for example, to iteratively evaluate networks of chambers of differing connectivities, with or without openings to the surrounding environment. In one possible configuration (Fig. 3C), multi-chamber microcavities could be designed to house multiple types of small cellular populations, such as adipocytes, macrophages, endothelial cells, etc. where chemotactic agents released by the populations would be allowed to compete against one another. A given set of well-defined conditions specified by a researcher (e.g., cell number, cell density) could be evaluated to determine relative chemotactic efficacy. In the example shown, chemotaxis induced by red cells dominate, either as a result of greater release levels, greater efficacy per molecule, or the metabolic state of the responding cells (here, the tan cells in the larger corral).

As we are at the infancy of the research using this technology, it is important to keep in mind that the current technology must be adapted for use in building mammalian models. Thus, it is of particular importance to use well-characterized systems with detailed information on their in vivo biology to optimize these models. We believe that the MKK4 metastasis suppressor system, as well as those of other metastasis suppressors or matched metastatic and non-metastatic variants is ideally suited for model optimization. In this application, metastasis suppressors would be used to identify specific genes, molecules, or cellular interactions which are required for efficient metastatic colonization. They could also be used to test the fidelity of the model during development. For example, if the in vitro model adequately recapitulates the important features of the milky spot microenvironment, the SKOV3ip.1-MKK4 cells should undergo reversible growth arrest when placed in it.

In addition to the need for optimization of biology, there are several other challenges that we are currently addressing. The first is an issue of scale. To date, the BSA microcavities have only been used on the scale of 2–6 pl, with dimensions suitable for the growth and proliferation of up to thousands of bacterial-sized cells. Although it is relatively straightforward to increase the lateral dimensions of microcavities (initially, $\sim$20 μm), a greater challenge is extending the *z*-axis (i.e., height) of the structure to house multiple layers of cancer cells. While protein-walled microcavities are probably limited to heights of a few tens of microns when fabricated at the highest possible resolution, this likely can be extended several fold via relatively modest sacrifices in minimum feature sizes. Consequently, it should be possible to create microcavities that can accommodate a thickness of at least 10–15 cells.

6. Conclusions

Metastasis suppressors have provided new insights into fundamental aspects of the biology of metastasis. We have now entered a phase where metastasis suppressors can be used as tools for discovery and targets for therapy. With regard to the former, we propose using MKK4 to disrupt cancer cell–microenvironment interactions to determine what is necessary and sufficient for metastatic colonization of specific target organs. We also are using this knowledge to develop models to study the early steps of dormancy and metastatic colonization. We believe that the development of such models is crucial, as trying to predict accurately where a particular metastatic cancer cell will lodge and whether or not that specific cell will survive and continue to proliferate is akin to playing a game of "whack-a-mole" blindfolded on a football field. For example, when considering ovarian cancer, we know that dormant metastatic cells are likely to lodge "on the football field" of the omentum, but we do not know the structural and/or biochemical features of the microenvironment that predict where the cells will lodge and when (or if) the cells will survive and ultimately grow. The ability to design and manipulate a microenvironment to test the functional role of specific molecules on the behavior of cancer cells would be a watershed. It is hoped that by sharing the approaches that we have used to generate our published findings, as well as our more speculative ongoing efforts toward model development, we can encourage others to investigate metastatic colonization as a crucial and understudied aspect of metastasis whose time has come.

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