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Deconstructing the role of the ECM microenvironment on drug efficacy targeting MAPK signaling in a pre-clinical platform for cutaneous melanoma

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Abstract

Therapeutics targeting the BRAF kinase in cutaneous melanoma have significantly improved patient survival. However, durable responses in the face of metastatic disease are rarely realized where the problem of brain metastases is generally growing in magnitude. Tumor and stromal cells dynamically remodel the extracellular matrix (ECM) during the establishment of a metastatic lesion. We reasoned that ECM composition strongly determines drug efficacy on cell motility, adhesion and viability rendering one drug more potent and another less so. To test this hypothesis, we constructed platforms recreating the ECM composition due to the stroma and tumor cells, mimicking the brain's perivascular niche and hyaluronic acid (HA) rich parenchyma. Using human melanoma cell lines, we observed that cell adhesion was minimally affected by BRAF inhibition but ablated by ERK inhibition. Cell motility was impaired for both drugs. We determined that the composition and architecture of the ECM niche modulated drug efficacy. In one series, potency of BRAF inhibition was blunted in 3D Fibronectin-HA hydrogels whereas Laminin-HA hydrogels protected against ERK inhibition. In the other series, Laminin blunted drug efficacy, despite both series sharing the same BRAF mutation. These data reinforce the importance of contextual drug assessment in designing future therapeutics.

Introduction

Widespread metastasis accounts for the high mortality and excessive resistance to therapeutic interventions in advanced cutaneous melanoma [1–4]. Disseminated tumor cells (DTCs) leave the site of the primary tumor to initiate the metastatic cascade. Before successful colonization of a distal organ, DTCs encounter different microenvironments that may induce epigenetic changes enabling *de novo* resistance. Tumor cells, as they proliferate, remodel, attach and rebuild a new microenvironment at the distant organ by releasing extracellular signaling molecules that promote tumor angiogenesis, extracellular matrix

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(ECM) remodeling and evasion of the immune system[5]. The composition and architecture of the ECM is dynamically tuned thereby remodeling the tumor microenvironment[5]. These changes in ECM composition potentiate oncogenic effects in various signaling pathways where perturbations in ECM synthesis, degradation, density, and rigidity promote cancer cell proliferation, migration, and invasion[6]. Similarly, stromal cells at this new site often alter their phenotypes to sustain the proliferation of neighboring cancer cells[7]. These stromal cells establish a mutually beneficial relationship with cancer cells; contributing to the ECM niche to facilitate organ colonization[5]. Hence the ECM niche at the site of metastasis is modified by contributions from both tumor cells and stromal cells. Therefore, drug treatment initially effective at the primary site may be rendered impotent by the alteration of the local microenvironment of the infiltrated organ. Thus understanding how ECM composition and topography influences cancer progression may help develop new therapeutic interventions by targeting the metastatic niche.

BRAF mutations have been implicated as a crucial step in the initiation of melanocytic neoplasia[1]. Specifically, mutations where the valine has been mutated to glutamic acid (BRAF^{V600E}) are present in ~40% of patients [8]. In particular, the prognosis of melanoma patients with brain metastases is poor, with a median survival of ~3 months post-diagnosis [9]. Brain metastases are largely diagnosed post mortem at autopsy, and are asymptomatic in roughly one-third of patients before succumbing to the disease [10, 11]. Current treatment strategies involve inhibitors designed to target mutant BRAF kinase such as Vemurafenib and Dabrafenib [12–14]. These drugs result in tumor shrinkage by inducing apoptosis and senescence in melanoma cells that harbor the BRAF^{V600E} variant [13]. Patients show an initial response, but frequently relapse and acquire resistance via reactivation of the MAPK pathway. ERK inhibitors are a potential solution to overcome resistance, and are currently undergoing investigation in clinical trials. Treatment of brain metastases, however, is complicated by poor penetration of the blood-brain barrier by chemotherapeutics, and other factors [15, 16]. In addition, the microenvironment is emerging as a critical factor in malignant progression, metastasis, tumor etiology and drug efficacy [17, 18]. Currently the mechanisms underlying contextual drug resistance remain elusive.

