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Regulation of melanoma cell survival and function by matricellular signaling and microenvironmental factors

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Regulation of melanoma cell survival and function by matricellular signaling and microenvironmental factors

by

Emi Yuriko Tokuda

B.S., University of Washington, 2009

A thesis submitted to the
Faculty of the Graduate School of the
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of the requirements for the degree of
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Department of Chemical and Biological Engineering

2015
This thesis entitled:
Regulation of melanoma cell survival and function by matricellular signaling and
microenvironmental factors
written by Emi Yuriko Tokuda
has been approved for the Department of Chemical and Biological Engineering

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Date ______________________

The final copy of this thesis has been examined by the signatories, and we find that both the
content and the form meet acceptable presentation standards of scholarly work in the above
mentioned discipline.
Metastatic melanoma is an aggressive, drug resistant form of skin cancer. Despite the recent development of several new therapeutics, patients typically relapse within 6 months of starting treatment. Consequently, researchers are working to understand the mechanisms of melanoma drug resistance and the key factors that impact its survival. Because cell-ECM (extracellular matrix) interactions alter outside-in signaling and relay information about the surrounding tissue to a cell, the cellular microenvironment can regulate cell function and this, in turn, may influence melanoma responsiveness to pharmacological inhibition. Preclinical studies have shown differential drug efficacy between traditional tissue culture-treated polystyrene (TCPS) and multicellular spheroids embedded within a soft collagen matrix. While researchers attribute more drug resistant behavior to three-dimensional (3D) cell culture, it is unclear whether interactions with soft (or stiff) matrices can promote this response, if dimensionality (2D versus 3D) of the culture environment matters, or whether the existence of cell-cell contacts is paramount to drug responsiveness.

This thesis research aimed to use hydrogel systems as synthetic ECM mimics to systematically study the influence of matrix elasticity and dimensionality on the behavior of melanoma cells at different stages of disease progression in response to the clinically relevant drug, Zelboraf (PLX4032, vemurafenib). Peptide-functionalized poly(ethylene glycol) (PEG) hydrogels provided control over bulk properties while probing specific questions regarding the local cell-ECM microenvironment and interactions. Human cell lines derived from radial growth phase and metastatic melanoma were cultured on hydrogel surfaces or encapsulated within synthetic ECM mimics with tunable elasticity and biological functionality (e.g., integrin binding epitopes, protease degradability). First, the role of matrix rigidity on PLX4032 sensitivity was assessed on 2D hydrogels
of varying moduli. The influence of dimensionality and cell-cell contacts on melanoma drug responsiveness to PLX4032 in 2D and 3D culture was measured. Finally, cells encapsulated within hydrogels were evaluated for both proteolytic activity in response to PLX4032 and the functional impact of this enzymatic activity. This thesis research demonstrated the importance of the tumor microenvironment in regulating PLX4032 responses and its implications for preclinical studies and clinical treatment.
Dedication

To my awesome family.
Acknowledgements

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Contents

Chapter

1 Introduction

1.1 Melanoma overview .............................................................. 1

1.1.1 Current clinical strategies and new inhibitors ...................... 2

1.1.2 In vitro studies of melanoma drug responsiveness .............. 4

1.2 Melanoma survival and drug resistance .................................. 5

1.2.1 Signaling pathways important to melanoma survival ........... 6

1.2.2 Intrinsic and acquired drug resistance ............................... 8

1.2.3 Cancer stem cells in melanoma tumor biology .................. 11

1.3 Strategies for in vitro studies of cancer cells ......................... 12

1.3.1 Hydrogels as 3D cell culture platforms to study cancer cells .... 15

1.3.2 PEG-peptide thiol-ene hydrogels ................................. 18

1.3.3 Matrix factors known to influence cancer cell signaling and function ... 20

1.4 Approach of this thesis ......................................................... 24

2 Objectives

3 Modulation of matrix elasticity with PEG hydrogels to study melanoma drug responsiveness

3.1 Abstract ................................................................. 30

3.2 Introduction .............................................................. 31
3.3 Materials and Methods ...................................................... 33
   3.3.1 Reagents ................................................................. 33
   3.3.2 Synthesis and characterization of macromers and peptides .......... 33
   3.3.3 Formation and characterization of PEG-norbornene hydrogels ........ 34
   3.3.4 Cell Culture ............................................................ 35
   3.3.5 Metabolic Activity .................................................... 36
   3.3.6 Apoptosis .............................................................. 36
   3.3.7 Proliferation ........................................................... 36
   3.3.8 Immunostaining ....................................................... 37
   3.3.9 Focal adhesion quantification ....................................... 37
   3.3.10 Statistical analysis .................................................. 38
3.4 Results ........................................................................ 38
   3.4.1 Regulation of cell-matrix adhesion by substrate elasticity .......... 38
   3.4.2 Effect of substrate elasticity on cell responsiveness to drug treatment ... 41
   3.4.3 Decoupling cytostatic and cytotoxic effects of drug treatment .......... 43
3.5 Discussion ................................................................. 45
3.6 Conclusions ............................................................... 49
3.7 Acknowledgements ....................................................... 50
3.8 Supplemental Information .................................................. 51

4 PEG-peptide hydrogels reveal differential effects of matrix microenvironmental cues on
melanoma drug sensitivity .................................................. 53
   4.1 Abstract .................................................................. 53
   4.2 Insight, Innovation, and Integration ...................................... 54
   4.3 Introduction .............................................................. 54
   4.4 Methods ................................................................. 56
      4.4.1 Reagents and Materials ............................................. 56
4.4.2 Cell culture .............................................................. 56
4.4.3 Hydrogel preparation for 2D and single cell encapsulations .......... 56
4.4.4 Spheroid formation and encapsulation .................................. 57
4.4.5 Metabolic activity, apoptosis, and DNA content ...................... 59
4.4.6 Statistical analysis .................................................... 59
4.5 Results ........................................................................... 60
4.5.1 Morphology and metabolic activity of 2D versus 3D cell culture ...... 60
4.5.2 Apoptosis as a function of inhibitor treatment in 2D versus 3D ...... 61
4.5.3 Single cells versus spheroids .......................................... 64
4.5.4 Drug responsiveness of single cells and spheroids ..................... 66
4.6 Discussion ..................................................................... 67
4.7 Conclusions .................................................................... 73
4.8 Acknowledgements .......................................................... 74
4.9 Supplemental Information ................................................... 75

5 Multifunctional bioscaffolds for 3D culture of melanoma cells reveal increased MMP activity and migration with BRAF kinase inhibition
5.1 Abstract ................................................................. 78
5.2 Significance Statement ..................................................... 79
5.3 Introduction ................................................................. 79
5.4 Results ........................................................................... 81
5.4.1 In situ screening of MMP activity and viability in three dimensions 81
5.4.2 Increased MMP activity correlates with changes in cell morphology and migration ......................................................... 86
5.5 Discussion ..................................................................... 89
5.6 Conclusions ................................................................. 94
5.7 Materials and Methods ..................................................... 94
5.8 Acknowledgements ........................................... 94
5.9 Supplemental Information ......................................... 95
5.10 SI Materials and Methods ........................................... 95
  5.10.1 Materials .................................................. 95
  5.10.2 Cell culture ............................................... 95
  5.10.3 Cell encapsulation ....................................... 95
  5.10.4 Metabolic activity ........................................ 96
  5.10.5 MMP activity ........................................... 96
  5.10.6 Zymography ............................................. 96
  5.10.7 Immunostaining ......................................... 97
  5.10.8 Migration ............................................... 97
  5.10.9 Data analysis and statistics .............................. 98
5.11 SI Figures ...................................................... 98

6 Conclusions and Recommendations .......................... 103

Bibliography ......................................................... 114

7 Appendix .......................................................... 134
  7.1 Introduction .................................................. 134
  7.2 Results ....................................................... 135
    7.2.1 Matrix elasticity regulates ERK and AKT signaling ... 135
    7.2.2 Matrix elasticity effects on FAK activity ................ 137
    7.2.3 FAK regulates AKT and influences melanoma drug responsiveness ... 138
  7.3 Discussion ................................................... 140
  7.4 Materials and Methods ....................................... 142
    7.4.1 Materials and reagents ................................ 142
    7.4.2 Cell culture ........................................... 142
7.4.3 Hydrogel formation ........................................ 143
7.4.4 Western blotting ........................................... 143
7.4.5 Metabolic activity ......................................... 144
7.4.6 Apoptosis .................................................. 144
7.4.7 Statistical analysis ......................................... 144
## Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Selected small molecule inhibitors against the ERK and AKT signaling pathways.</td>
<td>9</td>
</tr>
<tr>
<td>3.1</td>
<td>Macromolecular monomer compositions for gel formulations.</td>
<td>35</td>
</tr>
<tr>
<td>3.2</td>
<td>Measured values of modulus and mass swelling ratio.</td>
<td>35</td>
</tr>
<tr>
<td>3.S1</td>
<td>Change in equilibrium swelling compared to initial swollen state at polymerization.</td>
<td>52</td>
</tr>
<tr>
<td>7.1</td>
<td>Hydrogel formulations and properties.</td>
<td>135</td>
</tr>
</tbody>
</table>
Figures

Figure

1.1 Stages of melanoma progression. Healthy melanocytes reside in the basal layer of the epidermis. After a malignant transformation, melanoma cells begin to proliferate and spread laterally (radial growth phase, RGP) without penetrating into the dermis. The next stage of progression is vertical growth phase (VGP), where melanoma cells being to penetrate into the dermis, gain metastatic potential and is more difficult to remove by surgical resection. Finally, metastasis occurs where cells can leave the primary tumor and enter into the vascular system. Metastatic melanoma is very difficult to treat. Adapted from reference [1].

1.2 PET scan before and after PLX4032 treatment. Images are positron-emission tomography (PET) scans showing the uptake of 18F-fluorodeoxyglucose (FDG) in one patient before and after 15 days of PLX4032 treatment. Tumors uptake FDG, and in most patients, there was a marked decrease in FDG uptake by day 15. Image adapted from [2] (Phase 1, dose-escalation trial).

1.3 ERK and AKT signaling pathways. Both pathways can be activated by the binding of growth factors or focal adhesion formation. ERK and AKT are both considered survival pathways because they promote proliferation and suppress apoptosis.
1.4 Histological section of human skin. Keratinocytes, the most numerous type of cells in the skin, and melanocytes, which are the pigment-producing cells, reside in the basal layer of the epidermis. Melanocytes deposit melanin into keratinocytes to protect them from UV-induced damage. Image from www.apsu.edu/thompsonj, Epidermal Histology.

1.5 Complexity and relevance of cell culture models are related to dimensionality. Cell culture platforms can increase in relevance and complexity as dimensionality changes from 2D to 3D systems and with the addition of multiple cell types. The most relevant and complex system is an animal model. Image adapted from [3].

1.6 Correlation between gene expression changes in human squamous cell carcinomas (SCCs) dependent on culture conditions. The highest correlation among samples were between two human SCC samples. Notably, the highest correlation to patient-derived samples was a 3D in vitro organotypic model, while there was almost no correlation between human SCC samples and cells cultured on TCPS. Figure from [4].

1.7 Phase contrast images exhibiting the fundamental difference in cell morphology and architecture between 2D TCPS and cells embedded in Matrigel. Primary breast epithelial cells on TCPS (c, upper left), and acinar structures in Matrigel at lower (d) and higher magnification (e). Image adapted from Petersen et al. [5].
1.8 Thiol-ene reaction. (a) Four-arm 20 kDa PEG-norbornene was reacted with bi-functional cysteine containing MMP-degradable peptide crosslinker. The pendant adhesions peptide CRGDS can also be covalently incorporated to aid in cell adhesion, spreading, and survival. Thiol-ene networks form via step-growth polymerization in the presence of a photo initiator (LAP) and light, in this case, light centered around 365 nm. (b) Mechanism of thiol-ene reaction. A photoinitiator abstracts a hydrogen from a thiol group and the thiyl radical reacts across the double bond on norbornene. The norbornane radical then abstracts a hydrogen from another thiol to complete the thioether bond and regenerate a thiyl radical. Adapted from [6].

1.9 Effects of matrix stiffness on healthy mammary epithelial cells. Substrate elasticity affects many cell types, and as shown here, healthy mammary epithelial cells take on a more fibroblastic, malignant morphology as the culture substrate (PAA gels) is stiffened. The malignant phenotype is characterized by lumen filling, depolarization of apical-basal proteins, and spread, fibroblast-like morphology. Image from [7].

1.10 Comparison of 2D versus 3D cell morphology. In 2D culture, epithelial and mesenchymal cells grow as flat monolayers and maintain dorsal-ventral polarization. In 3D, however, epithelial cells form multicellular structures with apical-basal polarity and contain a lumen in the center, similar to in vivo structures. When cultured in 3D, mesenchymal cells lose the artificial polarization and instead exhibit a fibroblastic morphology. Image from [8].

3.1 Schematic of network formation utilizing cysteine-containing peptides and norbornene functionalized PEG.
3.2 Effect of substrate elasticity on overall cell morphology and focal adhesion formation. (A) Immunostaining for paxillin (green), f-actin (red) and nuclei (blue) on TCPS, stiff, medium, and soft gels of both cell types. Inset image is magnification of outlined area. (B) Quantification of focal adhesion area based on image analysis. n = 3 ± SEM. *p < 0.05. Scale bars, 20 µm. 40

3.3 Basal metabolic activity (ATP normalized to DNA content) measured under control conditions on all culture substrates. None of the samples were statically different. n = 3 ± SEM. 42

3.4 Relative fraction of metabolic activity for PLX4032-treated cells compared to cells cultured on each respective substrate without inhibitor. The dashed line represents the control samples. None of the treated samples for either cell type were statistically different metabolic activity based on culture substrate. n = 3 ± SEM. 42

3.5 Apoptosis measured via caspase 3 activity on different culture substrates. Each bar represents the PLX4032-treated sample normalized to its respective control showing the fold increase in caspase 3 activity with drug treatment. None of the A375 changes in apoptosis were statistically different. n = 3 ± SEM. *p < 0.05 . 43

3.6 Percent of cells entering S phase as quantified by EdU staining. White bars (left y-axis) represent control conditions; grey bars (right y-axis) represent PLX4032-treated samples. n = 3 ± SEM. Three fluorescent images were taken for each gel or TCPS well, the percent of positive EdU cells quantified, and then averaged for each sample. *p < 0.01 compared to TCPS control; #p < 0.0001 for PLX4032 samples compared to corresponding control; **p < 0.01. 44
3.7 Schematic of the effect of substrate stiffness on WM35 and A375 cells. WM35 cells exhibit dependence on substrate modulus by forming stiffness-dependent focal adhesions (green dots) and increase f-actin stress fiber formation (red lines). We hypothesize that for the WM35 cells, increased signaling from focal adhesion formation, perhaps an indication of stronger cell-matrix interactions, can increase survival signaling to inhibit apoptosis induced by PLX4032. In comparison, A375 cells show little dependence on the underlying matrix elasticity, always forming similarly sized matrix interactions. The lack of large focal adhesions, however, does not increase levels of apoptosis. Instead, A375 cells may have very different intracellular signaling that compensates for the lack of stiffness dependence for survival.

3.S1 Representative fluorescent images from EdU staining. Nuclei (blue) that are also positive for EdU (green) represent cells that have entered S phase in the cell cycle. On PLX4032 samples, there were fewer cells present to image, hence significantly lower cell density in the images. Scale bars, 100 \( \mu \text{m} \).
4.1 (a) Components of PEG-peptide hydrogels utilized for these studies. Four-arm norbornene-functionalized PEG (-ene) was crosslinked with an MMP-degradable peptide (KCGPQG↓IWGQCK) containing cysteines (-thiol). The PEG was used at a concentration of 3 mM (12 mM ene), and reacted 47% off stoichiometry (6.36 mM thiol, equivalently 3.18 mM crosslinker peptide) to form soft (0.4 kPa) hydrogels. The cysteines on either the MMP crosslinker or the pendant adhesion peptide CRGDS can react with norbornene to form a step-growth thiol-ene network. Integrins can engage the RGDS peptide, and combined with the proteolytically cleavable linker, aids in spreading and cell survival. (b) Cells can either be encapsulated or seeded on top of the PEG-peptide hydrogels. (1) Here, WM35 and A375 were tested on TCPS, on top of a hydrogel or encapsulated within the same formulation and tested for sensitivity to PLX4032 treatment. (2) The WM35 cells were then tested as single cells or multicellular spheroids against drug treatment to better understand what component of spheroid culture may contribute its more drug resistant nature.
4.2 Overall cell morphology and activity either on 2D or encapsulated within 3D hydrogels. (a) Immunostaining for focal adhesions was performed using two melanoma cell lines (WM35 and A375) cultured on TCPS or on 2D gel surfaces and embedded in 3D hydrogels after 24 hours of culture. Images of cells seeded on TCPS show cell spreading and more focal adhesions, while cells on the soft gels appear slightly more rounded. When encapsulated within the hydrogels, both cell types become very rounded, presumably due to the slower time scale for remodeling and degradation of the matrix. Paxillin (green), f-actin (red), nucleus (blue), scale bar, 100 µm. (b) Metabolic activity as measured by PrestoBlue of WM35 and A375 cells either seeded on TCPS, on top of a hydrogel, or encapsulated within a hydrogel after 48 hours of treatment. The WM35 cells exhibited a statistically significant increase in metabolic activity on a hydrogel surface compared to TCPS. No statistically significant differences were observed in metabolic activity between the culture conditions in the A375 cells. Dashed line represents values of the control samples. *p < 0.05.

4.3 WM35 and A375 cell apoptosis after 48 hours of treatment with PLX4032 in 2D and 3D microenvironments. (a) WM35 cells exhibited sensitivity to PLX4032 in TCPS and 2D and 3D hydrogel culture conditions. The 2D hydrogel PLX4032-treated sample was statistically higher than the TCPS PLX4032 sample. (b) A375 cells exhibited overall less sensitivity to PLX4032 treatment as observed by smaller increases in caspase 3 activity with PLX4032 treatment. The 3D control sample was significantly higher than the 2D hydrogel control sample, and the 2D hydrogel control was statistically lower than that of the TCPS control. (c, d) Fold change in caspase 3 activity over each respective control with PLX4032 treatment. *p < 0.05 compared to respective control, #p < 0.05 compared to corresponding condition in TCPS, **p < 0.05 between A375 2D and 3D control samples.
4.4 Procedure for forming spheroids. (a) A 50 µL layer of 1.5% Noble agar was plated in a 96-well plate and allowed to solidify at room temperature. 200 µL of WM35 single cell suspension (1.25 x 10⁵ cell/mL) was pipetted on top of the agar layer and left to grow for approximately 48 hours in normal growth media. After 2 days, the spheroids were transferred with a 1 mL pipette, and then encapsulated within PEG-peptide hydrogels. (b) A melanoma spheroid after 48 hours of culture on top of agar. Spheroids were approximately 1000 µm in diameter. Scale bar, 250 µm.

4.5 Overall morphology of WM35 single cells and spheroids encapsulated within PEG hydrogels. Live/Dead staining of single cells or spheroids within 0.4 kPa PEG-peptide gels in the presence or absence of PLX4032. Single cell encapsulations exhibited a very rounded morphology in the control conditions while many cells had small, thin protrusions in the presence of PLX4032. Inset image shows magnification of cells exhibiting rounded or elongated morphology. Spheroids cultured under control conditions formed relatively well defined edges with some cells loosely dissociated from the edge of the cluster. In contrast, spheroids treated with PLX4032 displayed many cells with an invasive morphology appearing to migrate away from the spheroid. Scale bar, 100 µm.
4.6 Overall drug responsiveness of melanoma cells to PLX4032 treatment after 48
hours. (a) Metabolic activity was measured and found to decrease with PLX4032
treatment for all conditions. (b) DNA content was measured to better understand
changes in cell number in the single cell encapsulations versus spheroid culture.
PLX4032 caused a significant decrease in cell number in both culture conditions
(∼68% and 55% in single cells and spheroids, respectively). (c) Apoptosis was
measured via caspase 3 activity and found to increase with PLX4032 treatment in
both culture conditions. The spheroid control and PLX4032-treated samples were
statistically different from the corresponding single cell encapsulation samples. *p
<0.05 compared to respective control, #p <0.05 compared to respective condition
in single cell encapsulation samples.

4.S1 Apoptosis of WM35 and A375 cells in stiff hydrogels (∼4 kPa) after 48 hours of
PLX4032 treatment. (a, b) Apoptosis was measured via caspase 3 activity. The
WM35 cells exhibited lower levels of caspase activity under control conditions on
top of or encapsulated within hydrogels compared to TCPS. A375s showed similar
basal levels of caspase 3 across all culture conditions tested. Both cell types ex-
hbit increases in caspase 3 in response to PLX4032 treatment. (c, d) Fold change
in apoptosis due to PLX4032 treatment compared to each respective control was
determined for each culture condition. *p <0.05 compared to respective control
condition, #p <0.05 compared to respective condition on TCPS, **p <0.05 be-
tween A375 2D hydrogel and 3D PLX4032 samples.

4.S2 Apoptosis of WM35 and A375 cells as a function of the adhesive ligand (fibronectin-
derived RGD versus collagen I P15 peptide). Apoptosis was measured via caspase
3 activity after 48 hours of drug treatment. *p <0.05 compared to respective control
condition.

4.S3 Brightfield images of spheroids encapsulated within PEG-peptide hydrogels. Scale
bar, 100 μm.
4.5 Fold change in apoptosis in single cells versus spheroids. When the caspase 3 activity was normalized to each respective control, the fold change was approximately the same.

5.1 MMP sensor peptide-functionalized hydrogels enable facile monitoring of 3D cellular proteolytic activity. The pendant MMP sensor peptide (Dab-GGPQG\textsubscript{\textdagger}IWGQK-Fl-AEEAc) is covalently incorporated into the PEG network. The MMP-sensitive peptide sequence is flanked by a quencher (dabcyl) on one side and a fluorophore (fluorescein). The intact peptide, with the quencher and fluorophore in close proximity, is weakly fluorescent. Upon cleavage by cell-secreted MMPs, the quencher can diffuse away from the fluorophore, resulting in an increase in fluorescence that can be measured on a standard plate reader. The fibronectin-derived pendant adhesion peptide CRGDS is also incorporated into the hydrogels. The network is crosslinked with an MMP-degradable peptide (KCGPQG\textsubscript{\textdagger}IWGQCK) to allow cell-mediated degradation of the network.