In vitro modeling of the diverse microenvironments encountered by malignant cells is crucial to reveal contextual drug responsiveness. Preclinical models allow the flexibility of deconstructing the contributions of individual components of the tumor microenvironment that cannot be readily accomplished using mouse xenograft models. 2D culture on tissue culture plastic remains the *de facto* platform used for pharmaceutical studies. However, cells often adopt physiologically irrelevant morphology and signaling, because they do not receive the external cues that allow them to “remember” and recapitulate their *in vivo* functions [19]. A common strategy is to use laminin-rich ECM or collagen type I hydrogels to provide 3D contextual cues[20, 21]. Laminin is primarily found in basement membranes[22], however if we were to extend these studies to other microenvironments, we must phenocopy both the physical and chemical composition of the tissue of interest. For example within the brain parenchyma, the major ECM component is hyaluronic acid (HA) [12,13], a non-sulfated glycosaminoglycan, which occupies a large fraction of the extracellular volume of the brain. We reasoned that the tumor-ECM niche mediated drug

sensitivity. To address this issue, we examined pharmaceutical efficacy in biomimetic platforms recapitulating both the *in vivo* topography and composition of distinct ECM microenvironments.

Using pairs of isogenic melanoma metastatic progression series harboring the BRAF^{V600E} mutation, we examined cell adhesion, proliferation and motility in biomimetic platforms. We recreated the distinct brain ECM microenvironments found in the perivascular niche and the brain parenchyma to assess drug efficacy for current treatment strategies such as BRAF (Dabrafenib) inhibitors and ERK (VX-11E) inhibitors in an effort to dissect underlying mechanisms [23, 24]. We also examined the effect of cisplatin, a common chemotherapy as a control as melanoma has been shown to be intrinsically resistant to platinum based drugs[25].

Cells adhere to the vasculature in the perivascular niche, adopting a 2D morphology where they are aligned along a vessel wall with axial restriction. To recreate this microenvironment, we engineered a 2D substrate to control cell-ligand densities, recreating *in vivo* topography and biochemical cell-vasculature interactions. Examination of cell motility and morphology revealed that we recapitulated *in vivo* phenomena such as “cell streaming”[26], thereby providing contextual accuracy for subsequent drug studies. We determined that pharmacological inhibition of the MAPk pathway, but not the chemotherapeutic agent cisplatin, reduced cell adhesion, collective cell migration, and cellular motility.

Post-extravasation, tumor cells may encounter the Hyaluronic Acid (HA) rich brain parenchyma during colonization [27, 28]. We embedded human melanoma cells in 3D HA hydrogels. Melanoma cells secrete copious amounts of ECM proteins including Fibronectin and Laminin [29, 30]. Therefore, to simulate contributions from both the stromal cells and the tumor cells in creating the ECM niche, we created hybrid HA gels with different ECMs to examine impact on malignancy and drug potency. Varying the ECM composition of the microenvironment and cell-ECM interactions prompted the less metastatic clone to approximate the observed tumor proliferation of the more metastatic clone in 3D brain-mimetic hydrogels. In one progression series (A375), tumor aggregates were protected from BRAF inhibition when cultured in 3D Fibronectin-HA hybrid hydrogels, but against ERK inhibition when cultured in 3D Laminin-HA hybrid hydrogels. In the other series (Wm793, 1205 LU), only Laminin was generally protective, enhancing growth under most conditions despite both series sharing the targeted BRAF mutation. These data reinforce the importance of contextual drug assessment and biomimetic platforms as an aid in drug screening and pre-clinical models to ensure success at the end of the drug pipeline.

Materials and Methods

1. Maintenance of cell lines

Human melanoma cell line A375 (cat. No. CRL-1619) was obtained from American Type Culture Collection (ATCC) (Manassas, VA) designated as A375P henceforth. A375 SM (super metastatic clone of the A375) was a kind gift from Dr. Glenn Merlino of the National Cancer Institute, National Institutes of Health. Briefly, they were obtained from serially

passaging the parental clone, A375 through mice to obtain an aggressively metastatic clone. WM793 and 1205Lu cell were obtained from Meenhard Herlyn at the Wistar Institute. Briefly, WM 793 and 1205Lu are clonal, where WM 793 is the parental line for the clone, and 1205Lu is isolated from a more metastatic clone in mice[31]. A375 cells were grown as monolayers in DMEM high glucose media (Life Technologies) supplemented with final concentration of 10% Fetal Bovine Serum (FBS), L-Glutamine and Penicillin Streptomycin. WM793 and 1205Lu cells were grown in MCDB153: Leibovitz L-15 4:1 media supplemented with a final concentration of 2% Fetal Bovine Serum (FBS), 1.68mM Calcium Chloride and 5µg/ml of insulin and that was refreshed every 2–3 days.

2. Pharmaceutical treatment

Pharmacological inhibitors, VX-11E (ERK inhibitor) and GSK2118436A (Dabrafenib) were obtained from Chemietek and dissolved in DMSO (Sigma-Aldrich) to a stock concentration of 10 mM. Cisplatin (aqueous) was a kind gift from Matthew Hall, LCB, NCI. Cells were incubated with media at final concentrations of 3µM and 30 µM for 24hrs and 4hrs respectively to determine ERK activity. Inhibitors were used alone and in combination.

3. Silanization of glass slides

Glass slides were submerged in 1 M KOH and sonicated for 20 minutes and then washed with ddH₂O 10 times, followed by washing with methanol twice, and submerged in a solution consisting of: 75 parts methanol, 1.5 parts aminosilane, and 3.75 parts acetic acid. These slides were incubated in the aminosilane solution for 10 minutes, sonicated for 1 minute, and incubated in the aminosilane for another 10 minutes. They were then washed twice with methanol, and then washed 10 times with ddH₂O. The slides were then air-dried using pressurized nitrogen to prevent spotting. After drying, the slides were placed in containers with a water reservoir to reduce evaporation for PEGylation.

4. PEGylation of glass slides

88 mg of PEG and 2 mg of bio-PEG was mixed with 360 µL of 100 mM sodium bicarbonate. This solution was vortexed, then centrifuged at 7200 rcf for 1 minute to remove any particulates. 80 µL of the solution was placed between 2 silanized slides to form a sandwich (without air bubbles) and incubated in containers (with water reservoirs) for 3 hours at room temp. The resultant PEGylated slide was washed extensively using ddH₂O and air-dried. The slides were stored at –20 C for up to two weeks, PEGylated side up.

5. Preparation of RGD Binding sites

After PEGylating, outlines of circles were created using a permanent marker on the non-PEGylated side to delineate attachment spots. Within these spots, 300ug/mL of neutravidin was added (PEGylated side) for an incubation time of 5 minutes. The excess neutravidin was then washed off with PBS three times (the slide should dry completely due to the hydrophobicity of the PEG), and then incubated with biotinylated-RGD spots at various concentrations (0.00089 µM (~10⁻⁵fmole/cm²) to 89 µM (~1fmole/cm²)). After a 5-minute incubation, the excess RGD was rinsed with copious amounts of PBS. Areas of biotinylated fluorescein and tetramethyl rhodamine labeled streptavidin were also prepared as a control

for cell-adhesion. The modified slide surface was enclosed with a plastic well and sealed with nail polish. This was allowed to dry at room temperature before use to minimize leaking.

6. Cell attachment

Cells that had been starved for a minimum of 8 hours (by removing FBS from media) were detached using 5 mM EDTA in PBS (Trypsin will digest extracellular receptors). They were then washed with serum free DMEM, centrifuged and re-suspended at a concentration of 450,000 cells per mL in serum free media. This cell suspension was placed in each well (12 spots per well, 450,000 cells per well) and the wells were incubated at 37C, 5% CO₂ for 4 hours either with media with DMSO control (0.01% v/v) or supplemented with pharmacological inhibitors. After 4 hours of incubation, unattached cells were gently aspirated and wells replenished with fresh serum free media.

7. 3D Cell Culture

A375P and SM cells were seeded at a density of 500,000 per mL of 3D Hyaluronic acid hydrogel following manufacturer's protocol (Hystem Hyaluronic Acid kit (GS1004, ESI-BIO). It was then supplemented with either: 30 µg of fluorescently labeled Laminin (LMN02, Cytoskeleton, Inc) or fluorescently labeled Fibronectin (FNR01, Cytoskeleton, Inc); or a cyclic-RGD (PCI-3696-PI, Peptides International) at 1.2 or 12 µM. 200 µL of each mix was distributed per well into 8-well glass-bottomed dishes (155411, Lab-Tek) and allowed to polymerize for 30 minutes in a 37C, 5% CO₂ incubator before media was added to each well. RGD seeded wells were further supplemented with soluble RGD- media at the same concentration as was seeded in the 3D matrix. After three days of growth, cells were imaged and then new media was added with no treatment (.1% DMSO control), 30 µM Dabrafenib, or 30 µM VX11e. Media was exchanged after 2 days, and the cells were imaged after 3 days of treatment using a live/dead viability cell toxicity kit (L3224, Life Technologies).

8. Imaging and Analysis

Bright field and fluorescent images were obtained on a Zeiss Axiovert 200 with an AxioCam MRm, using AxioVision LE 4.8.2 software. Images were analyzed using ImageJ to determine cell counts. Find Edges and Adjust Threshold macros were run prior to the Analyze Particles macro, used to measure cell #, cell surface coverage, and cell size. High-resolution images of fixed cells and cellular motility were obtained using a Zeiss 780 confocal, using a pinhole of 1 Airy Unit. Immunofluorescence was performed as described before, using 546 Phalloidin (actin), anti-Vinculin FITC (focal adhesions), and Hoechst (nuclei) respectively [32]. Sequential line scans were collected in three different filter bands, 415–500, 500–553, and 562–634 nm. Confocal images were processed using the despeckle command in ImageJ to remove noise. Significance was determined by 1-way ANOVA, comparing within conditions to control. Cellular motility was tracked by the Manual Tracking macro in Fiji.