5.2 Effect of drug treatment on MMP activity and metabolic activity in 3D culture. (a) Color map showing the effects of RAF/MEK inhibitors (corresponding columns) on metabolic activity and MMP activity in the four human melanoma cell lines tested (corresponding rows). Each drug-treated sample is normalized to the corresponding control within that cell type. Decreases in metabolic activity are indicated by a color shift from green (control, 100%) to yellow, then red (low metabolic activity, 50%) on the left side of each circle. Changes in MMP activity are indicated by a color shift from white (control) to blue (maximum of 1.7-fold increase) on the right side of each circle. Bar graphs showing quantification can be found in Fig. 5.S2a and b. n = 5, *p <0.05. (b) Gelatin zymogram of cultured media from A375 cells. (c) Quantification of MMP activity by zymography. Mean ± SEM, n = 3, *p <0.05.
5.3 Cell morphology changes with PLX4032 treatment. (a) A375 cells encapsulated within a hydrogel. Samples were stained for paxillin (green), F-actin (red), and DAPI (blue) to visualize overall cell morphology. (Scale bar, 20 μm.) (b) Aspect ratio of encapsulated cells. n > 1000 cells, *p < 0.05. (c) Fraction of elongated cells, as defined by having an aspect ratio greater than 1.5. Mean ± SEM, n = 3, *p < 0.05.

5.4 Effects of inhibitor treatment on cell motility. (a) The x-y positions of 10 sample cells were plotted with the origin being the initial cell position. (b) Fraction of migrating cells was defined as the portion of cells migrating >15 μm from its starting position. Mean ± SEM, n = 3, *p < 0.05. (c) Cell speed over the 8-h time frame centered around 24 h after treatment was calculated for all migrating cells. (d) Maximum displacement of migrating cells. The farthest radial distance from the starting point of each migrating cell was calculated. Mean ± 95% CI, *p < 0.05 by one-way ANOVA and Tukey posttests. (e-g) Cells were cultured in the presence of GM 6001 (10 μM), a broad spectrum MMP inhibitor, and the effects on cell motility with and without PLX4032 were assessed at 24 h after drug treatment. Mean ± SEM (e), mean ± 95% CI (f and g), *p < 0.05 by Students t-test. n.s., not significant; PLX, PLX4032; Sora, Sorafenib.

5.5 Verification of metabolic activity as a suitable measure for cell number. Both the WM35 and A375 cells were assessed to determine whether metabolic activity scaled with DNA content within each sample. Metabolic activity was measured 24 h after drug treatment. Gels were then degraded using collagenase, and the cell pellet was lysed to measure the DNA content. Metabolic activity was normalized to DNA content and found to scale with each other (i.e., the ratio between the two metrics was approximately 1 in each condition tested). Mean ± SD, n = 2, *p < 0.05 by one-way ANOVA and Tukey posttests.
5.S2 Quantification of metabolic activity and MMP activity. (a) Metabolic activity as measured by alamarBlue for each cell type and inhibitor. Bars represent percent metabolic activity normalized to the control. (b) MMP activity as measured by fluorescent MMP sensor peptide for each cell type and inhibitor. Mean ± SEM, n = 5, *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (DMSO) by one-way ANOVA and Tukey posttests.

5.S3 A375 cell migration metrics at 24, 48, and 72 hours. (a) Fraction of migrating cells as defined as having a displacement >15 μm. PLX4032 treatment caused a statistically significant increase in migrating cells compared to sorafenib at all time points and was higher than control samples. (b) Cell speed of migrating A375 cells. PLX4032 resulted in faster cell speeds at 24 and 48 hours after treatment. (c) Maximum displacement of migrating A375 cells. At 24 and 48 hours, A375 cells travelled farther with PLX4032 treatment compared to control or sorafenib samples. Mean ± SEM (± 95% CI for (b) and (c)) n = 3, *p < 0.05 based on two-way ANOVA and bonferroni posttests.

5.S4 Migration of WM35 cells at 24, 48, and 72 h. (a) Fraction of migrating cells as defined as having a displacement >15 μm. In the WM35 cells, the difference in the fraction of migrating cells became more apparent over time, with the largest difference in motility at 72 h with PLX4032 treatment. (b) Cell speed of migrating WM35 cells. Single cell speeds were not different until 72 h despite more migrating cells at each time point. (c) Maximum displacement of migrating WM35 cells. The average farthest displacement was most apparent at 72 hours in the WM35 cells, with no statistical difference at 24 and 48 h between control and drug treated samples. Mean ± SEM (± 95% CI for (b) and (c)) n = 3, *p < 0.05 based on two-way ANOVA and bonferroni posttests.
5.S5 MMP inhibitor does not affect metabolic activity. To verify that MMP activity is not confer a protective effect to A375 cells, the broad spectrum MMP inhibitor was used (10 \(\mu\)M) in conjunction with and without PLX4032 (1 \(\mu\)M) for 24 h. A decrease in MMP activity was observed with GM 6001 treatment, but there was no change in metabolic activity in the presence or absence of GM 6001. Mean \(\pm\) SEM, \(n = 4\), \(*p < 0.001\) two-way ANOVA and bonferroni posttests. There is no statistically significant difference in metabolic activity between the DMSO conditions (white bars) with and without GM 6001.

5.S6 A375 cells encapsulated within PEG hydrogels and treated with DMSO control. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 \(\mu\)m z-stack in the center of the hydrogel. Cells under control conditions do not exhibit high rates of motility. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 \(\mu\)m.

5.S7 A375 cells encapsulated within PEG hydrogels treated with 1\(\mu\)M PLX4032. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 \(\mu\)m z-stack in the center of the hydrogel. Cells cultured with PLX4032 exhibit increased migration speed and displacement compared to control or Sorafenib conditions. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 \(\mu\)m.

5.S8 A375 cells encapsulated within PEG hydrogels treated with 1\(\mu\)M Sorafenib. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 \(\mu\)m z-stack in the center of the hydrogel. Cells cultured with Sorafenib do not exhibit high rates of motility. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 \(\mu\)m.
6.1 The complex epidermal microenvironment. Keratinocytes (purple) make up the majority of cells in the epidermis and reside near melanocytes (gray). Keratinocytes and paracrine signaling from fibroblasts (red) can regulate melanocyte behavior. Once melanocytes undergo a malignant transformation, they escape keratinocyte control, increase invasiveness, and activate fibroblasts that produce growth factors and aid in proliferation. Image from [9].  

6.2 ERK and AKT signaling pathways important to cell survival. Focal adhesion formation and growth factor binding can cause increased signaling through the ERK and AKT pathways. Both pathways promote proliferation and suppress apoptosis, among other functions. ERK is also a known regulator of MMPs.  

6.3 Simultaneously visualizing cell migration and proteolytic activity. (A) Cells are typically rounded after encapsulation and fluorescence should be low due to little degradation of the fluorogenic sensor peptides. (B) As cells migrate through the hydrogel matrix, the peptide sensors will be cleaved and therefore increase in fluorescence that can be quantified either by a standard plate reader or potentially through microscopy. This will aid in localization as well quantification of enzymatic activity. Image courtesy of Jennifer Leight and Kristi Anseth from NIH R21 (#1551854) grant proposal.  

6.4 Co-culture of WM239A metastatic melanoma cell clusters cultured in the presence of fibroblasts. (a) Co-culture migration experimental setup. Healthy fibroblasts (red) were encapsulated within a collagen ring that surrounds the PEG hydrogel containing WM239A melanoma clusters (green). (b) Time lapse images of single cell migration away from a melanoma cluster. In the center of the images is a melanoma cluster encapsulated within a PEG hydrogel. A single cell broke away and was subsequently followed by other cells from the aggregate. The fibroblasts were located just beyond the scale bar in these images. Images were obtained every 20 minutes but sequential images from every 4 hours are shown. Scale bar, 40 µm.
6.5 Xenograft sample from a human patient and grown in a mouse. This xenograft sample was encapsulated within a PEG hydrogel and allowed to grow for approximately 24 hours. Time lapse images show outgrowth from the main body of the sample at approximately 8-hour intervals. Scale bar, 100 µm. Images courtesy of Michael Schwartz.

7.1 ERK activity of WM35 and A375 cells seeded on top of TCPS or hydrogels. Western blots were performed to assess levels of pERK (a), total ERK (b) or ERK activity (c) in both cell types. Image of a western blot (d), depicting the changes in expression levels. n = 4, *p < 0.05 compared samples within the same cell type, #p < 0.05 for indicated stiffness compared to WM35 cells.

7.2 AKT activity of WM35 and A375 cells seeded on top of TCPS or hydrogels. Western blots were performed to assess levels of pAKT (a), total AKT (b) or AKT activity (c) in both cell types. Image of a western blot (d). n = 6, *p < 0.05 for conditions within each cell type, #p < 0.05 for indicated stiffness compared to WM35 cells.

7.3 FAK activity of WM35 and A375 cells seeded on top of TCPS or hydrogels. Western blots were performed to assess levels of pFAK (a), total FAK (b) or FAK activity (c) in both cell types. Image of a western blot (d). n = 4, *p < 0.05 for conditions within each cell type, #p < 0.05 for indicated stiffness compared to WM35 cells.

7.4 Testing whether PF573228 or LY294002 inhibited AKT and affected PLX4032 sensitivity. (a) Western blots showing that pAKT was affected by PF573228 in both cell types, exhibiting decreased levels of expression. pERK and pFAK were also evaluated and affected by PF573228 or LY294002 for 4 hours on TCPS. n = 2 (b) Metabolic activity was measured after 48 hours of PLX4032 treatment. n = 3, *p < 0.05, **p < 0.01.
7.5 Apoptosis as measured by caspase 3 activity in the presence of PF573228 or LY294002 in response to PLX4032 treatment. n = 2.  140
Chapter 1

Introduction

1.1 Melanoma overview

Melanoma is an aggressive form of skin cancer that originates in melanocytes, the pigment-producing cells, in the basal layer of the epidermis. Skin cancer, melanoma in particular, is now considered a major public health problem in the United States, with the Surgeon General releasing a call to action in July 2014. Incidence rates of melanoma have been increasing for the last 30 years, and in 2014, an estimated 76,000 new cases will be diagnosed in the United States [10]. While melanoma accounts for approximately 2% of all skin cancers, it is responsible for nearly 75% of all skin cancer-related deaths [10]. Melanoma treatment and survival are heavily dependent on the stage of progression.

There are three distinct stages of melanoma progression: radial growth phase (RGP), vertical growth phase (VGP) and metastatic melanoma (Fig. 1.1). Early stage RGP spreads laterally, while VGP begins to penetrate into the dermis, and metastatic melanoma occurs when the cancer cells leave the primary tumor and enter the bloodstream or surrounding tissues. RGP legions can be easily treated by surgical excision [11]; however, once melanoma has progressed to VGP, it is more difficult to treat due to invasion and increased metastatic potential [12]. Metastatic melanoma is notoriously drug resistant [13] for reasons that are not completely understood. As of 2014, the 5-year survival rate for early stage melanoma is 98%, while for VGP and metastatic cases, the rates decrease to 62% and 16%, respectively [10]. In fact, the median survival time for metastatic cases is only 6-10 months with traditional chemotherapeutics [11,14]. Due to recent research efforts and
Figure 1.1: Stages of melanoma progression. Healthy melanocytes reside in the basal layer of the epidermis. After a malignant transformation, melanoma cells begin to proliferate and spread laterally (radial growth phase, RGP) without penetrating into the dermis. The next stage of progression is vertical growth phase (VGP), where melanoma cells being to penetrate into the dermis, gain metastatic potential and is more difficult to remove by surgical resection. Finally, metastasis occurs where cells can leave the primary tumor and enter into the vascular system. Metastatic melanoma is very difficult to treat. Adapted from reference [1].

FDA approval of new types of drugs for melanoma treatment, the median progression-free survival time is increasing.

1.1.1 Current clinical strategies and new inhibitors

Until 2011, the only FDA approved drugs for treatment of metastatic melanoma were interleukin-2 and dacarbazine, a chemotherapeutic with a response rate of only 7-12% and a median survival time of less than 8 months [15]; however, neither of these treatments were considered to improve overall survival [16, 17]. A major milestone was achieved in 2002 when Davies et al. reported that more than 60% of all melanomas were found to contain a specific BRAF mutation (V600E) that caused the protein to be constitutively active [18]. Through rational design, a small molecule in-
hibitor was identified and developed as a highly specific, allosteric inhibitor near the ATP-binding site of mutated BRAF [19]. Preclinical work showed that PLX4032 was capable of inhibiting mutated BRAF kinase activity, decreased signaling through the ERK cascade, and prevented proliferation in V600E BRAF cell lines [20–23]. When human melanoma tumors were transplanted into immunocompromised mice and treated with PLX4032, low doses prevented tumor growth and higher doses induced tumor regression [19]. Remarkably, Phase I clinical trials showed 81% of patients had partial or complete tumor regression, a groundbreaking result in melanoma treatment (Figure 1.2), but ultimately, all patients from the original study eventually relapsed [2, 24]. Subsequent clinical trials found an overall response rate of approximately 50% and the median overall survival time increased to 16 months [25, 26]. In 2011, the FDA approved PLX4032 (Vemurafenib, Zelboraf) for metastatic melanoma treatment. This drug was the first of its kind for melanoma treatment: a targeted therapy based on the mutational status of a specific protein.

Figure 1.2: PET scan before and after PLX4032 treatment. Images are positron-emission tomography (PET) scans showing the uptake of 18F-fluorodeoxyglucose (FDG) in one patient before and after 15 days of PLX4032 treatment. Tumors uptake FDG, and in most patients, there was a marked decrease in FDG uptake by day 15. Image adapted from [2] (Phase 1, dose-escalation trial).
Since the approval of Vemurafenib, several new drugs have been approved for clinical use in treating metastatic melanoma, including dabrafenib (Tafinlar, BRAF inhibitor), trametinib (Mekinist, MEK inhibitor), and ipilimumab (Yervoy, monoclonal antibody that inhibits T cells) [27–30]. Many other small molecule inhibitors against important survival pathways have since been developed and several entered clinical trials for melanoma treatment, which will be discussed in Section 1.2.1 [31](see Table 1.1 for a list of selected drugs either in clinical trials or approved by the FDA). For the duration of this thesis, PLX4032 will be the focus of subsequent studies, in part because of its clinical relevance and availability for wide usage in both clinical treatment and research labs.

1.1.2 In vitro studies of melanoma drug responsiveness

Since the landmark study by Davies et al. [18] and the identification of PLX4032 as a targeted therapy [19], there have been numerous studies to understand what effects inhibition of mutated BRAF might have on melanoma growth and progression. PLX4032 has been shown to cause potent cell cycle arrest and induce apoptosis in melanoma [19, 22, 24], as well as growth arrest in thyroid carcinoma cells [32, 33]. Studies have also identified the high specificity of PLX4032 for mutated BRAF; the inhibitor does not significantly affect wild type BRAF cell lines [19, 22–24].

Most preclinical studies include experiments performed on traditional tissue culture-treated polystyrene (TCPS), but there has been a growing argument for physiologically relevant culture systems with which to study cancer biology and drug responsiveness [34]. In an attempt to better characterize drug potency and potential melanoma drug resistance in vitro, researchers have taken advantage of 3D cell culture systems [35]. Most often, melanoma spheroids are formed and then embedded within collagen matrices [36–38]. Based on viability measurements, such as MTT assays, cells cultured on traditional TCPS are highly susceptible to drug treatment; however, when the same cell types are then treated as 3D spheroids, there is decreased efficacy [22, 38]. Based on live/dead staining, spheroids formed using metastatic melanoma cell lines tend to be less responsive to both MEK and BRAF inhibitors, often exhibiting less outgrowth from the spheroid and also less ethidium homodimer (dead) staining [22, 39]. In comparison, earlier stage RGP
and VGP spheroids are often still sensitive to drug treatment and exhibit high percentages of ethidium homodimer-positive cells and correspondingly high numbers of apoptotic cells [22,39]. Fewer studies have been performed using single cells embedded in 3D matrices, however, many speculate that the nature of 3D matrix interactions confers a more physiologically relevant microenvironment for cells and better correlates with in vivo responsiveness to drugs [8,40].

Many studies have been performed in recent years to characterize not only PLX4032, but the first generation of inhibitors to decrease signaling through the RAS pathway (Fig. 1.3). MEK inhibitors, which target the protein upstream of BRAF, have been widely studied, and CI-1040 was the first MEK inhibitor to enter clinical trials in 2004 for metastatic melanoma, though it was ultimately found to be ineffective [41,42]. AZD6244 was a 2nd generation MEK inhibitor with increased solubility and potency, but a Phase II study did not show significant differences in progression-free survival between AZD6244 and the control (temozolomide) [39,43]. Interestingly, with both MEK inhibitors, preclinical studies found that cells responded differently in 2D compared to 3D. When the same cell types were treated on TCPS compared to 3D spheroids embedded within collagen, experiments showed that the MEK inhibitors had a mostly cytostatic effect in 3D and prevented outgrowth from the spheroid [38,39]. However, when the inhibitor was washed out, spheroids would continue to grow; the 2D TCPS samples, on the other hand, often had already undergone apoptosis [39]. In contrast to MEK inhibitors, PLX4032 was observed to induce apoptosis and prevent outgrowth from aggregates in 3D [19,22], and this may be part of the reason for its potency and higher efficacy in clinical use.

1.2 Melanoma survival and drug resistance

Early stage melanoma is very simple to treat, however, metastatic melanoma has an ability to evade long-term remission in patients [2,11,26,44]. Unfortunately, the exact reasons for melanoma drug resistance are not well understood [45]. The difficulty with treating melanoma is that most common forms of anticancer drugs are ineffective, and this truly underscores the complex nature of melanoma drug resistance. Melanoma is unresponsive to DNA damage (via alkylation,
Figure 1.3: ERK and AKT signaling pathways. Both pathways can be activated by the binding of growth factors or focal adhesion formation. ERK and AKT are both considered survival pathways because they promote proliferation and suppress apoptosis.

crosslinking, or irradiation), microtubule destabilization, topoisomerase inhibition, and disruption of estrogen signaling (reviewed in [13], [46]). Drug resistance can occur in two different ways: intrinsic and de novo, or acquired, resistance. Melanoma has the capacity for both forms of resistance, and there has been evidence to suggest that both can occur in the same patients [13, 45, 47]. First, however, survival signaling inherent to not only melanoma, but most cell types will be discussed.

1.2.1 **Signaling pathways important to melanoma survival**

Analyzing the biochemical signaling of melanoma has led to many hypotheses related to drug resistance or possible ways in which melanoma can evade drug treatment. One important pathway is the extracellular regulated kinase pathway (ERK, also known as MAPK), which regu-
lates cell cycle progression, apoptosis, cell motility, and senescence [48, 49]. The ERK pathway responds to extracellular events such as growth factor binding, the formation of focal adhesions, or cell-cell interactions, and connecting these signals to the nucleus via a well established signaling cascade: \( \text{RAS} \rightarrow \text{RAF} \rightarrow \text{MEK} \rightarrow \text{ERK} \) [48, 49] (Fig. 1.3). ERK ultimately regulates transcription factors that promote or suppress numerous genes important for overall cell function and survival [48]. Over 30 BRAF mutations have been identified in human cancers, the majority of which up regulate ERK activity [50]. The ERK pathway is hyperactivated in approximately 30% of all cancers [51], and in particular, at least 66% of all melanomas contain a BRAF mutation and 15% have an activating RAS mutation [18]. Because of ERKs importance for overall cell survival and the constitutive activity of BRAF in many melanomas, researchers have hypothesized that the ERK pathway might be a suitable target for anticancer therapy. If cancer cells are oncogene addicted to ERK activity, they are dependent on its activity for survival, and therefore, inhibition of the ERK pathway would lead to apoptosis and halt proliferation [52]. The development of both MEK and BRAF inhibitors are predicated on this idea and have been shown to be potent \textit{in vitro} with many promising candidates entering clinical trials (reviewed in [53]). Yet, because of the lack of successful outcomes, one of the hypotheses for \textit{de novo} drug resistance is that melanoma cells compensate when the ERK pathway is inhibited, whether it is through activation of another pathway or by utilizing a different isoform of RAF that can aid in signaling through ERK (reviewed in [45]).

With respect to this point, another signaling pathway that is key to melanoma survival is the PI3K-AKT pathway (Fig. 1.3). This pathway is activated by receptor tyrosine kinases and regulates cellular functions such as proliferation, apoptosis, and cytoskeletal rearrangement, and is therefore important for not only melanoma, but most cell types (reviewed in [54]). The AKT pathway is often upregulated in cancer [55], and loss of PTEN, a suppressor of the AKT pathway, is found in 10-30% of melanomas [56]. In mice, activating BRAF mutations caused increased proliferation of melanocytic lesions, but only with combined loss of PTEN, all mice in the study developed invasive, metastatic melanoma [57]. Interestingly, recent work has shown how critical
AKT signaling can be for melanoma in particular. Hyperactivation of PI3K-AKT can arise from increased expression of receptor tyrosine kinases (RTKs), PDGFRβ, and EGFR have been identified so far, and block apoptosis through PI3K-AKT without rescuing ERK signaling [37, 58, 59]. As a result, several RTK inhibitors have been explored to suppress AKT activity and increase susceptibility to drug treatment [60, 61]. AKT also contributes to adhesion-dependent protection from anoikis [62], and drug resistant melanoma cell lines exhibit increased AKT levels [63]. The specific isoform AKT3 can rescue cells when viability is challenged with either siRNA for BRAF or pharmacological inhibitors; its expression is also adhesion dependent, where fibronectin, but not collagen, can protect the cells from chemical insult [63]. Several PI3K-AKT inhibitors have been developed and even used in combination therapy [64] (see Table 1.1 for a list of selected drugs), but because of AKT’s importance for normal cell homeostasis there is concern of whether doses will be tolerated to achieve a clinical benefit [55].

Because of the importance to not only melanoma cell survival, but most other types of cells, many inhibitors against the ERK and AKT signaling pathways have been developed for potential anticancer therapy. Table 1.1 shows a selection of ongoing and recent clinical trials for ERK/AKT pathways inhibitors as well as those that are FDA approved for melanoma treatment. As evidenced by the large body of research and number of clinical trials, the ERK and AKT signaling pathways are paramount to overall cell survival, but how these signaling pathways respond to microenvironmental changes and how those changes influence melanoma drug responsiveness remain unknown.

1.2.2 Intrinsic and acquired drug resistance

Some researchers and clinicians hypothesize that because melanoma arises from melanocytes, this type of cancer is “meant to survive” [13]. Melanocytes are a unique type of cells that produce pigment in response to UV exposure [65, 66]. Melanocytes reside in the basal layer of the epidermis (Fig. 1.4) and form long protrusions that extend to deposit vesicles of melanin into keratinocytes, the major cells found within the epidermis, to provide pigment and protection from UV damage [65, 66]. In contrast, most cells exposed to ionizing radiation either undergo pro-
Table 1.1: Selected small molecule inhibitors against the ERK and AKT signaling pathways.