9. Protein Analysis

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Thermo SCIENTIFIC, Rockford, IL, USA), 1X protease inhibitor cocktail (Calbiochem, Darmstadt, Germany) and 1X phosphatase inhibitor cocktail 2 & 3 (Sigma-Aldrich, St. Louis, MO, USA), sonicated for 10 seconds, followed by centrifugation (13,200 rpm, 4°C, 5 min), after which the supernatants were stored at -80°C until use. Protein concentrations were determined with the Pierce® BCA Protein Assay kit (Thermo SCIENTIFIC), using BSA as the concentration standard. Extracted proteins were then resolved using 4–12% Bis-Tris Gels (Life Technologies, Carlsbad, CA, USA) and subsequently transferred to PVDF membranes (Life Technologies). Membranes were washed and blocked with 1% BSA in TBS (20mM Tris-HCl, 150mM NaCl, pH 7.4) containing 0.1% Tween 20 (TBS-T) before incubation with primary antibodies. Next, they were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG or sheep anti-mouse IgG (GE Healthcare). Finally, the signals were visualized by means of an enhanced chemiluminescence system (Invitrogen, Carlsbad, CA, USA). The primary antibodies were used at the following dilutions: anti-phospho-p44/42MAPK (1:1,000), anti-p44/42MAPK (1:1,000), and anti-GAPDH (1:2,000) (Cell Signaling Technology, Danvers, MA) antibodies. Anti-GAPDH antibody was used as a loading control.

3. Results

3.1 A biomimetic platform recapitulates in vivo cell-vasculature interactions for contextual drug therapy

In murine models using intracardiac and orthotopic injections, melanoma brain-tropic cell lines align along the outside surface of a blood vessel [10, 33]. Tumor cells show an elongated morphology adhering to the vascular basement membrane via $\alpha_5\beta_1$ integrins and subsequently proliferate and invade. To recreate cell-vessel interactions, the surface of each slide was modified to adjust cell adhesion properties by systematically tuning ligand density (Figure 1A). We minimized non-specific binding by passivating the glass substrate through PEGylation. We could then independently control the adhesion by tuning the concentration of Arg-Gly-Asp (RGD) ligand density. Short RGD peptides act as surrogates for integrin adhesion ligands to mimic attachment to the vascular basement membrane [34]. The RGD peptide sequence is found naturally in ECM proteins including fibronectin, osteopontin and fibrinogen [35, 36]. By tuning the concentration, from 8.9 nM ($\sim 10^{-5}$ fmol/cm²) to 89 μ M (~ 1 fmol/cm²), we can interrogate weakly to tightly bound cells. Surface density of peptide is concentration dependent. We then confirmed that the peptide uniformly coated the surface, by examining fluorescence intensity of biotinylated fluorescein attached to the PEGylated surface (Figure 1A inset).

Then, we asked if our biomimetic platform recreated the *in vivo* morphology of melanoma cells as observed in murine models [10, 33]. We examined human cell lines that harbor BRAF^{V600E} (A375P and SM, WM793 and 1205Lu). Morphological comparison of the cells that lie within and adhere to a blood vessel and capillary show an elongated shape similar to that observed for cells seeded in the biomimetic platform (Figure 1B). Furthermore, when these same cells are cultured on plastic or glass, they show broad lamellipodia, in contrast to

when these cells are cultured on the RGD platform, where they attain a more spindle-like morphology after ~8 hrs. in culture (Figure 1B). On glass, cells were randomly motile with little displacement, displaying lamellipodia at the leading edge of movement (Supplemental Movie 1). They also tended to form largely immobile clusters. On RGD surfaces, behavior is significantly altered, with high levels of motility and collective migration of streaming cells over several micrometers observed for both melanoma clones (Supplemental Movie 2). This phenomenon is reminiscent of migrating melanoma [37], where long chains of cells span hundreds of micrometers (Supplemental movies 1 and 2).

3.2 The effect of pharmacological inhibition on cell adhesion as a function of metastatic potential

Having established that melanoma cells cultured on RGD surfaces show similar phenotypes to those observed *in vivo*, we then treated isogenic cell lines recapitulating metastatic progression with clinically relevant pharmaceutical inhibitors to determine drug efficacy in ablating cell adhesion to the vasculature. Dabrafenib (a BRAF inhibitor) has been shown to be potentially effective against metastatic disease especially in the brain [38]. However, patients frequently acquire drug resistance through the reactivation of the MAPk signaling pathway[39]. Thus, we also used VX-11E, which acts as a small molecule inhibitor against ERK1/2. In our platform, we examined drug interactions on an acute temporal scale. In order to delineate cell-adhesion effects where the environment wasn't significantly remodeled, we restricted our drug assays to four hours, long enough to detect changes in adhesion and kinase activity, but shorter than the time necessary for nascent extracellular matrix (ECM) proteins to be synthesized, secreted and assembled. Hence, alterations of the microenvironment were negligible. To compare acute treatment to longer-term treatment, we probed the activity of extracellular-signal-regulated kinases (ERK1/2) in response to treatment with Dabrafenib (BRAF inhibition) and VX11e (ERK inhibition) (Supplement Figure 1A). ERK1/2 is a downstream member of the signaling cascade regulated by the RAF kinases. We determined that ERK1/2 activity is comparable when we give an acute treatment of a high dosage (30 μ M) or long-term exposure to a lower dosage (3 μ M), which corresponds to the half maximal inhibitory concentration-IC₅₀ (Afasisheva et al Submitted) (Supplement Figure 1A). Thus we used this concentration for our acute studies.

As a secondary control, we treated cells with Cisplatin, a chemotherapy DNA cross-linking agent as melanoma is intrinsically resistant to platinum therapies [40]. Unsurprisingly, we observed that Cisplatin had a negligible effect on cell adhesion. Direct targeting of ERK activity showed a uniform decrease in adhesion for the full range of ligand densities in all cell lines (Figure 2A). We then tested the effect of BRAF inhibition on cell adhesion using our assay. We observed that in one isogenic metastatic pairing, A375, cell adhesion to the RGD surface was comparably decreased only in cases where Vx-11e was in combination with Dabrafenib (BRAF-inhibition) independent of metastatic potential. In contrast, Dabrafenib alone-reduced cell adhesion for the parental, WM793 cell line but against the metastatic clone, 1205Lu, was less effective for higher concentrations of RGD peptide (Figure 2A). Immunofluorescence revealed morphological changes due to pharmaceutical treatment, where cells showed altered cytoskeleton and focal adhesions. ERK inhibition diminished formation of focal adhesions as measured by vinculin punctates and actin stress

fibers, leaving cells with a more rounded, smaller area (Figure 2B). Vx-11e uniformly ablated vinculin punctuates for all cell lines for all concentrations of RGD.

Efficient metastasis is mediated by both cell adhesion and migration[6]. We next examined drug efficacy as a function of RGD ligand density on cell motility. Both A375 clones move at comparable velocities independent of substrate (glass vs. RGD) without treatment (Fig 3A–C). Net distance traveled by the A375SM cells indicated that they exhibited more directed motility. BRAF and ERK inhibitors reduced motility in A375P, A375SM, and WM793 cells. However A375SM cells had similar levels of motility under treatment as untreated A375P cells (Fig 3A–C). In general, the WM793 and 1205Lu were less motile than the A375 series, where 1205Lu remained largely immobile under any treatment condition. Note that even under treatment, cells were still as or more motile on RGD than on untreated glass.

3.3 Effects of modulating the tumor ECM microenvironment on tumor growth in a 3D HA hydrogel

During colonization of the brain parenchyma, tumor cells encounter a HA rich environment [33], which contains varying amounts of Fibronectin and Laminin [41]. However, there are other sources of these ECM proteins. In particular, the tumor cells also secrete copious amounts of ECM proteins, such as Fibronectin and laminin (Supplemental Figure 1B) that contribute to the tumor ECM niche[29, 30]. Therefore, we examined tumor growth in an HA-rich 3D matrix seeded with Fibronectin, Laminin or RGD ligand at differing concentrations to determine the effect of cell-microenvironment interactions on cell proliferation. Initial growth after 3 days was minimally affected by added ECM (Supplemental Figure 2). However, after another 3 days of growth, the number of clusters and cluster sizes of the more metastatic A375SM clone were significantly larger than those of the parental A375P clone although growth was largely independent of supplemented ECM or ligand density (Figure 4, Supplemental Figure 3). Interestingly, the cluster sizes of A375P cells cultured in the 3D ECM-HA composite hydrogels approached the size of A375SM clusters with the addition of RGD peptide (Supplemental Figure 3). In particular, direct visualization of clusters show that A375 cells incorporate the fluorescently labeled Fibronectin, but not Laminin, in punctae at the cell periphery in A375SM cells, more efficiently than the A375P cells (data not shown). Conversely, Laminin showed a more potent effect on the WM793 and 1205Lu cell lines where cells formed larger clusters in 3D laminin HA hydrogels (Figure 4).