<table>
<thead>
<tr>
<th>Target Protein (Total # Drugs)</th>
<th>Name</th>
<th>Applicable Treatment</th>
<th>FDA Approval, U.S. Brand Name</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF (6)</td>
<td>vemurafenib, PLX4032, RG7204, RO5185426</td>
<td>Unresectable or metastatic melanoma*</td>
<td>2011, Zelboraf</td>
<td>Accelerated approval for use with trametinib in Jan 2014</td>
</tr>
<tr>
<td></td>
<td>dabrafenib, GSK2119436</td>
<td>Unresectable or metastatic melanoma*</td>
<td>2013, Tafinlar</td>
<td></td>
</tr>
<tr>
<td>CEP-32496, AC013773</td>
<td>RO512054, PLX3303</td>
<td>Advanced unresectable melanoma and metastatic colorectal cancer*</td>
<td>Phase III</td>
<td>Phase I, closed</td>
</tr>
<tr>
<td>ARQ 736</td>
<td>Advanced solid tumors**</td>
<td>Phase I, closed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEK (16)</td>
<td>GSK1120212, JTP-74057</td>
<td>Unresectable or metastatic melanoma*</td>
<td>2013, MeKinst</td>
<td>Accelerated approval for use with dabrafenib in Jan 2014</td>
</tr>
<tr>
<td>selumetinib, ARRY-142866, AZD6244</td>
<td>Thyroid cancer, metastatic uveal melanoma, NSCLC, among others</td>
<td>Studies in Phase I, II, or III</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>trametinib, ARRY-162, ARRY-438162, MEK 1/2</td>
<td>Unresectable or metastatic melanoma*</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>TAK-733</td>
<td>Advanced nonhematologic malignancies</td>
<td>Phase I, closed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI3K (32)</td>
<td>idatasico, CAL-101, GS-1101</td>
<td>Relapsed chronic lymphocytic leukemia</td>
<td>Jul 2014, Zydelig</td>
<td>Accelerated approval for follicular B-cell non-Hodgkin lymphoma (FL) or relapsed small lymphocytic lymphoma (SLL)</td>
</tr>
<tr>
<td></td>
<td>buparlisib, BKM120, PI3K_inhibitor_BKM120</td>
<td>Combined treatment with vemurafenib in advanced melanoma*</td>
<td>Phase I, II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PX-866</td>
<td>Combined treatment with vemurafenib in advanced melanoma*</td>
<td>Phase I, II</td>
<td></td>
</tr>
<tr>
<td>AKT (11)</td>
<td>MK2206</td>
<td>Relapsed or refractory Hodgkin lymphoma or non-Hodgkin lymphoma</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GSK2141795</td>
<td>Combined treatment with trametinib in BRAF WT melanoma*, metastatic uveal melanoma, triple-negative breast cancer, multiple myeloma</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AZD5363</td>
<td>Combined treatment with dabrafenib in unresectable or metastatic solid cancers (e.g., breast, melanoma)</td>
<td>Phase I, II</td>
<td></td>
</tr>
</tbody>
</table>

*Data obtained from www.cancer.gov as of November 2014
*Must have BRAF mutation per FDA approved test
*Must have NRAS mutation
*BRAF WT - wild type, or non-mutated BRAF

grammed cell death (apoptosis) or pause their cell cycle in order to attempt DNA repairs [13]. Melanocytes, derived from the neural crest [65], have the ability to respond to UV damage and repair their DNA more effectively than most other healthy cell types found in the human body [66]. In addition, melanocytes possess a high amount of endogenous Bcl-2, an anti-apoptotic protein, that allows them to survive UV light-induced damage [13]. Some researchers theorize that after tumorigenic transformation, melanoma cells take advantage of their high levels of Bcl-2, along with other pro-survival signaling, that healthy melanocytes rely on for survival in order to resist drug treatment [13]. Overall, the inherent robustness of melanocytes may play an important role in melanoma drug resistance by providing many survival mechanisms to exploit.

In contrast to intrinsic resistance, acquired resistance can occur during or in response to drug treatment. Some research has reported an increase in drug efflux pumps, increased DNA
repair, altered drug metabolism, and deregulated signaling [47, 67]. Recent studies have focused on the acquired resistance of melanoma in response to BRAF inhibitors, due to the high rate of relapse in patients after starting PLX4032 [44, 68, 69]. One of the mechanisms thought to contribute to resistance to BRAF inhibitors is the re-activation of the ERK signaling pathway [70] by bypassing BRAF and the compensating roles for ARAF and CRAF, other isoforms of the RAF protein [37, 71]. Certain PLX4032-resistant cell lines revealed that the main mechanism of resistance was through ERK reactivation by RAS, and that RAS activation was due to enhanced FGF receptor 3 activation [72]. Additionally, a recent study found that new mutations after chronic exposure to PLX4032 can arise in BRAF that are not in the kinase domain and therefore are unresponsive to PLX4032 [73]. The ERK pathway promotes cell cycle progression through cyclin D1; if melanoma cells possess amplifications of cyclin D1, this may promote resistance to RAF inhibitors [45]. Smalley et al. showed that over-expression of cyclin D1 in a RAF inhibitor-
sensitive cell line decreases sensitivity to the inhibitor [74]. In another study, PLX4032 resistant $BRAF^{V_{600E}}$ melanoma cells were generated by chronic exposure to PLX4032, and upon analysis, an activating NRAS (Q61K) mutation was found [59]. The same mutation was found in two patient biopsies, and knockdown of NRAS restored PLX4032 sensitivity, supporting the hypothesis that NRAS activation can bypass BRAF to lead to persistent ERK activity. Another report suggested that melanoma cells become dependent on PLX4032 for proliferation and therefore clinical treatment should include discontinuous dosing to prevent resistance [75]. While PLX4032 has proven it induced apoptosis in melanoma cells, Haferkamp and coauthors showed PLX4032 resulted in stress-induced senescence, which might account for the lack of durable responses in patients [76]. EGFR-positive melanoma also exhibited induced senescence and that cycling PLX4032 treatment may prevent enrichment of the resistant subpopulation [77]. Additionally, preclinical studies have shown that ERK signaling may be heavily dependent on MEK, and has consequently led to clinical studies of combination therapies involving both MEK and BRAF inhibitors (reviewed in [68]).

However, another possible mechanism of resistance to drug inhibitors is the activation of other pathways to compensate for decreased signaling through ERK. Platelet-derived growth factor receptor $\beta$ (PDGFR$\beta$) tyrosine kinase [59], ErbB2, protein kinase C-ζ [70] and AKT [78] have all been implicated in melanoma resistance to RAF inhibitors. Of particular note is the role of PI3K-AKT signaling, which was discussed in Section 1.2.1.

### 1.2.3 Cancer stem cells in melanoma tumor biology

Another theory for melanoma survival relates to the idea of cancer stem cells. Cancer stem cells have been proposed for other types of cancer, such as breast cancer and chronic myelogenous leukemia (CML) [79] and may explain melanoma relapse [2, 26]. The majority of patients who receive PLX4032 initially exhibit significant tumor regression, but eventually metastases return and the patient relapses, typically within 6-7 months [2, 26, 80]. Cancer stem cells have been defined as having analogous characteristics to healthy stem cells: the ability for self-renewal, proliferation to promote tumor growth, and the capability to transform into any cell within a heterogeneous tu-
mor [79]. Fang et al. reported a subpopulation capable of self-renewing in suspension culture and maintaining the ability to differentiate into melanocytic, chondrocytic, or adipocytic lineages [81]. A cancer stem cell can also be considered to be a tumor-initiating cell [79], and there is evidence to suggest the existence of such cells in melanoma. Boiko et al. isolated a subpopulation of cells expressing the surface marker CD271, typically found on neural-crest-derived tissues, and showed strong evidence for the existence of cancer stem cells in melanoma [82]. The group found that CD271\(^+\) melanoma cells were much more likely to cause engraftment, melanoma tumor development, and metastases in mice compared to CD271\(^-\) cells from the same patient biopsies [82].

Another study identified a subpopulation of cells within melanoma tumors that had a protein called JARID1B [83]. Interestingly, JARID1B-positive cells resulted in highly proliferative daughter cells and exhibited a very slow cell cycle (>4 weeks), the opposite of most cancer cells, which often have a much faster doubling time [83]. The authors proposed that the conventional chemotherapeutic agent in use at the time (Dacarbazine) was not affecting JARID1B-positive cells because chemotherapeutics attack cells that proliferate quickly, and therefore would not target slow-cycling cancer cells [83]. While the study by Roesch et al. challenges the classic cancer stem cell concept [83], there are self-renewing and tumor-initiating cells within some melanomas that may be contributing to the relapse rate in patients and the difficulty in treating patients with late-stage melanoma.

1.3 Strategies for \textit{in vitro} studies of cancer cells

\textit{In vitro} studies for screening of cancer drugs are often initially performed on cells cultured on two-dimensional surfaces, such as tissue culture-treated polystyrene (TCPS) or plastic. While TCPS has advantages for facile access to cells, no substrate pretreatment required by the researcher, and well-established protocols, plastic dishes are orders of magnitude stiffer than soft tissues and can lead to physiologically irrelevant cell morphologies or responses [8, 84, 85]. As an alternative to traditional TCPS culture, researchers have utilized many different types of hydrogels to embed cells in three-dimensions; these highly swollen, elastic materials may better recapitulate certain
aspects of the native cell environment in soft tissues [8, 86–88]. Many researchers posit that soft hydrogels can serve as an intermediary between TCPS and expensive animal models, which introduce inherent variability, for screening of drugs, studying basic cell signaling mechanisms, and growing tumor models \textit{in vitro}.

While two-dimensional culture on plastic allows for high throughput screening of many dif-

Figure 1.5: Complexity and relevance of cell culture models are related to dimensionality. Cell culture platforms can increase in relevance and complexity as dimensionality changes from 2D to 3D systems and with the addition of multiple cell types. The most relevant and complex system is an animal model. Image adapted from [3].
Different experimental conditions, the studies are typically performed on a stiff, static material. A softer cell culture substrate, on the other hand, has a more physiologically relevant modulus, comparable to what cells in a native tissue might experience, whether as a 2D substrate or for 3D cell culture (Fig. 1.5). Three-dimensional systems have been shown to better mimic in vivo morphology and gene/protein expression profiles than TCPS [4, 89, 90] in the hopes of better recapitulating the complex, native environment [91]. Certainly, one might argue that the most relevant system to predict human outcomes is an animal model. But significantly, a recent study by Ridky et al., researchers found that 3D cell culture experiments and animal models had high levels of correlation to human gene expression profiles of spontaneous squamous cell carcinomas [4]. In contrast, traditional 2D cultures performed on plastic plates exhibited almost no correlation with patient-derived samples (Fig. 1.6) [4]. Thus, there are compelling arguments for understanding cell function and responses to stimuli and insults in vitro, as they can provide important pieces of evidence, allowing

![Figure 1.6](image.png)

Figure 1.6: Correlation between gene expression changes in human squamous cell carcinomas (SCCs) dependent on culture conditions. The highest correlation among samples were between two human SCC samples. Notably, the highest correlation to patient-derived samples was a 3D in vitro organotypic model, while there was almost no correlation between human SCC samples and cells cultured on TCPS. Figure from [4].
detailed hypothesis testing. However, more studies and detailed characterization are needed to see if these measurements of cell behavior in hydrogel cultures correlate to in vivo studies, and provide improvements over the TCPS standard.

1.3.1 Hydrogels as 3D cell culture platforms to study cancer cells

Naturally derived materials like collagen, Matrigel, or fibrin are compatible for culturing cells in three-dimensions and supporting their growth and function without modification, whether it is through inherent integrin-binding sequences, degradation and remodeling by cell secreted proteases, or by sequestration of endogenously or exogenously occurring local proteins [86, 92]. While naturally derived hydrogels offer unique properties that support cell growth and interactions (Fig. 1.7), they are not without limitations. For example, batch-to-batch variations are common; the matrix is often ill-defined with respect to its architecture and composition; cell-secreted and serum proteins can be locally sequestered unintentionally; and it can be difficult to achieve and
control matrix mechanical properties in physiological ranges [93, 94]. Collagen, for example, is often widely used for 3D culture; however, the matrix structure and overall composition of the material can be very different depending on the extraction method [95]. Matrigel (otherwise known as laminin-rich basement membrane or Engelbreth-Holm-Swarm mouse sarcoma basement membrane) has been widely used in cancer research for its ability to cause the spontaneous formation of multicellular acini structures (Fig. 1.7) [5]; however, Matrigel is harvested from the extracellular matrix surrounding tumors in mice. As a result, Matrigel is a complex milieu of basement membrane proteins and growth factors, which can lead to an ill-defined cell-matrix signaling environment (reviewed in [96]). Naturally derived materials often contain multiple cell-binding epitopes, but lack precise control over bulk properties like matrix elasticity [86, 93]. For example, in order to form stiffer collagen gels, a higher concentration of collagen is needed [7, 95, 97]; this, in turn, increases the concentration of binding epitopes and any potential growth factors sequestered in the material. The rate at which collagen fibrils form, which is dependent on collagen concentration, ionic strength, pH, and temperature, affect both matrix order and structure, and therefore, mechanical and transport properties of the collagen hydrogel [95].

Despite some of these limitations, naturally derived materials have provided valuable insight as an alternative cell culture platform for elucidating the importance of cell-matrix interactions and the resulting biological impact [98]. For example, cells cultured in 3D microenvironments using natural materials, like collagen [99–101] or Matrigel [102], often exhibit resistance or altered responses to drug treatment compared to 2D culture on TCPS. Specifically, the Bissell group showed β4 integrin-mediated polarity of both malignant and nonmalignant mammary epithelial cells conferred resistance to apoptosis; this indicated that both cell polarity and ECM composition rather than genotype dictated resistance [103]. There are, however, many inherent complexities of natural materials that can make interpretation of such results more difficult.

Synthetic materials provide an alternative method to natural extracellular matrix (ECM) derived materials. Synthetic hydrogels allow more precise control over macroscopic material properties, like modulus and network structure; however, because they do not inherently contain cell-
binding epitopes, these gels must be supplemented with some way of aiding cell survival in a non-biological material [86, 104]. Hydrogels can be designed to allow for user-defined changes in properties such as patterned substrates or bulk modulus [105–109], and they can also be laden with instructive cues to promote certain cellular responses [110–113]. Synthetic hydrogels have been synthesized from polymers such as poly(ethylene glycol) (PEG) [114], poly(vinyl alcohol) [115], or polyacrylamide [116]. Polyacrylamide gels, in particular, offer very precise control over substrate modulus, but cannot be translated to 3D encapsulations due to the toxic nature of gel formation process and its non-degradability [116–118]. But for 2D studies, the gel surface has been modified with proteins like laminin or fibronectin adsorbed or conjugated to the surface to promote cell attachment, without modifying the underlying gel mechanics [119, 120].

One of the most widely used synthetic materials for both 2D and 3D cell culture is PEG. PEG minimizes nonspecific protein adsorption, can be easily functionalized, has a range of tissue-relevant elastic properties, and can be crosslinked under cytocompatible conditions [121–123]. PEG can be end-modified to undergo various reactions, such as photoinitiated, chain polymerization of end-functionalized acrylated PEGs [121,124], or what will be the focus of this thesis, photoinitiated, step polymerization of norbornene-functionalized PEG polymerized with bi-functional cysteine-containing peptides [6]. In general, synthetic cell culture platforms afford researchers control of network structure and mechanics, but the materials inherently lack biological components to interact with cells [94]. Thus, specific cell-matrix interactions are routinely incorporated into synthetic networks to aid in cell survival, spreading, or adherence [6, 125–127]. Particular affinity peptide sequences can also be included to bind and sequester proteins of interest, such as growth factors [128]. In addition, cell-mediated degradation of PEG hydrogels can be incorporated by the use of specific peptide crosslinkers to be susceptible to proteases such as matrix metalloproteinases, cathepsins, or plasmins [114, 129, 130]. Overall, synthetic hydrogels afford more tunability, decrease inherent variability, and exemplify a minimalist approach to recapitulating the ECM as a 3D culture platform.
1.3.2 PEG-peptide thiol-ene hydrogels

This thesis focuses on the use of PEG-based hydrogels synthesized using a thiol-ene photopolymerization reaction to create cytocompatible peptide-functionalized gels [6, 123, 131, 132]. The PEG-ene component creates a “blank slate” that allows for a highly tunable, hydrated, and mechanically defined environment, whereas thiolated peptide components are designed to recapitulate important biological aspects of the ECM [94, 122, 123]. The thiol-ene “click” chemistry proceeds under cytocompatible polymerization conditions, which allows for facile translation between 2D

Figure 1.8: Thiol-ene reaction. (a) Four-arm 20 kDa PEG-norbornene was reacted with bi-functional cysteine containing MMP-degradable peptide crosslinker. The pendant adhesions peptide CRGDS can also be covalently incorporated to aid in cell adhesion, spreading, and survival. Thiol-ene networks form via step-growth polymerization in the presence of a photo initiator (LAP) and light, in this case, light centered around 365 nm. (b) Mechanism of thiol-ene reaction. A photoinitiator abstracts a hydrogen from a thiol group and the thiol radical reacts across the double bond on norbornene. The norbornane radical then abstracts a hydrogen from another thiol to complete the thioether bond and regenerate a thiol radical. Adapted from [6].
and 3D studies of cells [6]. The thiol-ene reaction is a radical mediated polymerization scheme that forms via step growth network formation, resulting in a relatively ideal network structure [6, 123]. For the materials used in this thesis, the radical is generated from the photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) that cleaves to form two radicals upon irradiation with light [133]. The radical initiates the reaction between thiol-containing peptides and norbornene-functionalized multi-arm PEGs (Fig. 1.8a) [6, 132, 134]. The radical abstracts a hydrogen from a thiol group, producing a thiol radical, which then reacts across the norbornene carbon-carbon double bond. The carbon-based radical cannot propagate through another norbornene, but instead abstracts a hydrogen from another thiol to form a thioether bond and regenerate a thiol radical (Fig. 1.8b). In this thesis work, 4-arm PEGs are typically crosslinked using a matrix metalloproteinase-degradable peptide (e.g., KCGPQG↓IWGQCK), which allows for cell-mediated remodeling of its local environment [114]. Any cysteine-containing peptide can be covalently reacted into the network, and for the work in this thesis, the fibronectin-derived pendant adhesion peptide CRGDS is incorporated to aid in cell adhesion and spreading [127].

Because nearly any peptide can be incorporated into the network, this approach was selected as it affords wide tunability of the material properties with which to answer fundamental biological questions. The network can be rendered non-degradable by exchanging the MMP-degradable peptide with a non-degradable sequence or a PEG-dithiol [94, 135, 136]. Additionally, the crosslinker can be designed such that it is degraded by specific cell-secreted enzymes, such as MMPs or cathepsins [114, 129, 130]. The adhesion peptide can be changed to mimic different extracellular matrix proteins like fibronectin, collagen, or elastin [135, 137, 138]. Additionally, bulk physical properties of the hydrogels, such as its modulus or equilibrium water content, can be controlled by changing the crosslinking density via the molecular weight, number of arms, or concentration of PEG [139–141]. Overall, thiol-ene “click” hydrogels provide a platform that can be engineered to study the effects of user-designed matrix properties to better understand the importance and role of certain extracellular matrix components on cellular responses.
1.3.3 Matrix factors known to influence cancer cell signaling and function

Clinically, tumor progression is associated with stiffer tissues [142–144], and therefore, researchers have asked whether matrix rigidity is important when studying cells in vitro. As discussed in the previous section, synthetic materials have been developed that allow precise tuning of substrate properties while avoiding confounding changes in cell binding moieties or sequestered growth factors. One widely used substrate is polyacrylamide (PAA), which can be modified to promote adsorption of whole proteins to the surface [119, 145] and the crosslinking density can be altered in order to change the bulk modulus [120, 146]. PAA gels have been utilized to study the effects of extracellular matrix proteins and matrix elasticity on healthy mammary epithelial cells [7]. Matrix elasticity is important for healthy acini structure formation, and if the matrix is stiffer than healthy tissue, the multicellular acini begin to take on a more malignant phenotype, characterized by depolarization of membrane proteins, a fibroblastic-like morphology, and altered survival signaling (Fig. 1.9) [7]. Additionally, matrix elasticity can regulate how cells respond to different molecules; for example, TGF-β can induce EMT (epithelial-to-mesenchymal transition) on stiff gels but cause apoptosis on soft gels in murine mammary gland cells [119]. Other times, cell-ECM interactions trump matrix stiffness such as mammary epithelial cells that undergo a malignant transformation on stiff matrices, but the effects are abrogated by the addition of basement membrane binding ligands [147]. Substrate rigidity has been implicated as an important factor to study in cancer biology and progression, but it also affects cell fate and phenotype, spreading, and intracellular signaling [146, 148–150].

In a landmark study, Engler et al. found that substrate stiffness can direct mesenchymal stem cell (MSC) differentiation [116, 151]. Importantly, soft materials similar to the elasticity of the brain caused neurogenic differentiation and stiff materials induced osteogenic markers [116]. Tissue rigidity is also associated with other pathologic conditions, such as aortic calcification, in which valves stiffen and cannot open and close properly to direct blood flow in the heart. However, the main cell population in heart valves, valvular interstitial cells (VICs), become activated
myofibroblasts when cultured on TCPS. Using PEG hydrogels, Benton et al. demonstrated that hydrogel stiffness could be used to control the fibroblast or myofibroblast phenotype of VICs [113], and further showed that VIC myofibroblasts can de-activate to quiescent fibroblasts when the matrix substrate is softened in situ [152]. Although tunable 2D culture substrates allow researchers to probe questions about the importance of matrix elasticity or adhesivity, there is a growing argument for the relevance and importance of 3D culture systems.

While two-dimensional hydrogel culture systems allow one to control matrix mechanics and composition, they are not immune to the concern of abnormal cell polarization and potentially altered cell proliferation, migration, and protease activity compared to cells embedded in a 3D matrix; all of these concerns are similar to cells cultured on TCPS. Thus, despite recapitulating certain aspects of the native extracellular matrix, hydrogel substrates do not simulate the dimensionality of native tissue namely, that tissues are 3D structures and cells exist and grow in a 3D environment.
Figure 1.10: Comparison of 2D versus 3D cell morphology. In 2D culture, epithelial and mesenchymal cells grow as flat monolayers and maintain dorsal-ventral polarization. In 3D, however, epithelial cells form multicellular structures with apical-basal polarity and contain a lumen in the center, similar to in vivo structures. When cultured in 3D, mesenchymal cells lose the artificial polarization and instead exhibit a fibroblastic morphology. Image from [8].

As a result, 3D hydrogel materials have been widely explored for recreating more physiologically relevant morphologies and environments (Fig. 1.10).