3.4 Drug efficacy as a function of microenvironmental changes via modulation of ECM composition in a brain mimetic HA matrix

We then interrogated drug efficacy as a function of microenvironmental changes. We used two metrics, the effect of drug treatment on cluster size and cluster number, to dissect efficacy in shrinking the tumor (cluster size) vs. eliminating the tumor entirely (# of clusters). The addition of RGD was marginally effective in the cells tested except in the case of the less metastatic clone, A375P, where we observed increased cluster growth (Figure 4 and Supplemental Figure 3). Both Fibronectin rich and laminin rich HA-gel enhanced proliferation compared to bare HA gels.

Generally, Laminin increased growth as determined by cluster number for both WM793 and 1205Lu independent of drug treatment (Figure 4). BRAF inhibition was generally ineffective in reducing cluster area (tumor shrinkage) for all cell lines except A375SM (Figures 5–6). However, A375SM were larger to begin with and drug treatment reduced cluster size to that observed in the parental A375P clone. For the A375 series, ERK inhibition universally reduced cluster size (tumor shrinkage) as a function of ECM composition. However, A375P clusters were mildly protected when embedded in the laminin rich HA gel (Figures 4). Live/Dead analysis revealed that VX11e has a much stronger effect on tumor clusters, generally affecting each cluster by inducing cell death throughout the cluster (Figures 5–6). Dabrafenib is less potent, with entire clusters remaining unaffected and cell death only occurring in isolated cells at the cluster periphery (Figures 5–6). These data suggest the complexity inherent to determining drug efficacy in distinct microenvironments.

Discussion

The mechanisms that govern contextual drug efficacy in the diverse microenvironments encountered by brain metastases are underexplored. In vitro preclinical models allow us to recreate in vivo complexity while maintaining the ability to delineate contributions from individual components. Here, we evaluated cells cultured on a 2D platform utilizing short RGD peptides to replicate the tumor-vasculature interface where we independently modulated adhesion strength. We also modeled the contributions of the tumor and stromal cells to building the ECM niche within in 3D Hyaluronic Acid (HA) hydrogel, one of the major ECM components found in the brain parenchyma. Using 2 pairs of isogenic, human models of metastatic progression harboring the BRAF^{V600E} mutation, we probed the potency of current pharmaceutical treatments. The physical and chemical signature of the microenvironment composition strongly determined the pharmaceutical induced changes on cell motility and cell adhesion, rendering one drug more potent and another less so. Disturbingly, examination of tumor clusters cultured in 3D HA-ECM hybrid hydrogels showed that they were largely insensitive to Dabrafenib (Figure 6). These data imply the tumor-ECM niche is a contributor to the potency of pharmaceutical treatments. Therefore ECM composition in addition to the mutational status must be considered when designing future drug therapies.

Tumor-brain vasculature interactions mediate the establishment of metastases, as the vasculature basement membrane acts as a substrate, facilitating adhesion and remodeling for invasion into the brain parenchyma [10, 16, 33, 40]. PEG surfaces have been extensively studied in the past [42, 43], along with binding to RGD motifs on polymers [35, 44, 45]. It has been shown that RGD stimulates adhesion when bound to a surface, inhibits adhesion when in solution, and alters cell morphology [36]. The RGD motif recognizes a myriad of integrin adhesion receptors that facilitate cross talk between cells and the ECM[34]. Previously, maximum cell spreading was observed on RGD coated surfaces corresponding to a surface concentration of 1fmol/cm² [46]. We observe similar modulation in cell adhesion but in our system, cells adopted spindle shapes and migrated as streaming sheets[26] on the completely passivated surface, illustrating a possible mode of invasion during early colonization. A recent study examined the morphology and drug efficacy when

melanoma cells were cultured on 2D PEG hydrogels of varying stiffness [47]. This study showed that a less metastatic clone underwent increased apoptosis when cultured on softer substrates. Acute ERK inhibition decreased adhesion independently of RGD adhesion strength, whereas BRAF inhibition showed negligible effects in one isogenic set, decreasing cell adhesion only when combined with an ERK inhibitor. However, BRAF inhibition reduced cell motility, notably disrupting collective cell streaming over longer periods of time (A375, Fig 3A–C). These data may be explained by the fact that cell adhesion and 2D cell migration are differentially affected by the MAPK signaling pathway [48–50]. MAP kinase-induced MLCK activity directly affects cell migration machinery with no effect on cell adhesion. These data suggest that Dabrafenib may modestly affect brain metastatic lesions by potentially reducing tumor migration [13]. They also reinforce that combined targeting of the same and different signaling pathways are needed to overcome drug resistance in metastatic melanoma [51].