As mentioned previously, both naturally derived and synthetic hydrogel systems can be used to encapsulate cells. Some of the first materials explored for 3D culture of cancer cells were natural hydrogels, like Matrigel, which contain a complex mixture of endogenous signaling factors and proteins [96]. In a pivotal study from the Bissell group, Matrigel was found to cause spontaneous formation of acini structures with mammary epithelial cells (Fig. 1.7) [5]. This was a revolutionary finding: that the extracellular matrix can direct and influence the overall structure and function of cells in vitro. Since then, many commentaries and reviews have suggested the use of more physiologically-relevant 3D culture systems to study cell function [8, 88, 153, 154]. For example, breast cancer stiffness was correlated with collagen crosslinking, which then, in turn, formed focal adhesions, AKT signaling, and invasion [155]. Additionally, recent work from Mooney and coworkers showed that 3D matrix rigidity can induce a malignant phenotype in mammary ep-
ithelial cells but that the presence of binding ligands can abrogate this effect [147]. Metastatic breast cancer cells exhibited less invasiveness in stiffer collagen gels and were less sensitive to chemotherapeutic treatment, suggesting matrix elasticity may have a protective effect on breast cancer drug susceptibility [156]. Several other studies have shown that cells in a 3D structure are less sensitive to drug treatment [157, 158] potentially due to integrin-mediated polarity and subsequent signaling [97, 103] or recapitulation of the heterogeneity of a tumor microenvironment where cells at the edge of a tumor have access to nutrients and cells at the center are hypoxic and nutrient starved [40]. There is now overwhelming evidence to support the fact that the extracellular matrix influences cell function and even may trump cellular genotype [159–163].

In cancer biology, many researchers choose to utilize spheroids to better recapitulate a tumor environment. A spheroid consists of cells that are aggregated together to form multicellular structures of varying size that can then be embedded within a 3D matrix [38, 159, 164] or even simply cultured on top of a material substrate [90, 102, 165]. Breast cancer derived cells, in particular, have been shown to respond to the same drug treatment differently when cells are cultured on traditional TCPS compared to an acini structure on top of Matrigel [102, 103]. Some researchers argue that cancer cells cultured in 3D are generally more resistant to drug treatment; this may be viewed as a more physiologically relevant response and a better system with which to study drug responsiveness and drug resistance in vitro [158]. The changes in intracellular signaling from a 3D environment to 2D surfaces may explain the difference in drug responsiveness; surface chemistry, matrix mechanics, and cell-cell contacts can all influence signaling [166]. Melanoma cells have been shown to have over 100 upregulated and 73 downregulated transcripts between spheroids and TCPS cultured cells [167].

Other drug resistant responses have been observed in melanoma spheroids that were embedded within collagen matrices [39, 40]. Based on live/dead staining, researchers observed that melanoma spheroids derived from metastatic lines are far less sensitive to drug treatment than cells from earlier stage melanoma; the early stage melanoma spheroids were observed to have extensive cell death and very little outgrowth, while the metastatic spheroids contained more viable cells
and some outgrowth from the aggregate was observed [39, 40]. Additionally, when compared to traditional TCPS, spheroids embedded within collagen typically exhibit different, if not less, sensitivity to drug treatment, as discussed previously in Section 1.1.2 [19, 39, 40]. Colony formation as measured by cluster formation on agar was inhibited and transwell migration was increased in BRAF wild type cells by PLX4032 but did not cause significant cell death; in contrast, traditional 2D culture revealed decreased metabolic activity [21]. Examples such as increased migration in response to PLX4032 treatment are often only observed in 3D culture but not 2D [21, 168].

Collectively, these examples provide evidence as to the importance of culture conditions, and how a cell’s microenvironment might play an important role in dictating cellular responses to drug treatment. Researchers hope that in using culture platforms besides TCPS, better insight will result, especially related to the effects of matrix biology on cancer progression. Despite the increasing complexity of assaying cells in 3D, perhaps more clinically relevant responses of melanoma in vitro will be found, which in turn, should aid in understanding what drives melanoma drug resistance and how it might be counteracted through new therapeutic strategies.

1.4 Approach of this thesis

The overall goal of this thesis is to exploit peptide-functionalized PEG-based hydrogels as a culture system for melanoma cells at various stages of disease progression to answer fundamental questions pertaining to microenvironmental influences on melanoma drug resistance and responsiveness. Melanoma drug resistance is a major clinical problem despite the development of several new treatment strategies. While pioneering studies have identified the differential responses between cells cultured on TCPS and embedded multicellular spheroids, this thesis aims to answer which aspects of these culture systems may be fundamentally important for drug sensitivity. The Specific Aims of this thesis, their rationale, and brief context are outlined in Chapter 2. The goals are to understand 1.) whether matrix stiffness is important to melanoma malignancy (Chapter 3), 2.) whether or not dimensionality of cell culture affects melanoma responsiveness (Chapter 4), and 3.) how drug treatment may affect protease activity and potential metastatic behavior (Chapter
Substrate rigidity has been identified to be important in mammary epithelial cells’ transformation to a malignant phenotype [7], but the importance of stiffness has not been widely studied in vitro for melanoma. Chapter 3 outlines efforts to understand whether matrix elasticity has an effect on melanoma drug responsiveness in 2D. We study how differential sensitivities to stiffness may arise depending on the stage of progression the melanoma cells are derived from. While TCPS versus 3D spheroid studies have shown distinct responses to drug treatment in melanoma and breast cancer, for example [39, 102], there are few studies analyzing whether single cells in 3D will also result in similar differences in drug responsiveness compared to 2D cell culture. In Chapter 4, experiments are designed to provide a more direct 2D versus 3D comparison of single cells cultured in matrix environments with the same stiffness/moduli, but of varying dimensionality. In Chapter 5, we continue to study single cells in 3D microenvironments and aim to determine the influence of drug treatment on proteolytic activity and cell motility. The questions of matrix elasticity, dimensionality and side effects on drug responsiveness have not been well studied in melanoma, despite the difficulty in treating patients and understanding melanoma drug resistance. We aim to increase knowledge of the microenvironmental influences on melanoma drug responses and potentially provide a culture platform with which researchers can further study the signaling pathways that might contribute to drug resistance. The major conclusions and recommendations for future research directions are summarized in Chapter 6.
Chapter 2

Objectives

Melanoma is an aggressive form of skin cancer that is difficult to treat in later stages of progression. Despite the development of new therapeutics for metastatic melanoma, patients, on average, relapse within 6 months of starting treatment [2, 26]. As a consequence, there is active research to understand the drug resistant nature of melanoma and what key factors impact its survival. As an example, the cellular microenvironment is important to cell function and may influence how melanoma cells respond to pharmacological inhibition [103, 169]. As discussed in Chapter 1, the local environment can affect cancer cell differentiation, malignant phenotypes, or drug sensitivity. Preclinical melanoma studies have shown differential drug responses between cells seeded on traditional tissue culture-treated polystyrene (TCPS) and those in multicellular spheroids embedded within a soft collagen matrix [38, 39]. While researchers attribute more drug resistant behavior to three-dimensional (3D) environments, it is unclear whether interactions with soft (or stiff) matrices can promote this response, whether dimensionality (2D versus 3D) of the culture environment matters, or whether the existence of cell-cell contacts is paramount to drug responsiveness.

Cell-ECM (extracellular matrix) interactions play an important role by altering outside-in signaling and relaying information about the surrounding tissue to a cell. Studies have shown that the ECM can promote melanoma survival [9]; for example, fibronectin, but not collagen, protects cells from undergoing apoptosis [63]. The goal of this thesis research is to use hydrogel systems as synthetic ECM mimics to systematically study the influence of matrix elasticity
and dimensionality on the behavior of melanoma cells at different stages of disease progression and their response to a clinically relevant drug, Zelboraf (PLX4032, vemurafenib). Specifically, peptide-functionalized poly(ethylene glycol) (PEG) hydrogels are utilized to provide control over bulk properties, while probing specific questions about local cell-ECM microenvironment and interactions. We hypothesize that matricellular signaling will influence melanoma cell responses to pharmacological inhibition that suppress proliferation and promote apoptosis. To test this hypothesis, human cell lines derived from radial growth phase and metastatic melanoma are cultured on hydrogel surfaces or encapsulated within these synthetic ECM mimics with tunable elasticity and biological functionality (e.g., integrin binding epitopes, protease degradability). To gain insight into melanoma responses as a function of these microenvironmental cues, the specific aims of this thesis research are to:

Specific Aim 1. Examine the effect of BRAF inhibition on melanoma cell function when cultured on PEG hydrogels of varying elastic modulus.

While the influence of matrix elasticity on mammary epithelial cells has been studied in order to better understand breast cancer progression [7, 97, 155], the same has not been evaluated in melanoma. Clinically, tumor progression is linked with tissue stiffness [142, 143], and therefore, we hypothesize that cells cultured on rigid substrates will result in more resistant responses to the drug PLX4032. The hydrogel modulus is changed by altering the crosslinking density and studied over a physiologically relevant range of 0.6 to 13 kPa. Cells are seeded on these compliant surfaces (2D cultures), and the influence of substrate modulus on melanoma cells is assessed by measuring cell morphology and overall metabolic activity. A radial growth phase (RGP) and metastatic melanoma cell line are tested in order to elucidate whether the stage of progression changes the basal response to substrate elasticity. Proliferation and apoptosis are evaluated in the presence or absence of PLX4032 to determine if matrix elasticity affects the level of response to the drug. PLX4032 is known to be both cytostatic and cytotoxic [19, 22], so it is important to determine if substrate elasticity affects the potency of this drug in both measures of drug efficacy.
Specific Aim 2. Assess the influence of dimensionality and cell-cell contacts on responsiveness of melanoma cells to PLX4032 treatment during 2D and 3D culture in highly defined PEG-peptide matrices.

As an intermediate between traditional 2D culture of cells on TCPS and multicellular aggregates of 3D spheroids, a material platform is developed and identified that allows culture of melanoma cells on 2D surfaces, as well as encapsulation as single cells or as multicellular spheroids in 3D. The focus of this aim is to determine if multicellular spheroids might exhibit decreased apoptotic responses to PLX4032 due to cell-cell contacts or strictly due to dimensionality. Studies have established that cells cultured on TCPS compared to those encapsulated as aggregates have very different morphologies and signaling [40,84]; therefore, this research aims to clarify the importance of dimensionality on PLX4032-induced apoptosis. Specifically, melanoma cells are seeded on TCPS as a reference point as well as on top of and encapsulated within identical gel formulations to provide a more direct 2D - 3D comparison with treatment, while measuring viability. The metabolic activity and apoptosis in the presence and absence of PLX4032 are measured to determine if a 3D environment confers a more drug resistant response. Single cell encapsulations are then compared to spheroids to introduce complexity and examine the influence of a multicellular 3D cellular architecture in apoptotic responses to drug treatment.

Specific Aim 3. Examine matrix remodeling by melanoma cells cultured in 3D environments and quantify how drug treatment influences proteolytic activity and cell motility.

Based on findings in Aim 2, we hypothesized that PLX4032 treatment influences melanoma protease activity and subsequent matrix degradation and chose to study this phenomenon in cultures of single cells embedded in 3D environments. Matrix metalloproteinase (MMP) activity is an important component of the metastatic cascade and mediates tumor invasion into surrounding tissue [170]. Cells embedded in covalently crosslinked PEG-peptide hydrogels must degrade their local matrix environment in order to spread and migrate, whereas cells seeded on 2D surfaces
do not encounter barriers to cell spreading and movement. To determine the level of proteolytic activity as a function of environment, matrix degradation is quantified using an MMP cleavable crosslinker (GPQG1IWGQ) modified with a fluorogenic substrate to detect activity in situ. Importantly, the impact on matrix degradation is evaluated for four different human melanoma cells lines with and without four different inhibitors of the ERK signaling pathway. Changes in metabolic activity due to drug treatment are also measured, along with corresponding changes in cell shape and motility due to inhibitor treatment.
Chapter 3

Modulation of matrix elasticity with PEG hydrogels to study melanoma drug responsiveness


3.1 Abstract

Metastatic melanoma is highly resistant to drug treatment, and the underlying mechanisms of this resistance remain unclear. Increased tissue stiffness is correlated with tumor progression, but whether increased tissue stiffness contributes to treatment resistance in melanoma is not known. To investigate the effect of substrate stiffness on melanoma cell treatment responsiveness, PEG hydrogels were utilized as a cell culture system to precisely vary matrix elasticity and investigate melanoma cell responses to a commercially available pharmacological inhibitor (PLX4032). The tensile moduli were varied between 0.6 and 13.1 kPa (E) and the effects of PLX4032 on metabolic activity, apoptosis, and proliferation were evaluated on human cell lines derived from radial growth phase (WM35) and metastatic melanoma (A375). The A375 cells were found to be stiffness-independent; matrix elasticity did not alter cell morphology or apoptosis with PLX4032 treatment. The WM35 cells, however, were more dependent on substrate modulus, displaying increased apoptosis and smaller focal adhesions on compliant substrates. Culturing melanoma cells on PEG hydrogels revealed stage-dependent responses to PLX4032 that would have otherwise been masked if cultured strictly on TCPS. These findings demonstrate the utility of PEG hydrogels as a versatile *in vitro* culture platform with which to investigate the molecular mechanisms of melanoma biology and treatment responsiveness.
3.2 Introduction

Melanoma is an aggressive form of skin cancer that is difficult to treat in later stages by conventional chemotherapeutics. Early stage radial growth phase (RGP) melanoma is easily treated by surgical excision [11], while vertical growth phase (VGP) has a propensity to metastasize [12]. Metastatic melanoma, however, is notoriously drug resistant [13], characterized by a median survival time of 6-10 months [11, 14]. One promising new drug, PLX4032 (Vemurafenib), was approved by the FDA in 2011 for metastatic melanoma treatment. PLX4032 is a small molecule inhibitor that has high specificity towards mutated BRAF proteins and has been shown to cause both cell cycle arrest and induce apoptosis in melanoma [12, 19, 22, 24] and potent growth arrest in thyroid carcinoma cells [13, 32, 33]. More than 60% of all melanomas have been found to contain a BRAF mutation (V600E) that renders this protein constitutively active [18]. Clinical trials showed marked patient responses to PLX4032 [2, 25] and follow up studies reported 16-month overall survival [26]. Yet while PLX4032 is among the most promising melanoma treatments, patients eventually relapse and the mechanisms and contributing factors of melanoma drug resistance remain elusive.

As one approach to aid in preclinical compound screening and better understanding of the molecular mechanisms that might contribute to melanoma drug resistance, improved in vitro culture systems are being explored. Traditional tissue culture-treated polystyrene (TCPS) is often the initial culture platform used for drug screening, but it is orders of magnitude stiffer than most soft tissues in the body and may lead to physiologically irrelevant cellular morphologies or responses [8, 84, 85]. Matrix elasticity has been shown to regulate cell function in a number of different cell types, such as mesenchymal stem cells [116] and smooth muscle cells [118], and clinically, tumors are often found to be stiffer than the surrounding or healthy tissues [142, 143]. In vitro, increased substrate elasticity has been shown to induce malignant morphology in healthy mammary epithelial cells and lead to increased invasion of breast cancer cells [7, 97]. The underlying substrate can also alter intracellular signaling [7, 155], which ultimately may change the efficacy of
drug treatments. In fact, Weigelt et al. showed that when breast cancer cells were cultured on TCPS or Matrigel, the reduction of proliferation to clinically available drugs was altered [102].

Many studies have shown the importance of matrix elasticity on breast cancer cells, but the same is not yet known for melanoma. Unlike epithelial-derived breast cancer cells, melanoma is derived from melanocytes which arise from the neural crest [65], and so it is difficult to assume melanocytes and epithelial cells will respond similarly to a microenvironmental change like substrate elasticity. We hypothesized that matrix elasticity is important for assessing melanoma responses to drug treatment and that softer materials may provide better insight into physiologically relevant cellular responses. To investigate melanomas dependence on substrate modulus, we utilized peptide functionalized poly(ethylene glycol) (PEG) hydrogels as a highly tunable, hydrated, and chemically defined cell culture substrate that can be designed to recapitulate important aspects of the extracellular matrix (ECM) [122, 123]. In particular, the thiol-ene “click” chemistry was exploited to form crosslinked networks via step-growth kinetics involving the reaction of an ene functionalized multi-arm PEG with cysteine-containing peptides (-thiol) [6]. Cell-matrix interactions can be altered by the concentration of ECM molecule peptide mimics, such as the fibronectin-derived peptide RGDS [122]. Matrix remodeling can be controlled by inclusion of matrix metalloproteinase (MMP) degradable peptide sequences, allowing cell-mediated degradation [114]; alternatively, the hydrogel can also be rendered nondegradable by the inclusion of crosslinkers such as PEG-dithiols [136]. Finally, bulk biophysical properties, such as modulus or equilibrium water content, can be controlled by changing the network crosslinking density, which may be tuned by changing the concentration, molecular weight, or number of arms of the PEG [6, 135]. This innate tunability of this biomaterial provides an attractive cell culture platform to answer fundamental questions about cellular responses to microenvironmental changes.

Here, we sought to answer whether matrix stiffness would alter melanoma cell morphology and responses to PLX4032 treatment using this synthetic ECM mimic. Formulations based on a 4-arm norbornene-functionalized PEG and bifunctional cysteine-containing MMP-degradable peptides were crosslinked using the thiol-ene photopolymerization approach. The matrix elastic-
ity was varied from 0.6 to 13.1 kPa (E, Youngs modulus) with the aim of spanning a range of mechanical properties reported for healthy and pathologic tissue, and the resulting gels were then seeded with either RGP or metastatic melanoma cells. Cell morphology and cell-matrix interactions were assessed via immunostaining and focal adhesion size then viability was challenged with PLX4032 treatment. To test cell responsiveness to this inhibitor as a function of the microenvironment, metabolic activity, apoptosis, and proliferation were quantified and correlated to substrate elasticity.

3.3 Materials and Methods

3.3.1 Reagents

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. Cell culture reagents were purchased from Life Technologies unless otherwise noted. Antibodies used: monoclonal paxillin (Y113; Millipore) and goat anti-rabbit Alexa-Fluor 488. TRITC-phalloidin (Sigma-Aldrich) stocks were prepared at 0.06 mg/mL and DAPI stocks were 0.1 mg/mL. PLX4032 (ChemieTek) stocks were dissolved in DMSO (Sigma-Aldrich) at 100 mM.

3.3.2 Synthesis and characterization of macromers and peptides

Four-arm PEG-norbornene (MW: 20,000) (Figure 3.1) was synthesized as previously described [6]. Briefly, norbornene acid was coupled to form norbornene anhydride in dichloromethane (DCM) via N,N'-diisopropylcarbodiimide (DIC) coupling. PEG hydroxyl (JenKem Technology USA) was dissolved in DCM and reacted with the norbornene anhydride in the presence of pyridine and 4-(dimethylamino)pyridine (DMAP) overnight. The product was precipitated in cold diethyl ether 3 times, dried, and characterized by proton NMR for degree of functionalization. In all studies, PEG with >95% functionalization was used. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as previously described [133]. Briefly, 2,4,6-trimethylbenzoyl chloride was added to dimethyl phenylphosphonite (Acros Organics) and
allowed to stir overnight. Lithium bromide and 2-butanone were then added to the reaction, heated to 50°C for 10 minutes, and then cooled. The product was filtered, washed and filtered 3 times with 2-butanone, and allowed to dry. The product was verified by proton NMR. All peptides were purchased from American Peptide Company, Inc. An MMP-degradable crosslinker (KCGPQG\textsubscript{\textbullet}IWGQCK) and pedant adhesion peptide RGD (CRGDS) were used.

### 3.3.3 Formation and characterization of PEG-norbornene hydrogels

Gels were formed between a glass slide that was dipped in Sigmacote and a thiolated coverslip to create flat hydrogels for cell seeding. Coverslips were passed through a flame to remove contaminates, and then reacted in 95% ethanol (pH \(\sim\)5.5) with 0.55% (v/v) 3-mercaptoptrimethoxysilane for 3 minutes. Coverslips were then rinsed with 95% ethanol and allowed to dry in an 80°C oven for 15 minutes.

Macromer solutions were prepared with the concentrations of reagents in Table 3.1 with 1.7 mM LAP. In a sterile cell culture hood, 30 \(\mu\)L drops of macromer solution were placed on sterile Sigmacote-coated slides and then covered with a thiolated 18 mm circle coverslip (Fisher Scientific). The solutions were placed under a UV lamp centered around 365 nm light at \(\sim\)5 mW/cm\(^2\) for 3 minutes. The formed gels were then allowed to swell in PBS for at least 2 hours, then sterilized with 5% IPA in PBS at room temperature for 1 hour. Before cell seeding, the gels
Table 3.1: Macromolecular monomer compositions for gel formulations.

<table>
<thead>
<tr>
<th></th>
<th>[PEG] (mM)</th>
<th>[Crosslinker] (mM)</th>
<th>[Pendant RGD] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiff</td>
<td>7.5</td>
<td>14.25</td>
<td>1.5</td>
</tr>
<tr>
<td>Medium</td>
<td>3</td>
<td>4.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Soft</td>
<td>3</td>
<td>3.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

were rinsed twice with PBS. Unless otherwise noted, 3 gels were formed per condition for each of 3 independent experiments.

For characterization, 30 µL gels were formed in the cut-end of a 1 mL syringe. The same gel formulations as those used for cell culture were made (Table 3.1). To determine the equilibrium swollen gel mass, the gels were allowed to swell overnight in PBS then weighed the next day. The mass swelling ratio q (Table 3.2) was calculated from the equilibrium swollen mass and the calculated theoretical dry mass. The water content (% Water) was calculated using the mass swelling ratio (1-q\(^{-1}\)) and converted to a percent. The shear modulus (G) was measured on a DHR-3 shear rheometer (TA Instruments) and converted to Young's modulus, where \(E = 2G(1 + \nu)\) assuming a Poisson's ratio (\(\nu\)) of 0.5 [171]. Strain and frequency sweeps were performed to ensure measurements were within the linear viscoelastic regime. Averages represent 3 independent experiments.

### 3.3.4 Cell Culture

Both cell lines were a generous gift from Professor Natalie Ahn, Department of Chemistry and Biochemistry, University of Colorado. All cell lines were cultured in 10% fetal bovine serum

Table 3.2: Measured values of modulus and mass swelling ratio.

<table>
<thead>
<tr>
<th></th>
<th>Equilibrium Mass Swelling Ratio q</th>
<th>Young's Modulus E (kPa)</th>
<th>Crosslink Density (\rho_{XL}) (mM)</th>
<th>% Water</th>
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<td>Stiff</td>
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<td>13.1 ± 0.4</td>
<td>5.17 ± 0.40</td>
<td>95.3 ± 0.2</td>
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<td>Medium</td>
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<td>97.7 ± 0.1</td>
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<tr>
<td>Soft</td>
<td>62 ± 1</td>
<td>0.6 ± 0.1</td>
<td>0.36 ± 0.05</td>
<td>98.4 ± 0.1</td>
</tr>
</tbody>
</table>

\(^1\)Crosslink density was calculated from rubber elasticity theory: \(\rho_{XL} = GQ^{1/3}(RT)^{-1}\)
(FBS) in RPMI 1640 without phenol red. For experiments, cells were seeded at 1 x 10^5 cells/cm^2 in 1% FBS in RPMI 1640 and subsequently cultured in 1% FBS for the remainder of any experiment. Cells were seeded on gels or TCPS and allowed to adhere overnight. DMSO control (0.01% v/v) or 1 µM PLX4032 diluted in media was then added to the corresponding samples and incubated for 48 hours.

### 3.3.5 Metabolic Activity

At the end of the 48-hour drug incubation time, CellTiter-Glo (Promega) was added to samples per the manufacturers instructions. Samples were placed on an orbital shaker for 2 minutes, and then 100 µL of lysate was transferred to a white 96-well plate and the luminescence was measured on a BioTek H1 Synergy plate reader (BioTek). To measure basal metabolic activity, standard curves of ATP and DNA were generated. After applying CellTiter-Glo to samples, the cell lysate was then diluted to quantify DNA content by Quant-it PicoGreen (Life Technologies).

### 3.3.6 Apoptosis

After 48 hours of PLX4032 treatment, the EnzChek Caspase-3 Assay Kit #2 (Life Technologies) was used to assess apoptosis. Briefly, floating cells were collected by centrifugation and attached cells were removed with TrypLE Select for 5-10 minutes at 37°C and then agitated to remove as many cells possible. Cell pellets were then lysed with the provided lysis buffer and then the company protocol was followed. DNA content was measured via PicoGreen (Life Technologies), and caspase 3 activity was normalized to the DNA content in a given sample. For analysis, each PLX4032-treated sample was normalized to its corresponding DMSO control sample to show a fold change in caspase 3 activity with PLX4032 treatment.

### 3.3.7 Proliferation

During the last 16 hours of incubation with or without the inhibitor, the cells were pulsed with EdU to detect cells entering S-phase. After a total of 48 hours, samples were processed
according to the protocol for the Click-iT EdU Alexa-Fluor 488 kit (Life Technologies). One exception was to invert the coverslips on 10 µL drops of the Alexa-Fluor 488 solution. For these experiments, 12 µL gels were formed on 12 mm circle coverslips (Fisher Scientific). Samples were then imaged on an inverted Nikon TE2000; three images per gel were taken and duplicate gels were prepared for each condition. Three independent experiments were performed. EdU quantification was performed using an ImageJ (NIH) macro to threshold images and count the number of nuclei positive for DAPI (blue) and EdU (green).

3.3.8 Immunostaining

For immunostaining experiments, cells were fixed after 24 hours of culture. Half the volume of media in the well was removed and replaced with 1:10 buffered formalin (Sigma-Aldrich) for 20 minutes at room temperature and rinsed twice with 0.05% Tween 20 (Sigma-Aldrich) in PBS. Samples were then permeabilized with 0.1% Triton-X (Fisher Scientific) for 5 minutes, washed twice, and then blocked for 30 minutes with 1% bovine serum albumin (BSA) in PBS. Samples were incubated with the primary antibody diluted in blocking buffer for 1 hour at room temperature, washed 3 times, and the secondary antibody was applied for 45 minutes at room temperature. Antibodies were used at: paxillin 1:400, goat anti-rabbit AlexaFluor 488 1:200, TRITC-Phalloidin 1:200, DAPI 1:1000. Samples were imaged on a Zeiss NLO LSM 710 (Zeiss) at 40X.

3.3.9 Focal adhesion quantification

ImageJ (NIH) was used to quantify the focal adhesion area on a per cell basis. For each cell that formed visible focal adhesions, the cell was cropped from the image and the RGB channels separated. If there was high cytoplasmic staining of paxillin in green, then the background was subtracted using a rolling ball radius of 15 pixels. The image was thresholded, made binary, and the area was measured using the Analyze Particles function. At least 10 different cells were analyzed for focal adhesion size from each of 3 independent experiments.
3.3.10 Statistical analysis

To compare metabolic activity and apoptosis samples, a one-way ANOVA was performed in Prism 5 (GraphPad Software, Inc). To compare treated and control EdU staining samples within each stiffness condition, a two-way ANOVA was performed with Bonferroni posttests in Prism. The data was then transposed and a two-way ANOVA with Bonferroni posttests was performed that compared the control samples for each stiffness to each other and then compared the treated samples for each stiffness to each other. For focal adhesion quantification, a Shapiro-Wilk test for normality was performed in R for each cell type/condition from each independent experiment. The distributions were found to be non-normal, and therefore a modified Levene’s test was performed in R to determine if the variances among the experiments for each condition were different. Because the samples were deemed to have statistically similar variances, we pooled the data (>30 cells for each condition) from all three experiments and used a Kruskal-Wallis test in Prism to compare culture conditions within each cell type.

3.4 Results

3.4.1 Regulation of cell-matrix adhesion by substrate elasticity

To assess the effects of matrix elasticity on melanoma responses to drug treatment in a range of physiologically relevant moduli, PEG-based hydrogels were engineered such that the matrix moduli were systematically altered through changes in the network crosslinking density. Specifically, in these studies, 4-arm 20 kDa norbornene-functionalized PEG was crosslinked with a bi-functional cysteine-containing MMP-degradable peptide (Figure 3.1). Gels were formed via the thiol-ene polymerization reaction, which was photoinitiated with LAP under ~5 mW/cm² of UV light centered around 365 nm. To promote cell adhesion, 1.5 mM of the pendant fibronectin-derived RGDS peptide was incorporated. Fibronectin is present in melanoma tumors and can promote invasion [172], and the integrin pair αvβ3, which readily binds to RGDS, is upregulated in tumorigenic melanoma cells [173]. An MMP degradable crosslinker was selected to better re-
capitulate cell-mediated degradation of the local microenvironment observed *in vivo* and to enable comparisons to 3D studies where degradability is necessary for cell spreading, survival, and proliferation. While the mechanical properties of the gels may change with cells present, due to matrix deposition and/or MMP degradation, changes in modulus were minimized by the use of PEG (to minimize protein adsorption), low cell density, and relatively short culture times. Additionally, by using a range of moduli over three orders of magnitude, the mechanical properties of the gels may change over the course of the experiment, but the large relative difference between the soft and rigid gels remains, as evidenced by the differences in cell spreading observed at the beginning and end of the experimental timeline.

The bulk modulus of the materials was varied by changing either the concentration of PEG (7.5 mM or 3 mM) or altering the concentration of the MMP crosslinker (Table 3.1). Using these formulations, we were able to achieve a range of moduli from approximately 13.1 kPa (E, Youngs modulus) to 0.6 kPa. The crosslinking density was calculated from rubber elasticity theory [171](see Table 3.2 footnote), and the equilibrium mass swelling ratio was also measured to determine the water content of each gel (Table 3.2). The modulus of skin and melanoma tumors is not well established in the literature, so we chose a range of moduli: the softest gel formulation of 0.6 kPa (soft), 1.6 kPa (medium), and 13.1 kPa (stiff). This modulus range encompasses physiologically relevant tissues from breast tissue (0.1-0.5 kPa) to relaxed muscle (approximately 10 kPa) [116, 174]. Because increased tissue stiffness is often correlated with tumor formation [175], several gel elasticities were used for subsequent studies. TCPS samples were included as controls in order to compare results to the established literature related to studying melanoma cell responses to BRAF inhibition.

To evaluate whether substrate modulus affected cell morphology and cell-matrix interactions, immunostaining for the actin cytoskeleton and the focal adhesion component, paxillin, was performed on all substrates under control conditions (Figure 3.2A). Increased focal adhesion formation has been linked to increased ERK signaling [7, 97], an important survival pathway. Therefore, we reasoned that melanoma cell morphology and cell-matrix interactions could be regulated
Figure 3.2: Effect of substrate elasticity on overall cell morphology and focal adhesion formation. (A) Immunostaining for paxillin (green), f-actin (red) and nuclei (blue) on TCPS, stiff, medium, and soft gels of both cell types. Inset image is magnification of outlined area. (B) Quantification of focal adhesion area based on image analysis. n = 3 ± SEM. *p < 0.05. Scale bars, 20 µm.

by matrix elasticity, where stiff substrates induce a more spread morphology and mature focal adhesion formation compared to soft substrates.

The RGP WM35 cells were well spread on TCPS with distinct f-actin fibers around the peripheral edges and punctate paxillin staining (Figure 3.2A). In contrast, when cultured on softer PEG gels, the WM35 cells appeared more rounded and exhibited fewer f-actin stress fibers. Depending on the stiffness of the gel, approximately 50-75% of cells exhibited punctate paxillin staining. Of the WM35 cells that had distinct focal adhesion formation, cells formed much smaller adhesions as the gel elasticity was decreased. Focal adhesion area was quantified using image analysis, and there was a general downward trend of decreasing focal adhesion size with decreasing
substrate modulus (0.70 \(\mu\text{m}^2\) on the stiffest to 0.44 \(\mu\text{m}^2\) on softest gels) (Figure 3.2B). Overall, results demonstrate that the WM35 cells are sensitive to the underlying substrate elasticity and consequently form stiffness-dependent focal adhesions.

In contrast to the WM35 cells, the metastatic A375 cells were much smaller and had more irregular morphologies, often exhibiting f-actin patterns that indicated lamellipodia formation on TCPS, stiff, and medium gels. Furthermore, when cultured on gels, the A375 cells appeared slightly rounded though still able to form focal adhesions, based on punctate paxillin staining. Again, focal adhesion size was quantified and found to be approximately the same (between 0.44-0.54 \(\mu\text{m}^2\)), independent of the underlying substrate elasticity, while the size of the adhesions were generally smaller than that observed for the RGP WM35s (Figure 3.2B).

### 3.4.2 Effect of substrate elasticity on cell responsiveness to drug treatment

To determine if basic cell function, with no drug treatment, was altered due to substrate elasticity-induced changes in morphology, basal metabolic activity was assessed on TCPS and stiff, medium, and soft gels. Basal metabolic activity was measured by normalizing ATP to DNA content within a sample to provide a measure of metabolic activity on a per cell basis (Figure 3.3). For both cell types, the basal metabolic activity was not significantly different between the different culture conditions.

To evaluate how matrix elasticity regulates the response of melanoma cells to drug treatment, WM35 and A375 cells were cultured on TCPS and stiff, medium, and soft gels and treated with PLX4032. As an initial measure of drug-induced effects, metabolic activity was measured after 48 hours of 1 \(\mu\text{M}\) PLX4032 treatment (Figure 3.4). The metabolic activity in each treated sample was normalized to its respective control (i.e., the TCPS PLX4032-treated sample was normalized to a TCPS DMSO-containing sample) to yield relative metabolic activity present after drug treatment. The WM35 cells exhibited a significant response on all substrates as measured by a reduction in metabolic activity with drug treatment to approximately 0.55 of the control conditions. Similarly, the A375 cells exhibited a significant decrease in metabolic activity to no more than 0.43 of the
control conditions on all culture substrates. Among the different substrates, however, the change in metabolic activity was not significant. Because the WM35 cells exhibited stiffness-dependent focal adhesion formation, we had hypothesized that there would be increased cell death on soft substrates. However, here we observed that PLX4032 drastically reduced metabolic activity on both TCPS and gels and there was no significant change with substrate stiffness. Metabolic activity measurements, however, cannot differentiate between the cytotoxic (cell death) and cytostatic

Figure 3.3: Basal metabolic activity (ATP normalized to DNA content) measured under control conditions on all culture substrates. None of the samples were statically different. n = 3 ± SEM.

Figure 3.4: Relative fraction of metabolic activity for PLX4032-treated cells compared to cells cultured on each respective substrate without inhibitor. The dashed line represents the control samples. None of the treated samples for either cell type were statistically different metabolic activity based on culture substrate. n = 3 ± SEM.
(anti-proliferative) effects of PLX4032 and may not capture the specific effects of PLX4032 treatment. Therefore, we next measured cell death directly via apoptosis.

### 3.4.3 Decoupling cytostatic and cytotoxic effects of drug treatment

Apoptosis was measured via caspase 3 activity, and treated samples were normalized to their respective controls (i.e., the TCPS PLX4032-treated sample was normalized to a TCPS DMSO-containing sample) to show the fold change in caspase 3 activity (Figure 3.5). Interestingly, the WM35 cells exhibited increased sensitivity to PLX4032 as the substrate modulus decreased. On TCPS there was a 2-fold increase in caspase 3 activity with drug treatment, while on the soft gels, there was a nearly 3-fold increase. In contrast, the A375 cells did not exhibit major changes in the levels of apoptosis as a function of substrate properties. There was a smaller increase in apoptosis on TCPS (approximately 1.7-fold), but no significant increases were observed for cells cultured on gels. Based on this evidence, we concluded that the WM35 cells were more susceptible to PLX4032 treatment on gels as compared to the A375 cells. Because PLX4032 has been shown to both induce apoptosis and cause cell cycle arrest, we next investigated whether or not the decrease in A375 metabolic activity could be the result of PLX4032s antiproliferative effect rather than its

![Figure 3.5: Apoptosis measured via caspase 3 activity on different culture substrates. Each bar represents the PLX4032-treated sample normalized to its respective control showing the fold increase in caspase 3 activity with drug treatment. None of the A375 changes in apoptosis were statistically different. n = 3 ± SEM. *p <0.05](image-url)
cytotoxic effects.

To measure proliferation, an EdU assay was used and the percentage of cells that were stained positive was quantified (Figure 3.6). In general, drug treatment led to less than 1% of cells undergoing DNA synthesis on any substrate. The WM35 cells cultured on stiff and soft gels, as well as the A375 cells on soft samples, that were treated with PLX4032 had no cells entering S phase. Interestingly, in both cell types, there was an increase in the percent of proliferating cells when

![Figure 3.6](image)

Figure 3.6: Percent of cells entering S phase as quantified by EdU staining. White bars (left y-axis) represent control conditions; grey bars (right y-axis) represent PLX4032-treated samples. n = 3 ± SEM. Three fluorescent images were taken for each gel or TCPS well, the percent of positive EdU cells quantified, and then averaged for each sample. *p < 0.01 compared to TCPS control; #p < 0.0001 for PLX4032 samples compared to corresponding control; **p < 0.01.
cultured on top of gels without drug treatment. Specifically, we observed \(~39\%\) of A375 cells on TCPS undergoing proliferation, but that number increased to as high as \(68\%\) on the gel formulations. The WM35 cells exhibited a less dramatic increase from \(46\%\) on TCPS to a maximum of \(64\%\) on medium gels, but the increase was statistically significant.

3.5 Discussion

Here, using PEG hydrogels as a 2D cell culture platform, we assessed how substrate elasticity affects melanoma cells’ sensitivity to drugs and whether or not the treatment responsiveness was tumor stage-dependent. The modulus of healthy and tumorigenic skin tissue is not well established [175], and so a range of elasticities was chosen to capture both states. Previous studies have measured that a range in Young’s modulus from 0.2 kPa to 1.2 kPa will elicit vastly different morphologies in mammary epithelial cells in vitro [7]. Breast tissue, however, is among the softest tissues, so here we used a broad range of elastic moduli in an effort to encompass both healthy and tumorigenic tissue stiffness (0.6 to 13.1 kPa).

To examine the effect of drug treatment on melanoma cell function, metabolic activity was chosen as an initial indication of drug sensitivity, however, it ultimately was not indicative of the phase-dependent responses to substrate modulus. The WM35 cells showed large decreases in metabolic activity in response to PLX4032, and further analysis revealed this decrease was due to increased apoptosis on compliant gels coupled with almost complete inhibition of proliferation. The increase in apoptosis on more compliant substrates appeared to counteract the increased proliferation that we observed, resulting in similar losses of metabolic activity with PLX4032 treatment.

In comparison, the A375 cells exhibited slightly larger decreases in metabolic activity than the WM35 cells. Similarly to the WM35 cells, the observed decrease in metabolic activity showed an incomplete picture of the cellular responses to drug treatment. We found that A375 levels of apoptosis did not change with matrix elasticity. When cultured on any of the gels, A375 cells proliferated more than when cultured on TCPS; more cells were entering S-phase based on EdU staining. PLX4032 treatment induced potent cell cycle arrest, where very few cells were undergoing
DNA synthesis. Taken together, A375 metabolic activity might have been low on PLX4032-treated hydrogel substrates due in large part to inhibition of proliferation rather than increasing levels of apoptosis on compliant gels.

We note that many reports have demonstrated that 3D cellular architecture or culture platforms are more physiologically relevant (reviewed in [86] and [87]) and can elicit very different cellular responses to the same stimuli when compared to TCPS culture [158]. Cells cultured in 3D microenvironments using synthetic hydrogels [158] or naturally derived materials like collagen [99, 100], exhibit resistance to drug treatment as compared to 2D TCPS culture. While naturally derived hydrogels, such as reconstituted basement membrane (Matrigel), can promote more physiologically relevant multicellular acini structures [99] and altered responses to clinically available drugs compared to TCPS samples [102], 3D cell culture is not without complexity. One difficulty relates to performing experiments on encapsulated samples; in order to perform certain assays, the gels must be degraded in order to collect the cells. A second drawback of 3D culture is that cells locally remodel their environment in 3D (e.g., degrading the local matrix to allow morphological changes), which can make it difficult to control and quantify the local material properties in the pericellular region. Thus, we decided to first study melanoma cells on the surfaces of hydrogels, as a simple alternative to TCPS or encapsulated cells and explore the mechanisms of melanoma responses to matrix elasticity and drug treatment in a highly controlled environment. Specifically, the substrate elasticity is tunable and constant, and the drug treatment is introduced in a relatively homogeneous manner. While the PEG hydrogel system is completely compatible with future 3D cell culture studies, by using a 2D system, assays such as western blotting and apoptosis can be performed similarly to TCPS experiments.

Although 2D cell culture has its benefits, there are many excellent studies that exploit the advantages of 3D culture to recapitulate important aspects of cell-cell adhesion or a 3D microenvironment to study melanoma drug responsiveness [21, 40]. Three-dimensional melanoma spheroid studies have provided platforms with which to observe decreased efficacy of drugs in vitro that may prove crucial to elucidating drug resistant mechanisms or ineffective drugs in preclinical stud-
ies. For example, metastatic melanoma cells survive in 3D aggregates with the MEK inhibitor UO126 while radial and vertical growth phase cells are more susceptible [38]. Additionally, another MEK inhibitor AZD6244 was shown to be cytostatic on collagen-embedded spheroids, and wash out experiments showed the effects were reversible [39]. Unlike the MEK inhibitors, however, PLX4032 was found to be effective in 3D to induce apoptosis and prevent aggregate outgrowth in collagen [19,22]. While spheroid studies show V600E mutated melanoma susceptibility, clinical efficacy suffers from patient relapse [2,26]. Therefore, better methods are needed to study melanoma in vitro to provide new insight into treatment responsiveness and cellular mechanisms.

While naturally derived materials such as collagen and reconstituted basement membrane provide physiologically relevant moduli, there is also inherent variability in the material, sequestration of growth factors, and ill-defined chemistry [93]. Alternatively, PEG hydrogels serve as a highly defined bioinert scaffold that allows precise tuning of properties such as modulus or cell-mediated degradation [93]. Using PEG hydrogels as a 2D substrate for cell culture, we observed stiffness-independent apoptotic responses in the metastatic A375 cells while the RGP WM35 cells exhibited increased sensitivity when cultured on more compliant substrates. These findings correlate with previous 3D culture studies, but using the 2D PEG hydrogel system, we were able to investigate how matrix stiffness specifically affects treatment responsiveness in melanoma cells.

To better understand the effect of substrate rigidity on apoptosis, cell morphology and focal adhesion size were examined for a possible correlation between cell-matrix interactions and response to PLX4032 treatment. Metastatic A375 cell morphology, focal adhesion size, and levels of apoptosis showed a weak dependence on matrix elasticity compared to the RGP WM35 cells. The WM35 cells formed smaller focal adhesions on softer substrates and appeared more rounded. Despite the different morphologies, this did not significantly alter basal metabolic activity. However, when cell survival was challenged with drug treatment, increased levels of apoptosis were observed in cells cultured on more compliant gels. Matrix stiffness revealed different responses to PLX4032 between the two cell lines that were not seen on TCPS. Taken together, the metastatic cells were able to adhere and survive equally well on soft (0.6 kPa) and very stiff (TCPS) substrates,
exhibiting stiffness-independent survival while the WM35 cells were dependent on the underlying substrate modulus when cell survival was challenged with drug treatment.

Based on our findings, we hypothesize that the WM35 cells interact with their microenvironment by forming matrix stiffness-induced focal adhesions. As a result, the WM35 cells retain the ability to sense the rigidity of the underlying substrate, and on softer materials, they form fewer, smaller focal adhesions, decreasing survival signaling, and ultimately increasing PLX4032 sensitivity as indicated by increased apoptosis (Figure 3.7). Metastatic A375 cells, on the other hand, appear to have decreased mechanosensing abilities, and changes in substrate compliance did not alter focal adhesion formation or PLX4032 sensitivity. The A375 cells were able to maintain similar levels of cell-matrix interactions on all substrates and showed little differences in apoptosis.

Focal adhesion formation may regulate susceptibility to PLX4032 treatment based on survival signaling through BRAF-MEK-ERK and/or PI3K-AKT. Matrix elasticity can regulate focal adhesion formation in breast cancer and increase ERK signaling [7, 97]; the same phenomenon may occur in melanoma. In addition, PI3K signaling via AKT has been correlated with increased survival of melanoma with MEK or BRAF inhibitor treatment [63]. Boisvert-Adamo and Aplin showed that AKT contributes to adhesion-dependent protection from anoikis in melanoma [62], and that increased AKT levels have been found in drug resistant melanoma cell lines [63]. Focal adhesion signaling can regulate both BRAF-MEK-ERK and PI3K-AKT signaling, and thereby affect treatment responsiveness. Perhaps PI3K-AKT signaling is increased in the A375 cells, which promotes an anti-apoptotic phenotype, while the WM35 cells are heavily oncogene addicted to BRAF-MEK-ERK. The ability to sense the local microenvironment, however, may be stage-dependent in melanoma. The interaction between pathways, involvement of focal adhesion formation and the subsequent signaling cascade may provide clues to melanoma drug responses. Ultimately, this interplay between pathways and the interaction of melanoma tumor cells with the microenvironment may alter the efficacy of BRAF and MEK inhibitors.
Figure 3.7: Schematic of the effect of substrate stiffness on WM35 and A375 cells. WM35 cells exhibit dependence on substrate modulus by forming stiffness-dependent focal adhesions (green dots) and increase f-actin stress fiber formation (red lines). We hypothesize that for the WM35 cells, increased signaling from focal adhesion formation, perhaps an indication of stronger cell-matrix interactions, can increase survival signaling to inhibit apoptosis induced by PLX4032. In comparison, A375 cells show little dependence on the underlying matrix elasticity, always forming similarly sized matrix interactions. The lack of large focal adhesions, however, does not increase levels of apoptosis. Instead, A375 cells may have very different intracellular signaling that compensates for the lack of stiffness dependence for survival.