Melanoma cells are able to infiltrate brain parenchyma and form lesions[52]. Hyaluronic acid is one of the most abundant ECM proteins found in the adult brain parenchyma, which is largely devoid of Laminin and Fibronectin, as they are typically restricted to near the vasculature [53–55]. However, tumor cells are able to modulate their microenvironment by secreting and assembling endogenous ECM proteins such as fibronectin and laminin[30, 56] therefore contributing to the tumor-organ ECM niche. Architecture has been shown to be important for determining drug responses when cells are cultured in 3D compared to tissue culture plastic [57, 58]. We simulated this complexity by utilizing 3D ECM-HA hybrid hydrogels to more closely replicate how tumors remodel their local microenvironment. Previous studies have utilized HA-based hydrogels to assess the effect of stiffness and signaling on stem cell differentiation, brain tissue engineering and tumor proliferation and invasion for cases of glioblastoma multiforme [59–62]. Here, 3D architecture mitigated drug efficacy, where only a partial killing was achieved despite using concentrations at IC 50 values higher than that calculated using 2D proliferation assays. We find that altered ECM composition and RGD ligand density of the 3D HA hybrid gels regulated tumor growth, with the cluster size of the less metastatic A375P clone in 12 μ M RGD approaching that of the more metastatic clone. Also, cells are protected against ERK inhibition when cultured in Laminin-rich gel, and are resistant to BRAF inhibition when cultured in Fibronectin rich HA-gel. Conversely, Laminin rich HA-gels offered no protection against BRAF inhibitor, but increased the number of viable clusters for both WM793 and 1205Lu cell lines. As Dabrafenib and VX1le are approximately the same molecular weight (~500 Da), differential efficacy cannot be explained by diffusion-mediated drug uptake. These discrepancies can be due to the origin of the cell lines where the A375 SM were repeatedly passage through mice to obtain the most aggressive clone that colonized several organs whereas the 1205 Lu was obtained from a lung metastatic lesion in the mouse. However, these results highlight the complexity of designing effective therapies despite the fact that these cell lines harbor the same mutant kinase. The cross talk between extracellular matrix (ECM) and integrins regulate gene expression associated with cell growth, differentiation and survival via a reciprocal inside-out bi-directional signaling[6]. In vitro studies have shown the addition of laminin mediates drug resistance in small cell lung cancer cells[63]. Similarly, studies show that non-small cell lung cancer cells induced FN biogenesis in response to treatment with

Cetuximab (targeting the EGF receptor) [64]. This response was determined to blunt the cytotoxic effects of Cetuximab and reduce sensitivity to radiotherapy for *in vitro* and *in vivo* murine models. A dynamic tumor microenvironment may not only contribute to systemic metastasis, but also significantly modify drug efficacy and these data may explain why metastatic lesions that overexpress ECM proteins are resistant to drug therapy [18, 65].

The recent expansion of small-molecule inhibitors of the MAP kinase pathway has led to many possible combinatorial treatments that necessitate dissecting how inhibition of different parts of the MAPK pathway affects the various steps of the metastatic cascade. MAP kinases regulate a huge array of cellular functions, including proliferation, differentiation, and apoptosis. The two inhibitors studied here have differential effects on cell-RGD adhesion and motility, and growth in HA matrix, indicating that combinatorial treatment with Dabrafenib and VX11e may exert synergistic effects on the inhibition of melanoma metastasis.

5. Conclusion

Here, using biomimetic platforms capable of reconstituting the tumor-vasculature interface, and combinatorial ECM niches, we determined that the composition and architecture of the tumor-ECM niche directly affected drug efficacy rendering one drug more potent and another less so. This platform can be tailored for any type of cancer that metastasizes to the brain to study drug interactions. The ability to interrogate contextual drug efficacy using *in vitro* platforms provides an attractive intermediate step towards the development of successful cancer therapies. Future studies incorporating additional drugs and ECM motifs may yield additional insight on designing effective therapeutic strategies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cutaneous melanoma cells show elongated spindle morphology when cultured on a 2D biomimetic platform of the tumor-vasculature interface

A) Analysis showed increasing surface coverage as a function of concentration as displayed in graph of fluorescence intensity vs. biotin-fluorescein concentration from the lowest concentration-zero to 89 μM ($\sim 1 \text{ fmole}/\text{cm}^2$). Inset shows uniform surface coverage as measured by fluorescence intensity, 89 μM ($\sim 1 \text{ fmole}/\text{cm}^2$). B) 2D morphology of cells grown on RGD adopt a more elongated, spindle shape whereas cells grown on glass show a round shape as indicated by aspect ratio analysis where round ~ 1 and elongated $\sim >2$.