3.6 Conclusions

We investigated the use of PEG-based hydrogels for 2D cell culture to determine how extracellular matrix elasticity affects melanoma responses to PLX4032 treatment. Metastatic A375
cells can better mitigate the cytotoxic effects of PLX4032 on more compliant substrates, which led to decreased proliferation more than an increase in apoptosis. Conversely, early stage RGP melanoma cells exhibited increased susceptibility to PLX4032 on compliant substrates due to increased apoptosis. We hypothesize that these differences may be linked to the ability to form cell-matrix interactions via substrate rigidity-regulated focal adhesion formation. Overall, culturing melanoma cells on compliant hydrogel platforms may provide a more physiologically relevant culture system than TCPS and provide better insight into what microenvironmental factors contribute to the drug resistant nature of melanoma.

3.7 Acknowledgements

The authors thank Dr. Natalie Ahn (University of Colorado, Boulder) for kindly providing the melanoma cell lines. The authors also wish to thank Dr. Huan Wang and David Cate for helpful technical discussions, Dr. Chelsea Kirschner and Dr. William Wan for statistical analysis advice, and Caitlin Jones for technical assistance. This work was supported by the National Institutes of Health (# R01 CA132633) and the Howard Hughes Medical Institute. This work was done in collaboration with Dr. Jennifer Leight.
3.8 Supplemental Information

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Figure 3.S1: Representative fluorescent images from EdU staining. Nuclei (blue) that are also positive for EdU (green) represent cells that have entered S phase in the cell cycle. On PLX4032 samples, there were fewer cells present to image, hence significantly lower cell density in the images. Scale bars, 100 µm.
Table 3.S1: Change in equilibrium swelling compared to initial swollen state at polymerization.

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Chapter 4

PEG-peptide hydrogels reveal differential effects of matrix microenvironmental cues on melanoma drug sensitivity

Prepared for submission to Integrative Biology, April 2015

4.1 Abstract

Metastatic melanoma is highly drug resistant, though the exact mechanisms of this resistance are not completely understood. One method to study melanoma drug responsiveness \textit{in vitro} is through the use of multicellular spheroids, which have been found to exhibit decreased drug sensitivity compared to traditional 2D culture on various substrates. Because it is unclear whether dimensionality and/or cell-cell contacts may influence melanoma drug responsiveness, we utilized a synthetic PEG-based hydrogel to compare the response of cells cultured on top of or encapsulated within the matrix. We found that depending on the stage of progression with which the melanoma cells were derived, the cells responded differently to PLX4032 treatment, a commercially available melanoma drug. Specifically, early stage cells were insensitive to dimensionality (i.e., 2D versus 3D culture), while metastatic cells exhibited decreased responsiveness in 3D compared to 2D. To further understand the role of the microenvironment on early stage melanoma cells, we tested single cells and multicellular spheroids in 3D. Results revealed that the spheroids were less sensitive to melanoma treatment compared to single cell encapsulations. Collectively, this study implicates the role of dimensionality on metastatic melanoma drug responsiveness, and the potential influence of cell-cell contacts in early stage melanoma on the apoptotic response to PLX4032.
4.2 Insight, Innovation, and Integration

Using a tunable, fully synthetic, and cytocompatible hydrogel scaffold, we compared melanoma cells seeded on top of soft hydrogels (2D) or encapsulated within the same hydrogel formulation as a single cell suspension (3D) to assess the role of dimensionality on melanoma apoptotic responses to drug treatment. Current *in vitro* methods to evaluate drug responsiveness include culture on traditional plastic (2D) and multicellular spheroids embedded within a matrix (3D). While 3D spheroid models have shown decreased drug responsiveness in cancer cells compared to 2D culture, it is difficult to determine whether dimensionality, cell-cell contacts, or matrix elasticity are the most important microenvironmental factors affecting drug responsiveness. Here, we elucidate which factors may be important to study melanoma drug resistance *in vitro*.

4.3 Introduction

Traditional two-dimensional (2D) culture of cells on tissue culture-treated polystyrene (TCPS) has allowed invaluable characterization and improved the quantitative understanding of basic cell signaling and function. However, these 2D surfaces are often aphysiologically stiff (six orders of magnitude stiffer than most soft tissues), can un-naturally polarize cells, and do not necessitate any matrix remodeling for proliferation and migration as occurs *in vivo* [86, 176]. As a result, three-dimensional (3D) hydrogel culture platforms have evolved to present a more *in vivo*-like microenvironment [94,177–179]. For example, a pioneering study by Petersen *et al.* illustrated a fundamental difference in breast epithelial cells, where they proliferated indefinitely as a monolayer on TCPS but formed *in vivo*-like multicellular acinar structures when embedded in Matrigel [5]. Since this landmark publication in 1992, 3D matrix environments have become more common in the study of cancer biology and drug responsiveness, as key parameters of matrix signaling can be quite important in predicting *in vivo* outcomes from *in vitro* screens [3,4,157].

In cancer research, 3D models often utilize multicellular spheroids, where cells are either aggregated or allowed to proliferate when embedded within hydrogel microenvironments, which
are typically composed of collagen or Matrigel [38, 99, 159]. Numerous studies have reported altered or increased resistance to drug treatment in these multicellular aggregates compared to traditional 2D culture on TCPS [38, 39, 102, 157, 158, 180]. Researchers have hypothesized that a 3D environment better recapitulates the native environment that cancer cells might experience, where cell-matrix and cell-cell interactions can promote survival [8, 13, 40, 179, 181]. As a result, the use of 3D models has advanced to become a more standard method to better evaluate and predict drug candidate efficacy before studying their effects in animal models [88].

While experiments using 3D spheroids have shown differential responses to the same drug treatment compared to cells in monolayer culture, numerous differences exist between cell aggregates and cells on hard plastic surfaces [87]. For instance, on TCPS, cells are unnaturally polarized, are exposed to a sink of nutrients or drugs without any diffusion length scale, and cell-matrix interactions are generally extensive [84, 86, 160]. This is in stark contrast to 3D spheroids, where spatial positioning of the cells can matter, cell-cell interactions are numerous, and the elasticity of the microenvironment is dramatically different than TCPS [166, 182].

With this in mind, we sought to explore the role of dimensionality, in a more controlled manner, on melanoma apoptotic responses to clinically available drugs. We utilized fully synthetic PEG-based hydrogels in order to simplify the culture system compared to naturally derived 3D systems, such as collagen [93]. These PEG-peptide hydrogels were formed via the thiol-ene “photoclick” reaction through step-growth network formation [123] between norbornene-functionalized multi-arm PEG and cysteine containing peptides [6]. The thiol-ene reaction is cytocompatible and therefore allows for culture as both a 2D and 3D culture platform with wide tunability of bulk properties [135, 139]. Previously [183], we reported that early stage radial growth phase melanoma cells (WM35) were sensitive to substrate elasticity in 2D, and this in turn, affected their drug responsiveness to PLX4032 (clinically, Zelboraf or vemurafenib). Metastatic A375 cells, however, appeared less reliant on substrate elasticity, with little changes in drug responsiveness as a function of the stiffness of the local environment. In order to make more direct comparisons between 2D and 3D environments, WM35 or A375 cells were seeded on top of and also encapsulated within hydrogels
of the same composition as a single cell suspension. Viability was then challenged with PLX4032 for 48 hours and metabolic activity and apoptosis were measured. Finally, studies comparing single cell encapsulations with embedded spheroids were performed. Collectively, experiments were designed to better understand what aspects of the spheroid “tumor-like” environment contribute to its more “drug resistant” phenotype that is often studied and described in the literature.

4.4 Methods

4.4.1 Reagents and Materials

Unless otherwise noted, all cell culture reagents were purchased from Life Technologies and all chemicals were purchased from Sigma. PEG was purchased from JenKem and functionalized with norbornene as previously described [6]. All peptides were purchased from American Peptide Company. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was synthesized as previously described [133]. PLX4032 was purchased from Chemietek and made to a stock of 10 mM in DMSO.

4.4.2 Cell culture

Radial growth phase human melanoma cells (WM35) and metastatic A375 cells were a generous gift from Professor Natalie Ahn (University of Colorado, Department of Chemistry and Biochemistry). Cells were cultured in RPMI 1640 without phenol red in 10% FBS at 37°C and 5% CO₂. Experiments were performed with 1% FBS.

4.4.3 Hydrogel preparation for 2D and single cell encapsulations

Hydrogels were prepared similarly as previously described [183]. To allow for cell-mediated degradation of the network, matrix metalloproteinase-degradable peptides (KCGPQG↓IWGQCK, down arrow denotes site of cleavage) were used to crosslink the hydrogel, while the fibronectin-derived adhesion sequence RGD (CRGDS) was covalently tethered to aid in cell adhesion and
spreading. Macromer solutions were prepared with 3mM four-arm 20 kDa norbornene-functionalized PEG, 3.18 mM MMP crosslinker, 1 mM RGD, and 1.7 mM LAP. Two-dimensional hydrogels were prepared by placing a thiolated 12 mm circular coverslip on top of a 12 µL drop of macromer solution, polymerized for 3 minutes with UV light (XX Series) centered around 365 nm light, ∼5 mW/cm². Coverslips were first cleaned by passing them through a flame and then functionalized with 0.55% (v/v) (3-mercaptopropyl)trimethoxysilane in 95% ethanol (pH ∼5.5) to result in thiol-functionalized coverslips. The 2D culture gels were prepared the day before seeding; on the day of seeding, gels were sterilized for one hour at room temperature in 5% isopropanol in PBS under a UV light, rinsed twice with PBS, and then seeded at 1 x 10⁵ cells/cm². Cells were encapsulated at 2 x 10⁶ cells/mL as previously described (Fig. 4.1) [184]. Briefly, cells were suspended in PBS to achieve the desired cell concentration and added to the macromer solution described above. 2D hydrogels were seeded the same day as 3D encapsulations and both conditions used the same cell solution stock. To characterize the modulus of the hydrogels, 30 µL gels were formed in the cut end of a 1 mL syringe and allowed to swell overnight. The moduli of all hydrogels were measured using an Ares DHR3 (TA Instruments) shear rheometer to quantify the shear elastic modulus and then converting to Young’s modulus, where $E = 2G(1 + \nu)$ assuming a Poisson’s ratio ($\nu$) of 0.5. The hydrogels were found to have a modulus of approximately 0.4 kPa.

4.4.4 Spheroid formation and encapsulation

Spheroids were formed via the liquid overlay method [36, 38]. Briefly, 1.5% Noble agar (Fisher Scientific) dissolved in dH₂O was heated in a microwave until liquid. Then, 50 µL of the solution was pipetted into the bottom of a 96-well plate and allowed to solidify. 25,000 cells in 200 µL, as a single cell suspension, were added on top of the agar and allowed to cluster and grow for 2 days in 10% FBS growth media. Spheroids were transferred using a 1 mL pipette to minimize their dissociation, and three spheroids were collected in a 1.7 mL Eppendorf tube and spun down for 5 minutes at 3000 RPM. As much media as possible was aspirated from the spheroids, which were then re-suspended in the macromer solution defined above and polymerized. For measuring
Figure 4.1: (a) Components of PEG-peptide hydrogels utilized for these studies. Four-arm norbornene-functionalized PEG (-ene) was crosslinked with an MMP-degradable peptide (KCGPQG↓IWGQCK) containing cysteines (-thiol). The PEG was used at a concentration of 3 mM (12 mM ene), and reacted 47% off stoichiometry (6.36 mM thiol, equivalently 3.18 mM crosslinker peptide) to form soft (0.4 kPa) hydrogels. The cysteines on either the MMP crosslinker or the pendant adhesion peptide CRGDS can react with norbornene to form a step-growth thiol-ene network. Integrins can engage the RGDS peptide, and combined with the proteolytically cleavable linker, aids in spreading and cell survival. (b) Cells can either be encapsulated or seeded on top of the PEG-peptide hydrogels. (1) Here, WM35 and A375 were tested on TCPS, on top of a hydrogel or encapsulated within the same formulation and tested for sensitivity to PLX4032 treatment. (2) The WM35 cells were then tested as single cells or multicellular spheroids against drug treatment to better understand what component of spheroid culture may contribute its more drug resistant nature.
metabolic activity and apoptosis, samples were prepared in a 1 mL syringe with the end cut off and made as 30 µL gels. 15 µL of the macromer solution was polymerized in the bottom of the syringe for 30 seconds, then the solution containing 3 spheroids was added on top and polymerized for 3 minutes. Single cell encapsulations were prepared with 2.5 x 10^6 cells/mL to yield ~75,000 cells in both the spheroid and single cell samples for comparisons. Both single cell and spheroid encapsulations were performed the same day with the same passage number of cells.

4.4.5 Metabolic activity, apoptosis, and DNA content

After seeding cells on 2D gel surfaces or encapsulating them in 3D gel samples, the cells were allowed to adhere to the materials and recover overnight. The media was then changed to introduce drug treatment (1 µM PLX4032) or as a negative control (DMSO). The 2D gel samples were moved to clean plates before adding fresh media. Cells were treated for 48 hours. PrestoBlue (Life Technologies) was used to measure metabolic activity; samples were allowed to incubate for 1 hour and then read on a plate reader (Biotek Synergy H1). The media was saved to capture any potential floating cells, and 2 mg/mL collagenase (Type 1, Worthington Biochemicals) was used to dissolve the gels and recover the embedded cells (3D gels required ~1 hour to degrade completely). Samples were spun down for 4 minutes at 1100 RPM, rinsed with 2 mL PBS, and then centrifuged again. The cell pellet was lysed using the lysis buffer provided by the EnzCheck Caspase 3 Kit #2 (Life Technologies). The cell lysate was then exposed to one freeze-thaw cycle to ensure complete lysis, spun down at 7000 RPM for 7 minutes, and followed by company protocol to measure caspase 3 activity. DNA content from the same lysate was measured by taking 10 µL of the sample and using the Quant-iT PicoGreen assay kit (Life Technologies). Both caspase 3 and DNA content were measured on a Biotek Synergy H1 plate reader.

4.4.6 Statistical analysis

All experiments were performed at least 2-3 times with a minimum of 2 technical replicates. Graphs were produced in Graphpad Prism 5. Statistical analysis was performed in Graphpad Prism
5 by a two-way ANOVA and Bonferonni posttests for \( p < 0.05 \) (Fig. 4.3, 4.6); one-way ANOVA with Tukey posttests for \( p < 0.05 \) (Fig. 4.2b).

4.5 Results

4.5.1 Morphology and metabolic activity of 2D versus 3D cell culture

Hydrogels were synthesized from 3 mM four-arm PEG-norbornene (MW: 20,000) crosslinked with 3.18 mM MMP-degradable peptides (KCGPQG\( \downarrow \)IWGQCK), and 1 mM pendant CRGDS (fibronectin-derived adhesive sequence), in the presence of 1.7 mM LAP (photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate) and \( \sim 5 \) mW/cm\(^2\) light centered around 365 nm and resulted in a final Young’s modulus of approximately 0.4 kPa (Fig. 4.1). This formulation was selected based on previous work showing that 1 mM RGDS promoted cell attachment and spreading while the MMP-crosslinker allowed for cell-mediated degradation and possible motility of both encapsulated primary and cancer cells [136, 185–187]. To study the effect of dimensionality (i.e., 2D vs 3D) on cell function, two different human melanoma cell lines were selected: an early stage radial growth phase (RGP) cell line (WM35), and a metastatic line (A375). Both WM35 and A375 cells were seeded on the surface of the hydrogels (2D) at a density of \( 1 \times 10^5 \) cells/cm\(^2\), as well as encapsulated within the same hydrogel formulation at a density of \( 2 \times 10^6 \) cells/mL (3D). Overall cell morphology was assessed via immunostaining (Fig. 4.2a), and cells cultured on TCPS were included as a reference for comparison. On TCPS, the WM35 cells were spread, exhibited clearly defined F-actin stress fibers, and formed distinct focal adhesions as indicated by punctate paxillin staining (green), a focal adhesion component. When cultured on top of the relatively soft hydrogels, the cells became more rounded and focal adhesions appeared smaller; encapsulated cells were mostly rounded with only cytoplasmic paxillin staining patterns. The metastatic A375 cells were not as large as the WM35 cells on TCPS and formed small lamellipodia structures with smaller focal adhesions. When cultured on the surface of the hydrogels, some of the cells maintained small lamellipodia, but others were more rounded. A375 cells encapsulated in the hydrogel matrices
were typically rounded, though a few began to form short, wide protrusions, indicative of local matrix degradation.

To understand the influence of PLX4032 treatment on the cultured melanoma cells, metabolic activity was measured after 48 hours of exposure to 1 µM of PLX4032, and results are reported as a percent of activity relative to the control conditions (no PLX4032 treatment, Fig. 4.2b). While both cell types exhibited an overall decrease in absolute metabolic activity in the presence of PLX4032, the WM35 cells revealed a more sensitive response to drug treatment when cultured on TCPS compared to either the 2D or 3D hydrogel conditions; the reduced response in the 2D hydrogel condition was statistically significant. There were no statistically significant differences in metabolic activity as a function of dimensionality in the A375 cells (i.e., 2D versus 3D culture). In all A375 drug-treated samples, the metabolic activity was between 40% and 60% compared to the untreated control conditions. While metabolic activity provides a general screen of cell health, PLX4032 has been shown to be cytostatic and cytotoxic, so we next measured apoptosis in order to better understand whether there were any differential effects of PLX4032 as a function of the culture environment.

4.5.2 Apoptosis as a function of inhibitor treatment in 2D versus 3D

Apoptosis in both radial growth (WM35s) and metastatic (A375s) melanoma cells was measured based on caspase 3 activity after 48 hours of drug treatment. Caspase 3 activity was normalized to the TCPS control (i.e., the TCPS control had a value of 1), and cells were seeded on TCPS and on top of or encapsulated within hydrogels. The WM35 cells were previously shown to respond to PLX4032 exposure in a manner that was dependent on the underlying substrate elasticity [183]. Here, WM35s exhibited sensitivity to PLX4032 that also was independent of dimensionality though perhaps coupled to the effects of the substrate stiffness (Fig. 4.3a). In both 2D and 3D hydrogel samples, PLX4032 treatment caused an increase in apoptosis (~4.3 and 4.1-fold increases compared to respective controls) compared to a 2.4-fold increase on TCPS (Fig. 4.3c). Despite the lower absolute values of caspase 3 activity in both 2D and 3D samples compared to
Figure 4.2: Overall cell morphology and activity either on 2D or encapsulated within 3D hydrogels. (a) Immunostaining for focal adhesions was performed using two melanoma cell lines (WM35 and A375) cultured on TCPS or on 2D gel surfaces and embedded in 3D hydrogels after 24 hours of culture. Images of cells seeded on TCPS show cell spreading and more focal adhesions, while cells on the soft gels appear slightly more rounded. When encapsulated within the hydrogels, both cell types become very rounded, presumably due to the slower time scale for remodeling and degradation of the matrix. Paxillin (green), f-actin (red), nucleus (blue), scale bar, 100 µm. (b) Metabolic activity as measured by PrestoBlue of WM35 and A375 cells either seeded on TCPS, on top of a hydrogel, or encapsulated within a hydrogel after 48 hours of treatment. The WM35 cells exhibited a statistically significant increase in metabolic activity on a hydrogel surface compared to TCPS. No statistically significant differences were observed in metabolic activity between the culture conditions in the A375 cells. Dashed line represents values of the control samples. *p <0.05.
TCPS, the fold changes in caspase 3 were much higher in the hydrogel conditions.

![Figure 4.3: WM35 and A375 cell apoptosis after 48 hours of treatment with PLX4032 in 2D and 3D microenvironments.](image)

(a) WM35 cells exhibited sensitivity to PLX4032 in TCPS and 2D and 3D hydrogel culture conditions. The 2D hydrogel PLX4032-treated sample was statistically higher than the TCPS PLX4032 sample. (b) A375 cells exhibited overall less sensitivity to PLX4032 treatment as observed by smaller increases in caspase 3 activity with PLX4032 treatment. The 3D control sample was significantly higher than the 2D hydrogel control sample, and the 2D hydrogel control was statistically lower than that of the TCPS control. (c, d) Fold change in caspase 3 activity over each respective control with PLX4032 treatment. *p < 0.05 compared to respective control, #p < 0.05 compared to corresponding condition in TCPS, **p < 0.05 between A375 2D and 3D control samples.

Apoptosis was also assessed in the metastatic A375 cells (Fig. 4.3b), and overall, the differential changes in caspase 3 activity were much lower (~1.8, 1.4, and 1.1-fold increases over the controls in TCPS, 2D and 3D gels, respectively, Fig. 4.3d) compared to the radial growth phase WM35s (up to a ~4.3-fold increase). For the A375 cells cultured on TCPS and 2D gels, PLX4032 induced a significant increase in apoptosis compared to the control, though the basal level of caspase 3 activity in the 2D hydrogel control was statistically significantly lower than the TCPS control (Fig. 4.3b). Additionally, the level of apoptosis caused by PLX4032 treatment for A375s cultured on 2D gel surfaces or embedded in 3D gels was significantly lower than that of
the TCPS PLX4032 condition. In fact, the 3D hydrogels did not exhibit a statistically significant increase in apoptosis in the presence of PLX4032, and instead when comparing the fold change with respect to the controls, A375s embedded in 3D hydrogels exhibited a statistically significantly lower level of apoptosis compared to TCPS (Fig. 4.3d).

Apoptosis was also evaluated for both cell types in a stiffer hydrogel formulation (≈4 kPa) (Fig. 4.S1), as matrix elasticity can be important for cell function and subsequent signaling [7, 116, 149, 151]. WM35s remained sensitive to PLX4032 in the stiff hydrogels (≈4.2 and 4.8-fold increases in caspase 3 over corresponding controls in 2D gels and 3D, respectively) though there were no significant differences between the PLX4032-treated samples in 2D and 3D gels (Fig. 4.S1a, c). The A375s exhibited approximately a 1.4-fold increase in caspase 3 activity when compared to respective controls in both 2D gels and 3D (Fig. 4.S1b, d). Again, the maximum change in caspase 3 activity was only ≈1.8 on TCPS in the A375s compared to ≈4.8 in the WM35 cells in 3D. Cell death was also measured in soft hydrogels with either RGDS, derived from fibronectin, or a P15 collagen I peptide mimic. Collagen I is one of the most abundant proteins in the dermis and spheroid studies often embed cells within a collagen matrix; therefore, we asked whether the adhesive ligand might change the apoptotic response to PLX4032. Both WM35 and A375 cells responded similarly to PLX4032 regardless of the adhesive peptide used (Fig. 4.S2).

4.5.3 Single cells versus spheroids

Since we observed slightly increased levels of apoptosis in radial growth phase WM35 cells cultured in 3D compared to 2D hydrogels or TCPS, as well as the large volume of literature comparing aggregates to single cells, we next asked whether multicellular spheroids embedded in hydrogels might confer protection from PLX4032-induced apoptosis compared to single cells. In order to study spheroids, we first utilized the liquid underlay method to form spheroids [36, 38]. Briefly, 200 µL of a WM35 single-cell suspension at a concentration of 1.25 × 10⁵ cells/mL was pipetted on top of an agar-coated 96-well plate and cultured for 2 days (Fig. 4.4a). The resulting spheroids were then collected, and sizes were observed to be approximately 1000 µm in dia-
A typical phase image shows that the melanoma spheroids were generally rounded and had clearly defined edges (Fig. 4.4b). The spheroids were next encapsulated within the soft hydrogel formulation used in the 2D versus 3D studies (0.4 kPa) and compared to WM35s encapsulated as single cells. The WM35 cells were selected for this study based on their stronger and differential response to drug treatment despite dimensionality.

After successfully forming spheroids, their overall shape was assessed via confocal microscopy (Fig. 4.5). Live/dead images show both single cells and spheroids encapsulated within hydrogels with and without PLX4032 after 48 hours of treatment. In the single cell control, cells were rounded and many appeared to proliferate significantly over the relatively short time course of this experiment (as indicated by numerous cells in close proximity to one another). In the PLX4032-treated sample, there were fewer “paired” cells and many had small protrusions. Interestingly, in the spheroids under control conditions, the aggregates had a defined edge with a few loosely dissociated cells near the perimeter. However, the PLX4032-treatment led to what appeared to be a more invasive morphology, as characterized by an elongated cell shape and perimeter cells moving large distances away from the aggregate (up to ~200 μm). Brightfield images of the spheroids confirm the compact shape of the control spheroid and the potential invasiveness of the drug-treated spheroid (Fig. 4.S3).
**Figure 4.5**: Overall morphology of WM35 single cells and spheroids encapsulated within PEG hydrogels. Live/Dead staining of single cells or spheroids within 0.4 kPa PEG-peptide gels in the presence or absence of PLX4032. Single cell encapsulations exhibited a very rounded morphology in the control conditions while many cells had small, thin protrusions in the presence of PLX4032. Inset image shows magnification of cells exhibiting rounded or elongated morphology. Spheroids cultured under control conditions formed relatively well defined edges with some cells loosely dissociated from the edge of the cluster. In contrast, spheroids treated with PLX4032 displayed many cells with an invasive morphology appearing to migrate away from the spheroid. Scale bar, 100 µm.

### 4.5.4 Drug responsiveness of single cells and spheroids

To further assess the effects of PLX4032 treatment on WM35 single cells and spheroids, metabolic activity, DNA content, and apoptosis were measured (Fig. 4.6). Metabolic activity was measured after 48 hours of culture with or without PLX4032, and in both cases, it caused a decrease in the average metabolic activity (approximately 30% and 20% decreases in single cells and spheroids, respectively) (Fig. 4.6a). The spheroids exhibited a 20% lower basal level of metabolic activity in the control condition compared to the single cell encapsulation. When PLX4032 treatment is assessed, drug treatment may be less effective in spheroids because of the smaller change in metabolic activity compared to the respective control conditions. The DNA content of single cells and spheroids was also measured. The DNA content of spheroid controls was significantly higher
Figure 4.6: Overall drug responsiveness of melanoma cells to PLX4032 treatment after 48 hours. (a) Metabolic activity was measured and found to decrease with PLX4032 treatment for all conditions. (b) DNA content was measured to better understand changes in cell number in the single cell encapsulations versus spheroid culture. PLX4032 caused a significant decrease in cell number in both culture conditions (~68% and 55% in single cells and spheroids, respectively). (c) Apoptosis was measured via caspase 3 activity and found to increase with PLX4032 treatment in both culture conditions. The spheroid control and PLX4032-treated samples were statistically different from the corresponding single cell encapsulation samples. *p < 0.05 compared to respective control, #p < 0.05 compared to respective condition in single cell encapsulation samples.

(1.8-times) than that of single cell encapsulations (Fig. 4.6b). With drug treatment, the DNA content significantly decreased in both culture conditions (70% and 44% lower compared to controls in single cells and spheroids, respectively), and the DNA measured in PLX4032 spheroids was significantly higher (approximately 3-times higher) than that of the single cell PLX4032 conditions. Lastly, the level of cell death via apoptosis was also assessed. In both the control and PLX4032-treated samples, the level of caspase 3 activity was significantly different between the single cells and spheroids (Fig. 4.6c), where the spheroids exhibited approximately 40% less apoptosis under control conditions. With drug treatment, caspase 3 increased (1.4-fold in single cell encapsulations and 1.3-fold in spheroids, Fig. 4.S4); however, the relative levels of apoptosis between single cells and spheroids were very different.

4.6 Discussion

Overall, we aimed to determine whether dimensionality of the cellular microenvironment (i.e., 2D versus 3D culture) might influence the apoptotic response of melanoma cells to PLX4032. Previous reports have compared cells cultured on TCPS to spheroids and their sensitivity to different types of cancer drugs [38, 39, 102, 158]. These results have demonstrated that aggregated cells
can respond differently, often in a less sensitive manner, when cultured as a multicellular structure embedded within collagen or seeded on top of Matrigel compared to traditional 2D TCPS culture [157, 188]. While these studies are needed to understand the role of the tumor microenvironment and aid in the development of proper in vitro drug screening cell culture platforms, several variables are changing when single cells are plated on TCPS versus those that are aggregated into spheroids [87]. For example, spheroids introduce cell-cell contacts, a heterogeneous 3D cellular architecture with respect to diffusional distances, as well as a 3D matrix platform that may have a change in modulus or chemistry of the surrounding environment (e.g., adhesion ligands, sequestered growth factors [86, 93, 180]). Therefore, experiments were designed to better understand how certain aspects of a cell’s microenvironment might create a more drug resistant response as measured by activity and survival. Specifically, we investigated whether radial growth phase (WM35) or metastatic (A375) cells, when cultured as single cells, would respond differently to PLX4032 treatment when cultured on TCPS, a soft 2D hydrogel environment, or embedded in the same hydrogel system. Both metabolic activity and apoptotic responses were evaluated with PLX4032 treatment. Then, the possible role of cell-cell contacts in melanoma drug responsiveness was inferred in comparing the single cell studies to spheroid cultures. Overall, the metastatic A375 melanoma cells were less responsive to drug treatment regardless of culture conditions. In contrast, the early stage radial growth phase cells were found to be more sensitive when cultured on 2D hydrogels and in 3D culture compared to TCPS, but when aggregated into multicellular structures, they too, became less responsive to PLX4032.

We first assessed overall morphology of cells seeded on TCPS and on top of or encapsulated within hydrogels (Fig. 4.2a). Not surprisingly, both cell types were spread and formed focal adhesions on TCPS, indicating a high level of cell-substrate interactions. On hydrogel substrates, cells were able to attach, but did not spread to the same degree, possibly due to the softness of the underlying substrate [7, 189, 190]. Upon encapsulation, the melanoma cells were generally rounded, in part because they are completely surrounded and confined by the hydrogel network, which necessitates that the cells degrade the local matrix to allow spreading. Perhaps not enough
time had elapsed to allow the cells to spread (e.g., degrade the local matrix) and more elongated morphologies would be observed at longer culture time [187].

Next, cellular responses due to PLX4032 treatment were assessed. Metabolic activity showed similar responses to PLX4032 treatment in the A375 cells for each of the culture conditions (Fig. 4.2b). The WM35 cells had a significantly lower metabolic activity compared to the 2D hydrogels. PLX4032, however, can induce apoptosis and prevent proliferation [22, 191], and so the differences in metabolic activity reflect both the cytotoxic and cytostatic effects of drug treatment on melanoma cells. To better understand how drug treatment affected melanoma cells, the apoptotic response to PLX4032 was evaluated. We hypothesized that dimensionality would lead to differential cell responses, and the results showed an increase in apoptosis in WM35 cells cultured on top of or encapsulated within gels compared to TCPS (Fig. 4.3a). In the case of the radial growth phase WM35 cells, 3D culture by itself was not sufficient to protect the cells from apoptosis compared to traditional monolayer culture. Instead, a soft environment, whether cells were on or encapsulated within a hydrogel, induced an increased apoptotic response. We previously showed a correlation between smaller focal adhesions and increased apoptosis in the WM35 cells [183], and perhaps even in a 3D environment, WM35s are unable to form mature focal adhesions and therefore receive decreased survival signaling from the local environment.

The metastatic A375 cells were generally less responsive to PLX4032, regardless of the microenvironment, as evidenced by the fold changes in caspase 3 activity (approximately 1.8 to 1.1-fold increases) (Fig. 4.3b, d), compared to the ~4.3-fold increases observed for the WM35 cells (Fig. 4.3c). Interestingly, the A375 cells exhibited decreased levels in caspase 3 induction from TCPS to soft 2D hydrogels to 3D culture (Fig. 4.3d); there was a statistically significant difference between the fold changes in caspase 3 activity between TCPS and 3D culture. This data suggests that 3D cell culture may help to protect the A375 cells from PLX4032 treatment. We hypothesize that for the metastatic A375 cells, 3D culture is sufficient to cause a lower apoptotic response, and perhaps this only contributes to more drug resistant spheroid studies. The A375 cells also appeared to have a slightly lower overall apoptotic response to PLX4032, but the basal
(control) level of caspase 3 in 3D environments was higher than that on 2D hydrogels. While the materials and encapsulation conditions have been used for numerous types of primary cells [139, 140, 192], perhaps the process of encapsulation is slightly harsher on the melanoma cells compared to their seeding in 2D, and this may account for the elevation in caspase 3. Future work might look at time points later than 48 hours to assess the rates of proliferation in 2D versus 3D and how they are affected by PLX4032 treatment.

Though we observed differences between TCPS and 2D hydrogel cultures, we must note that there are several differences between the two culture conditions. TCPS does not inherently contain binding moieties, is orders of magnitude stiffer than soft tissues, and nonspecifically adsorbs serum proteins. The 2D hydrogel platform used in these studies is nearly six orders of magnitude softer than TCPS, can allow the diffusion of nutrients to both sides of the cells, and generally resists non-specific protein adsorption. The 2D hydrogels maintain a polarized binding environment similar to TCPS in that cells can only bind on one of their surfaces, however, a direct comparison of TCPS to any hydrogel system is often difficult due to the multiple variables that can change.

We also investigated the possible role of the adhesive ligand in conferring survival. Most published work on 3D spheroids embeds them in commercially available collagen gels. This motivated us to examine differences between an RGDS adhesive peptide, derived from fibronectin, to a P15 sequence, derived from collagen I. However, the results indicated no change in how WM35 or A375 cells responded to PLX4032 treatment as a function of these different integrin-binding epitopes (Fig. 4.S2). We had hypothesized that a collagen mimic might increase susceptibility to drug treatment [63], but perhaps these particular melanoma cell lines are less sensitive to specific cell-ECM interactions in 3D compared to other cells, such breast cancer cells [90, 103].

Because the WM35 cells appeared to have increased susceptibility to drug treatment on both 2D hydrogels and 3D culture conditions as a single cell suspension, we focused on these radial growth phase cells and asked whether aggregation into spheroids might confer a protective effect from apoptosis. Interestingly, we observed a more elongated morphology with PLX4032 treatment of 3D single cells and in cells around the periphery of drug-treated spheroids (Fig. 4.5). Cells
embedded in these molecularly crosslinked gels must locally remodel their environment to allow spreading and motility. It is worth noting that the invasive phenotype seen in PLX4032-treated spheroids was using a RGP melanoma cell line, whereas previous outgrowth from spheroids has been observed in metastatic cell lines [39, 193]. Two previous studies have observed increased metastatic melanoma invasion with MEK or BRAF treatment via transwell assays or spheroid outgrowth [21,168]. Here, we show a change in morphology associated with migration in an early stage melanoma cell line, which could have important implications for melanoma treatment with the clinically available PLX4032 drug.

Lastly, we analyzed viability metrics for drug responsiveness in single cells versus spheroids. Beyond activity and apoptosis, the DNA content of the control spheroids was significantly higher than that of single cell encapsulations (Fig. 4.6b), which may point to increased proliferation rates of cells (and the effect of high cell-cell contacts) within the spheroid. We hypothesize that perhaps cells in the outer shell of the spheroid are proliferating more than those in the potentially nutrient-deprived core [194]. In these studies, the spheroids were approximately 1 mm in diameter. As such, with spheroid culture conditions, there is an inherent heterogeneity among the cells sampled when measuring apoptosis or DNA content. A spheroid contains some cells that are in contact with the ECM, some that are nutrient deprived at the core, and others that have numerous cell-cell contacts [195, 196]. When assessing metrics for the spheroid population, it is difficult to determine what components are contributing to this signaling cell-cell contacts or cell-matrix interactions of the outer ring of cells in the spheroid. Here, caspase 3 activity, DNA content, and metabolic activity (Fig. 4.6) were measured based on the average of the collective population. For metabolic activity and live/dead staining, the experiments may likely only measure the outer shell of cells that come into contact with PLX4032, as cells at the periphery can consume most of the probe molecules and imaging in the 3D interior of large aggregates is difficult. With this in mind, we hypothesize that the observed decreased apoptosis in spheroids (Fig. 4.6c) is likely due to two issues [197]. First, the inner mass of cells may be protected from exposure to drug treatment by the outer cells [165], and second, cell-cell contacts in spheroids may promote survival
Further experiments are needed to spatially identify where apoptotic cells reside in the aggregates, and this may necessitate new methods to form small aggregates of more uniform size [199]. In addition, understanding the growth of these spheroids in real time (e.g., proliferation markers, tracking cell numbers) would aid in understanding why the DNA content within spheroids is higher than that of single cells, even though both encapsulation conditions started with the same number of cells. By measuring the proliferation and subsequent growth of the spheroids over time, one might better understand why the spheroids respond differently to drug treatment compared to single cells.

Despite some of the complexities in understanding the heterogeneity that exists within spheroids and how that might affect cell responses, spheroids may be the most appropriate in vitro models of apoptotic responses to drug treatment. Spheroids can bridge the gap between traditional 2D TCPS culture and animal models [3, 8], and they recapitulate aspects of the natural heterogeneity of a tumor that contains a protected core of cells and an outer ring that may be exposed to a drug [200]. According to our study analyzing apoptosis in response to PLX4032, spheroids produced a more “drug resistant” response in otherwise sensitive melanoma cells. Even so, single cell encapsulations and soft 2D hydrogel cell culture platforms still have importance in deconvoluting potential effects of matrix signaling, which is likely important to cancer biology and drug responses.

For example, single cells embedded in 3D hydrogels are useful for studying cell migration [201], as 3D environments necessitate matrix remodeling, which occurs in vivo [1, 202]. When combined with drug treatment, these hydrogel systems can encompass protease activity and functional motility in response to drug treatment, and are not limited to metabolic activity and apoptosis. While aspects of this could be studied on 2D substrates, cells are less likely to secrete proteases, as there is no barrier to proliferation or migration on planar substrates [176]. We have also learned important facets of cell behavior from 2D studies; for instance, 2D stiffness studies have elucidated the role in matrix elasticity in mesenchymal stem cell differentiation [116], mammary epithelial cell transformation [7], and myofibroblast activation associated with aortic calcification [152]. In addition, 2D studies on soft substrates and single cell encapsulations com-
plement spheroids by allowing for control of cell density, and allowing one to focus on cell-matrix interactions and signaling analysis, as each cell would theoretically experience the same type of environment. Conversely, monolayer and dispersed cell encapsulation studies may not be as physiologically relevant because they lack the cell-cell contacts inherent to an in vivo tumor. Overall, single cell encapsulations can work in concert with spheroid studies by simplifying the local environment and help with the interpretation of results from spheroid experiments.

Here, we aimed to design experiments to reduce the complexity and better control some of the variables between 2D and 3D culture conditions. Rather than using TCPS culture as the sole 2D comparison, we exploited hydrogel substrates for cell seeding. For the 3D comparison, we used both a single cell suspension and spheroids that could be encapsulated within the same hydrogel formulation used as the 2D hydrogel substrate. In doing so, we were able to maintain the same modulus, use the same material chemistry for both 2D and 3D studies, and control cell-matrix interactions (single cell experiments) or allow cell-cell contacts (spheroid experiments). Collectively, these results contribute to the broader characterization of the role of dimensionality on cells, and particularly melanoma apoptotic responses to PLX4032 treatment.

4.7 Conclusions

Experiments were designed to test whether or not dimensionality may alter PLX4032-induced apoptosis in an early stage radial growth phase (WM35) and a metastatic (A375) melanoma cell line. The metastatic A375 cells were not as sensitive to PLX4032 treatment as the WM35 cells and exhibited decreased apoptosis in 3D compared to TCPS or 2D hydrogel culture conditions. Interestingly, the WM35 cells appeared to be more sensitive to PLX4032 when cultured on soft 2D hydrogels and in 3D, exhibiting a higher level of apoptosis compared to cells cultured on TCPS (4.3 and 4.1-fold increases on 2D and 3D hydrogel samples). Due to the increased sensitivity of the WM35 cells cultured in hydrogel matrices, especially compared to TCPS, we then asked whether the addition of cell-cell contacts through spheroid formation might confer anti-apoptotic protection from PLX4032. Indeed, the WM35 spheroids exhibited less apoptosis and potentially
increased proliferation in response to PLX4032. At this time, WM35 melanoma spheroids appear to help protect the cells from PLX4032 treatment that would otherwise be very sensitive. The mechanisms are not completely understood, and it is difficult to determine if the decreased sensitivity is due to a protected core of cells, cell-cell contacts confer survival signaling, or if it is a combination of both components. Overall, 3D culture in and of itself is not sufficient to confer significantly decreased apoptotic responses to pharmacological inhibition in RGP WM35s; however, it may protect metastatic cells. We also confirm the paradigm that spheroid culture results in more “drug resistant” responses in early stage melanoma cells, though we cannot conclude it is due to 3D culture alone, but perhaps instead due to cell-cell contacts or spheroid heterogeneity.

4.8 Acknowledgements

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4.9 Supplemental Information

Figure 4.S1: Apoptosis of WM35 and A375 cells in stiff hydrogels (~4 kPa) after 48 hours of PLX4032 treatment. (a, b) Apoptosis was measured via caspase 3 activity. The WM35 cells exhibited lower levels of caspase activity under control conditions on top of or encapsulated within hydrogels compared to TCPS. A375s showed similar basal levels of caspase 3 across all culture conditions tested. Both cell types exhibit increases in caspase 3 in response to PLX4032 treatment. (c, d) Fold change in apoptosis due to PLX4032 treatment compared to each respective control was determined for each culture condition. *p < 0.05 compared to respective control condition, #p < 0.05 compared to respective condition on TCPS, **p < 0.05 between A375 2D hydrogel and 3D PLX4032 samples.
Figure 4.S2: Apoptosis of WM35 and A375 cells as a function of the adhesive ligand (fibronectin-derived RGD versus collagen I P15 peptide). Apoptosis was measured via caspase 3 activity after 48 hours of drug treatment. *p <0.05 compared to respective control condition.

Figure 4.S3: Brightfield images of spheroids encapsulated within PEG-peptide hydrogels. Scale bar, 100 µm.
Figure 4.S4: Fold change in apoptosis in single cells versus spheroids. When the caspase 3 activity was normalized to each respective control, the fold change was approximately the same.
Chapter 5

Multifunctional bioscaffolds for 3D culture of melanoma cells reveal increased MMP activity and migration with BRAF kinase inhibition

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5.1 Abstract

Matrix metalloproteinases (MMPs) are important for many different types of cancer-related processes, including metastasis. Understanding the functional impact of changes in MMP activity during cancer treatment is an important facet not typically evaluated as part of preclinical research. With MMP activity being a critical component of the metastatic cascade, we designed a 3D hydrogel system to probe whether pharmacological inhibition affected human melanoma cell proteolytic activity; metastatic melanoma is a highly aggressive and drug resistant form of skin cancer. The relationship between MMP activity and drug treatment is unknown, and therefore, we utilized an in situ fluorogenic MMP sensor peptide in order to determine how drug treatment affects melanoma cell MMP activity in three dimensions. We encapsulated melanoma cells from varying stages of progression within PEG-based hydrogels to examine the relationship between drug treatment and MMP activity. From these results, a metastatic melanoma cell line (A375) and two inhibitors that inhibit RAF (PLX4032 and Sorafenib) were studied further to determine whether changes in MMP activity led to a functional change in cell behavior. A375 cells exhibited increased MMP activity despite an overall decrease in metabolic activity with PLX4032 treatment. The changes in proteolytic activity correlated with increased cell elongation and increased single-cell migration. In contrast, Sorafenib did not alter MMP activity or cell motility, showing the changes induced by
PLX4032 were not a universal response to small molecule inhibition. Therefore, we argue the importance of studying MMP activity with drug treatment and its possible implications for unwanted side effects.

5.2 Significance Statement

Matrix metalloproteinases (MMPs) are a family of enzymes that enable cell-mediated remodeling of the local environment. Because of their ability to degrade the extracellular matrix, MMPs are an important component of the metastatic cascade. However, the effect of cancer drug treatment on MMP activity is not well-established. This study reveals that treatment with a clinically relevant BRAF protein kinase inhibitor (PLX4032) correlates with increases in melanoma MMP activity in 3D culture, which consequently leads to increased cell migration. Although PLX4032 initially causes significant tumor shrinkage, patients typically relapse due to metastatic lesions. This work may contribute to our understanding of how initially promising therapeutics ultimately lead to poor patient outcome through drug-induced effects on MMP activity and cell motility.

5.3 Introduction

Proteolytic cleavage of the tumor microenvironment is an important mediator of cancer metastasis, which is the primary cause of mortality in cancer patients. Remodeling of the local environment involves a complex balance of cellularly secreted enzymes that promote and inhibit matrix degradation (reviewed in [203]). As part of this process, the matrix metalloproteinase (MMP) family of zinc-dependent enzymes plays a key role in this remodeling by degrading extracellular matrix (ECM) proteins, which promotes tumor cell invasion and colonization of distant metastatic sites, stimulates blood vessel infiltration, and releases numerous growth factors [204–208]. MMP expression is elevated in nearly all solid tumors, including breast, colon, pancreas, and melanoma, and increased expression is often correlated with decreased survival [209]. Additionally, targeted
overexpression of MMP-3, -7, or -14 in vivo results in increased mammary carcinogenesis [210], and several knockout mouse models have decreased tumor incidence, angiogenesis, and metastasis [211].