Figure 2. BRAF and ERK inhibition differentially affects cell adhesion in the 2D platform
 Only ERK inhibition uniformly disrupts cell adhesion in 2 different pairs of isogenic human metastatic progression series harboring the BRAF^{V600E} mutation. (A) Graphs showing cell adhesion as quantified by percentage area covered as a function RGD concentration plotted on log scale, under acute treatment (~ 4 hrs) with Dabrafenib (BRAF-i), Vx-11e (ERK-i), combination of Dabrafenib (BRAF-i) and Vx-11e (ERK-i), Cisplatin, and DMSO control for 2 pairs of isogenic BRAF^{V600E} metastatic progression series (A375P, N=5, and the more metastatic clone A375SM, N=7, Wm793 N=4, 1205Lu, N=4, where N are independent experiments). Significance was determined by 1-way ANOVA, comparing within conditions

to control where * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates a $p < 0.0001$. (B) Representative micrographs show immunofluorescence of actin (cytoskeletal organization, red), vinculin (focal adhesions, green) and nuclei (DNA, blue) for cells adhered to lowest concentration of RGD ($0.089 \mu\text{M}$ ($\sim 10^{-3} \text{fmole/cm}^2$), adhered to maximum concentration ($89 \mu\text{M}$ ($\sim 1 \text{fmole/cm}^2$) and treated with Vx-11e (ERK-i). Note, cell adhesion was completely obliterated for WM793 cells when cultured on the highest concentration at concentrations used

Figure 3. BRAF and ERK inhibition significantly reduce motility on 2D biomimetic platform for both series

Graphs showing the (A) total distance traveled in 12 hours, (B) average velocity, and (C) average step size for cells adhered to glass compared to those adhered to RGD peptides, with drug treatment and vehicle control. A375SM cells show significantly higher motility compared to A375P cells. Note that after drug treatment, observed motility of the more metastatic clone, A375SM is comparable to what is observed on glass for the A375P cells. In contrast, WM793 and 1205Lu cells both show lower levels of motility where a significant increase in motility is only observed for WM793 cells cultured on RGD. N> 30 cells for

each condition. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates a $p < 0.0001$, compared using ANOVA where RGD is compared to glass, and both drug treatments are compared to RGD.

Figure 4. The effects of added ECM in 3D HA hydrogel on drug efficacy: tumor growth (cluster size) and apoptosis (# of clusters)

Cells were grown for 3 days in 3D HA with no RGD, 1.2 μM, 12 μM RGD, Fibronectin, or Laminin and then were treated for 3 days with 30 μM Dabrafenib (BRAF-i), VX11e (ERK-i) or 1 % DMSO control. Cells were then stained live (green, cytoplasm)/dead (red, DNA), and cluster size and cluster number were measured, to determine the effect of the addition of soluble RGD or ECM proteins. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates a $p < 0.0001$, compared using ANOVA. Only A375P and WM793 had significant effects from RGD treatment. 2 independent experiments for each cell line, 4 independent fields of view per experiment, N values of >500 cells.

Figure 5. ERK inhibition significantly increases the number of dead cells in clusters where clusters are largely insensitive to BRAF inhibition in the A375 isogenic series in 3D hybrid-HA gels

Drug treatment's effect on the A) A375P and B) A375SM cell lines was examined in differing microenvironmental conditions lines show that for BRAF-i clusters are largely insensitive to apoptosis where ERK-i show a significant increase in the number of clusters where dead cells are present as determined by cluster number. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates a $p < 0.0001$, compared using ANOVA). 2 independent experiments for each cell line, 4 fields of view per experiment N values of > 500 cells. C) Representative micrographs of live/dead cell assay for both cell lines show that for BRAF-i clusters are largely insensitive to apoptosis where ERK-i show a significant increase in clusters where dead cells are present.

Figure 6. Drug treatment has negligible effects on WM793 and 1205Lu cell series in HA gels
Drug treatment's effect on the A) WM793-B) 1205Lu cell series was examined in differing microenvironmental conditions. As these cells were harvested from a lung tropic tumor, it is possible they are quiescent in brain-like ECM, and their lack of growth rendered them unresponsive to treatment. Cluster number and cluster size were measured. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, **** indicates a $p < 0.0001$, compared using ANOVA). 2 independent experiments for each cell line, 4 fields of view per experiment. C) Representative micrographs of live/dead cell assay for both cell lines show that for BRAF-i clusters are largely insensitive to apoptosis where ERK-i show a significant increase in clusters where dead cells are present.