Although clearly important during cancer progression, MMP activity is not routinely evaluated during in vitro preclinical screening for potential cancer therapeutics. This is due partly to the many layers of regulation that control the final MMP activity, which include gene expression, zymogen activation, localization, and secretion of endogenous inhibitors. To capture all of these levels of regulation, it would be advantageous to measure MMP activity at the site of action when it is occurring within the sample of interest. Additionally, mounting evidence indicates that microenvironmental cues (e.g., dimensionality, matrix stiffness, adhesion ligand presentation) also regulate MMP activity [184, 212–215], so there is an increasing need for three-dimensional (3D) in vitro culture systems in which MMP activity can be measured quickly and simply to bridge the gap between traditional 2D cell culture and in vivo models. Three-dimensional culture systems are quite relevant to evaluate how drug candidates affect cellular MMP activity, because they provide a more physiologically relevant context, which necessitates breakdown of surrounding matrix to allow migration and invasion in a dense ECM-like environment.

To address this need, a recent approach has extended the use of tethered fluorescent MMP degradable substrates, developed for visualization of MMP activity using microscopy, to quantify MMP activity within 3D microenvironments with a standard plate reader [184]. In this approach, poly(ethylene glycol) (PEG) hydrogels were functionalized with a fluorogenic MMP substrate, enabling precise control over microenvironmental parameters (e.g., matrix stiffness, adhesion ligands, degradability) as well as monitoring of in situ MMP activity easily and quickly with a plate reader. Here we used this MMP fluorogenic sensor-functionalized hydrogel system to investigate how melanoma cell viability and MMP activity were affected in 3D culture environments when treated with inhibitors of the ERK pathway.

Melanoma is a type of skin cancer derived from pigment-producing cells called melanocytes. While melanoma accounts for only 4% of all skin cancers, it is responsible for ~75% of all skin
cancer-related deaths with a 5-y survival rate of less than 15% for metastatic cases [10]. A break-through in drug development and treatment for melanoma came with the discovery that nearly two-thirds of all melanomas contain a BRAF mutation within the RAS/RAF/MEK/ERK pathway, rendering BRAF constitutively active [18]. In 2011, PLX4032, a specific BRAF$^{V600E}$ inhibitor, was approved by the Food and Drug Administration (FDA) for metastatic melanoma treatment; despite promising clinical trials, however, patients often relapse within 6 mo of treatment [26]. Metastatic melanoma is particularly drug-resistant, and may potentially be aided by constitutive activity within the ERK pathway, which influences cell growth, proliferation, apoptosis, and MMP activity [48]. Additionally, a number of studies have found increased MMP expression, specifically expression of collagenases (MMP-1 and -3) and gelatinase (MMP-2), is correlated with a poor patient prognosis, and that an MMP-1 activated signaling axis promotes invasion and metastasis of vertical growth phase melanoma [216–219]. Because ERK is a known regulator of MMPs and MMPs are an important component of the metastatic cascade, we hypothesized that commercially available BRAF and MEK inhibitors would decrease cell viability and MMP activity, therefore impacting metastatic potential. With a possible link between drug treatment and MMP activity, we conducted a screen of four melanoma cell lines and four pharmacological inhibitors to test the feasibility of a 3D screening approach to investigate the effect on MMP activity. An innovative biomaterial system that allows culture of cells in three dimensions and simultaneous real time measurement of MMP activity was used [184]. Changes in MMP activity were correlated with other standard measures of cell function, including metabolic activity, morphology, and migration.

5.4 Results

5.4.1 In situ screening of MMP activity and viability in three dimensions

Given the high incidence rates of mutations in the RAS pathway in melanoma and the role of this pathway in regulating MMP activity, we first investigated whether pharmacological inhibition of BRAF would impact MMP activity of melanoma cells cultured in a 3D microenvi-
ment. To assess MMP activity, we used a recently developed functionalized hydrogel platform in which covalent incorporation of a fluorogenic MMP-sensitive peptide (Dab-GGPQG↓IWGQK-Fl-AEEAc-C where the arrow denotes site of cleavage) enables facile measurement of in situ MMP activity and cell viability within the same 3D hydrogel sample [184]. The GPQG↓IWGQ sequence

Figure 5.1: MMP sensor peptide-functionalized hydrogels enable facile monitoring of 3D cellular proteolytic activity. The pendant MMP sensor peptide (Dab-GGPQG↓IWGQK-Fl-AEEAc) is covalently incorporated into the PEG network. The MMP-sensitive peptide sequence is flanked by a quencher (dabcyl) on one side and a fluorophore (fluorescein). The intact peptide, with the quencher and fluorophore in close proximity, is weakly fluorescent. Upon cleavage by cell-secreted MMPs, the quencher can diffuse away from the fluorophore, resulting in an increase in fluorescence that can be measured on a standard plate reader. The fibronectin-derived pendant adhesion peptide CRGDS is also incorporated into the hydrogels. The network is crosslinked with an MMP-degradable peptide (KCGPQG↓IWGQCK) to allow cell-mediated degradation of the network.
is derived from type I collagen, and is cleaved by several MMPs, including MMP-1, -2, -3, -7, -8, and -9 [220]. Because collagen I is abundant in the tumor stroma and increased expression of several collagenases (MMP-1 and -3) and gelatinases (MMP-2) is associated with melanoma progression, degradation of this molecule is of particular importance to melanoma invasion and metastasis [6,216,219]. Cleavage of the peptide by cell-secreted MMPs separates the quencher (dabcyl; Dab) from the fluorophore (fluorescein; Fl), and an increase in fluorescence can be measured (Fig. 5.1). These PEG-peptide gels are formed through a radical-mediated thiol-ene “bio-click” reaction via step-growth polymerization kinetics [6]. In this reaction, any cysteine-containing (thiol) peptide can be incorporated into the network by reacting with the norbornene-functionalized PEG (-ene) in this system. The network is cross-linked by bifunctional cysteine-containing MMP-degradable peptides (KCGPQG↓IWGQCK) and four-arm PEG-norbornene to complete the thiol-ene reaction in the presence of a photoinitiator and light (365 nm at ~5 mW/cm²). This functionalized hydrogel was demonstrated to be highly specific to measuring MMP activity, and use of this sensor enabled facile measurement of MMP activity in 3D culture environments using a standard plate reader.

Four human melanoma cells lines (WM35, WM115, WM239A, A375) were encapsulated in functionalized hydrogels and treated with DMSO or 1 µM of a BRAF/MEK small molecule inhibitor (PLX4032, Sorafenib, AZD6244, CI-1040) (Fig. 5.2a). Resazurin, a cell permeable non-fluorescent molecule that is reduced by living cells to the fluorescent molecule resorufin, was added 18 h postencapsulation for metabolic activity measurements. Fluorescence readings were then collected at 24 h postencapsulation to measure metabolic activity and MMP activity within the same wells. Because one of the primary cell functions assessed during in vitro development of new cancer therapeutics is the effect of a drug on cell viability, the utility of this assay is greatly increased by the inclusion of a similar readout. Here, metabolic activity, as measured by a resazurin-based assay, was used to assess effects on cell viability. The resazurin assay enables facile measurement of metabolic activity, which is important for screening a large number of samples. Additionally, the excitation/emission spectrum of the assay (excitation 560 nm/emission 590 nm) was distinct from
Figure 5.2: Effect of drug treatment on MMP activity and metabolic activity in 3D culture. (a) Color map showing the effects of RAF/MEK inhibitors (corresponding columns) on metabolic activity and MMP activity in the four human melanoma cell lines tested (corresponding rows). Each drug-treated sample is normalized to the corresponding control within that cell type. Decreases in metabolic activity are indicated by a color shift from green (control, 100%) to yellow, then red (low metabolic activity, 50%) on the left side of each circle. Changes in MMP activity are indicated by a color shift from white (control) to blue (maximum of 1.7-fold increase) on the right side of each circle. Bar graphs showing quantification can be found in Fig. 5.S2a and b. n = 5, *p < 0.05. (b) Gelatin zymogram of cultured media from A375 cells. (c) Quantification of MMP activity by zymography. Mean ± SEM, n = 3, *p < 0.05.

the MMP fluorogenic sensor (excitation 494 nm/emission 521 nm), enabling concurrent measurements in the same well, reducing the number of samples needed. Although measuring metabolic activity was convenient for these experiments, cancer cells have been shown to use both metabolic respiration and glycolysis (i.e., the Warburg effect) to produce energy, so measuring metabolic activity with this assay may not fully reflect changes in total cell number. As a control experiment,
DNA content with and without drug treatment was measured and compared to metabolic activity measurements. Similar changes were observed using either method (Fig. 5.51) and therefore validated the use of metabolic activity as a quick readout of cell viability.

In response to drug treatment, WM35 cells were observed to have the most dramatic decrease in metabolic activity, a ~50% decrease in viability [as indicated by a shift from green (control) to red (loss of metabolic activity)], compared to untreated cells upon exposure to three of the four drugs tested (PLX4032, AZD6244, and CI-1040) (Fig. 5.2a, see Fig. 5.52 for numerical data, error, and statistical analysis). There was also a significant decrease, ~30%, in metabolic activity when A375 cells were treated with the drugs tested, including Sorafenib which did not significantly affect metabolic activity of the WM35 cells. The WM115 cells appeared to be less responsive to drug treatment, with a ~20% decrease in metabolic activity in response to only two drugs, PLX4032 or AZD6244. The WM239A cell line, however, was resistant to all of the drugs tested, with no significant decrease in metabolic activity for any of the drugs tested.

Concurrent with measurements of metabolic activity, MMP activity was also measured using the fluorogenic MMP sensor-functionalized hydrogels. Although it was hypothesized that inhibition of the RAF/MEK/ERK pathway would decrease MMP expression and activity, interestingly, MMP activity increased with drug treatment in several conditions [indicated by a shift from white (control) to blue]. To more easily compare changes in metabolic activity and MMP activity with drug treatment, results are indicated side-by-side with a color map (Fig. 5.2a; see Fig. 5.52 for numerical data, error, and statistical analysis). WM35 cells had the largest increase in MMP activity, an ~70% increase in three out of four drug treatment conditions. For comparison, growth factor stimulation has previously been shown to increase MMP activity by ~50% increase [184]. There was no significant increase in MMP activity for either the WM115 or WM239A cell lines with drug treatment. Whereas increased MMP activity appeared to negatively correlate with metabolic activity in these observations, A375 cells, which had a significant decrease in metabolic activity with all of the drugs tested, showed a significant increase in MMP activity only with PLX4032 or CI-1040 treatment but not with Sorafenib or AZD6244. To confirm the observed changes in MMP
activity, a standard MMP activity assay, gelatin zymography, was also used to measure proteolytic activity of A375 cells treated with the DMSO control, PLX4032, or Sorafenib. PLX4032 induced approximately a 1.4 fold increase compared with the DMSO control and no change in activity was observed with Sorafenib treatment (Fig. 5.2b and c).

5.4.2 Increased MMP activity correlates with changes in cell morphology and migration

When cells are embedded in covalently crosslinked hydrogels and cultured in three dimensions, they are typically confined to a spherical morphology, until their local environment is degraded and remodeled to allow spreading and matrix interactions. Therefore, because we observed an increase in MMP activity with PLX4032 treatment, we next investigated whether this increased MMP activity affected cell morphology in A375 cells. To assess cell shape, samples were stained for F-actin and the focal adhesion component paxillin with and without drug treatment. Qualitatively, A375 cells were more elongated in 3D matrices, with long, thin protrusions observed when treated with PLX4032 for 24 h (Fig. 5.3a). In contrast, control DMSO samples were more rounded, with some cells containing shorter and wider protrusions (Fig. 5.3a). To quantify these changes in cell morphology, the aspect ratio was measured; A375 cells were found to have an increased aspect ratio (more elongated morphology) in the presence of PLX4032 (Fig. 5.3b). When elongated cells were defined as having an aspect ratio of $>1.5$, the fraction of elongated cells increased from $0.21 \pm 0.05$ to $0.29 \pm 0.05$ (mean $\pm$ SD) in drug-treated samples (Fig. 5.3c).

To determine whether increased MMP activity and elongation led to a functional change that could be important for metastasis, cell motility was assessed under control (DMSO), PLX4032, and Sorafenib conditions (Fig. 5.4 and Fig. 5.S3). We hypothesized PLX4032 treatment would increase migration due to the observed increased MMP activity, whereas Sorafenib, which caused a similar decrease in metabolic activity but did not induce an increase in MMP activity, would not affect migration. A375 cells were encapsulated and allowed to recover overnight ($\sim16$ h) and then treated with DMSO or the RAF inhibitors for 3 d. Time-lapse images were acquired every 20 minutes on a live-cell microscope, and then cells were tracked using MetaMorph. Examples
Figure 5.3: Cell morphology changes with PLX4032 treatment. (a) A375 cells encapsulated within a hydrogel. Samples were stained for paxillin (green), F-actin (red), and DAPI (blue) to visualize overall cell morphology. (Scale bar, 20 µm.) (b) Aspect ratio of encapsulated cells. n >1000 cells, *p <0.05. (c) Fraction of elongated cells, as defined by having an aspect ratio greater than 1.5. Mean ± SEM, n = 3, *p <0.05.

of A375 cell migration paths from an 8-h window (centered around 24 h, 20-28 h after treatment) were plotted on x-y scales (Fig. 5.4a and representative movies, Mov. 1-3, Fig. 5.S6, 5.S7, 5.S8). The tracked cell positions were used to calculate cell speed and displacement as a function of time after treatment; Fig. 5.4 shows calculations for 24 h after drug treatment. To determine whether the proportion of migrating cells (defined as having a displacement greater than 15 µm, approximately the length of a cell body) changed with inhibitor treatment, the fraction of migrating cells was calculated. Sorafenib treatment caused a significant decrease compared to the control and PLX4032 samples (Fig. 5.4b). In contrast, in PLX4032-treated samples, not only did the fraction of migrating cells increase, but the average speed of migrating cells increased nearly 150% from 11.6 µm/h, 95% CI [10.4, 12.7] and 10.1 µm/h, 95% CI [7.0, 13.2] for control and Sorafenib.
conditions respectively, to 16.8 µm/h, 95% CI [15.2, 18.5] (Fig. 5.4c). A similar trend of increased migrating cells, cell speed, and displacement in the presence of PLX4032 was observed at 48 and 72 h after treatment (Fig. 5.53). Qualitatively, PLX4032-treated samples had longer paths (Fig. 5.4a), and when the maximum displacement was calculated, PLX4032 samples travelled the farthest over the 8-h time frame compared to either control or Sorafenib samples (Fig. 5.4d). An increase in cell elongation, fraction of migrating cells, and migration speed were also confirmed in the radial growth phase WM35 cell line (Fig. 5.54), which also exhibited an increase in MMP activity in the presence of PLX4032. To confirm whether MMP activity was necessary for the

Figure 5.4: Effects of inhibitor treatment on cell motility. (a) The x-y positions of 10 sample cells were plotted with the origin being the initial cell position. (b) Fraction of migrating cells was defined as the portion of cells migrating >15 µm from its starting position. Mean ± SEM, n = 3, *p <0.05. (c) Cell speed over the 8-h time frame centered around 24 h after treatment was calculated for all migrating cells. (d) Maximum displacement of migrating cells. The farthest radial distance from the starting point of each migrating cell was calculated. Mean ± 95% CI, *p <0.05 by one-way ANOVA and Tukey posttests. (e-g) Cells were cultured in the presence of GM 6001 (10 µM), a broad spectrum MMP inhibitor, and the effects on cell motility with and without PLX4032 were assessed at 24 h after drug treatment. Mean ± SEM (e), mean ± 95% CI (f and g), *p <0.05 by Students t-test. n.s., not significant; PLX, PLX4032; Sora, Sorafenib.
observed increase in cell migration with PLX4032 treatment, MMP activity was blocked with a broad-spectrum pharmacological inhibitor (10 μM GM 6001). Treatment with the MMP inhibitor abrogated the previous changes observed in cell migration, and no significant difference in cell migration (as measured by the fraction of migrating cells or cell speed) was observed with or without PLX4032 treatment (Fig. 5.4e-g). In addition, metabolic activity was measured in the presence and absence of GM 6001 to determine whether MMP activity may confer a protective effect on melanoma cells. Regardless of whether GM 6001 was present, the metabolic activity was approximately the same (Fig. 5.S5).

5.5 Discussion

While decreasing the total number of cells is a critical aspect in determining a cancer drug’s effectiveness, here we demonstrate that other cell functions can also be regulated by drug treatment and have unintended consequences related to cell migration and possibly metastasis. By screening a number of cancer cell lines and drugs and their influence on MMP activity, we encountered some interesting conditions that warrant further study related to the influence of drug treatment on cell migration. Based on changes in viability alone, we would not have been able to identify these conditions. Here we used an in situ measure of MMP activity that allowed for more rapid screening of conditions in a 3D matrix compared with what might have otherwise been possible with traditional measures of proteolytic activity (e.g., PCR, zymography). Because these MMP sensor-functionalized hydrogels can be read directly on a plate reader within a well plate, sample processing time is significantly decreased and enables real-time measurement of MMP activity, allowing researchers to understand how degradation of a cell-laden matrix changes over time with varying culture, substrate, and/or treatment conditions. PLX4032 treatment was associated with increased MMP activity, which was correlated with elongated cell morphology and, ultimately, increased cell motility; this response to PLX4032 was robust and maintained at both 48 and 72 h after treatment (Fig. 5.S3 and Fig. 5.S4). Further studies are needed to determine whether the observed of increased MMP activity and cell migration are relevant in vivo. Cell migration is a
critical aspect of metastasis, much of which is driven by MMP degradation of the local ECM but is not routinely evaluated when screening drug candidates. Compound specificity and cytotoxicity are the most common effects studied, and are critical to drug efficacy and success; however, we argue the potential importance of studying proteolytic activity as a result of drug treatment and propose a simple method with which to study it.

Although increased invasion has been observed previously with MEK inhibition and PLX-4032 [21, 168], we note increased MMP activity as a potential cause of this enhanced motility and consequent displacement. Additionally, in the presence of a general MMP inhibitor, no changes were observed in cell migration with or without PLX4032 (Fig. 5.4e-g). As further evidence of MMP-mediated motility, we did not observe increased MMP activity in the A375 cells with Sorafenib treatment nor was there an increase in migration, showing that the changes induced by PLX4032 were not a universal response to small molecule inhibition. Sorafenib is a multi-kinase inhibitor that targets BRAF and CRAF (also termed Raf-1) and was later found to have affinity for other pathways, including VEGFR and PDGFRβ [221, 222], whereas PLX4032 is a specific V600E BRAF inhibitor [19]. We hypothesize that targeting the other isoforms of RAF with Sorafenib potentially prevents melanoma cells from compensating for BRAF inhibition (as with PLX4032 treatment) by using CRAF to continue signaling through MEK and ERK. There has been previous evidence to suggest the role of ARAF and CRAF in aiding in melanoma resistance to BRAF inhibition through their up-regulation in BRAF inhibitor-resistant cells (reviewed in [45]). The differential responses that we observed between PLX4032 and Sorafenib may be influenced by the changing kinome response to kinase inhibition. For example, MEK2 inhibition can be overridden by receptor tyrosine kinases (RTKs) that are stimulated by MEK inhibition, therefore increasing ERK signaling and resulting in drug resistance [223]. We hypothesize that the compensation mechanisms in the melanoma kinome may contribute to the up-regulation of MMP activity with PLX4032 treatment; perhaps certain kinase inhibitors stimulate RTKs or other pathways to increase ERK signaling, which leads to increased MMP activity. Although perhaps Sorafenib does not induce the unwanted side effect of cell migration, this is an incomplete picture
of its efficacy because ultimately Sorafenib failed clinical trials for not achieving clinical activity in patients [222, 224]. Interestingly, the maximum displacement was significantly increased with PLX4032 treatment, indicating that more cells are motile and tend to travel farther distances. The fact that melanoma cells increase overall migration in response to PLX4032, a clinically available drug, is an effect worth noting in conjunction with the high rate of relapse in patients who use PLX4032 (clinically, Vemurafenib or Zelboraf).

Previous reports have observed increased invasion of wild type BRAF melanoma cells via transwell migration, however in 3D matrices, melanoma cell with mutated V600E BRAF were observed to have increased invasion [21, 168]. Perhaps the addition of a 3D environment causes differential responses between wild type and mutated BRAF melanoma cells to inhibitor treatment. Previous studies have shown that treatment with the same drug can induce differential effects in three dimensions compared to traditional 2D culture [102, 158]. Two-dimensional cell culture, although easier to screen through many conditions, can lead to artificial polarization and morphologies and potentially elicits different responses to growth factors and cytokines present in media compared to a 3D environment [86]. In addition, 2D cell culture does not pose a barrier to cell migration, as there is no matrix that needs to be degraded in order for a cell to move. In using a 3D matrix, we were able to assess motility that requires proteolytic degradation, which is more physiologically relevant and may have important implications when evaluating drug treatments. Within the PEG-peptide network used in this study, part of the matrix must be significantly degraded in order for a cell to spread, because the mesh size is estimated to be only tens of nanometers, much smaller than the size of a cell. The use of proteolytic degradation to aid in cell motility is a characteristic of classic mesenchymal migration; however, cells were also observed to move with a rounded morphology and appeared to squeeze through gel defects that are created by cellular-degradation of the MMP-cleavable crosslinks (Mov. 2, Fig. 5.S7). Human fibrosarcoma HT1080 cells have previously been observed to use a mixed-mode of migration within these matrices [186].

This study focused on the degradation of one substrate: a peptide based on type I collagen,
the most abundant protein in the body. Collagenases and gelatinases are particularly relevant in melanoma because primary tumors arise in the dermis, which consists mostly of collagen. As a result, studying the MMPs relevant to the degradation of collagen is of particular relevance to melanoma metastasis. Although not studied here, other proteases are also likely important in melanoma progression. For example, cathepsin B is overexpressed in melanoma, and so studying the effects on cathepsins and ADAMs would be extremely relevant and important to the overall response of these cells to clinically relevant drugs. Additionally, because the Ets family of transcription factors (phosphorylated by ERK1/2) regulates cysteine cathepsins, and ETS1, in particular, has been linked to several MMPs (including MMP-1 and -9) and uPA (urokinase-type plasminogen activator) (reviewed in [225]), we hypothesize that if MMPs are up-regulated, other proteases may be as well. ADAMs and cathepsins have also been shown to be important to cell invasion and dissemination through the cleavage of cell-cell interactions such as E-cadherin [226], and if up-regulated, may further promote cell scattering and motility [227, 228]. Therefore, future studies aimed at synthesizing other peptide sequences and sensors will prove useful in elucidating the responses and roles of other proteolytic enzymes and greatly contribute to the understanding of the response of metastatic melanoma to treatment.

This preliminary screen also highlights heterogeneity in response to drug treatment, as certain cell lines were more sensitive than others. For example, the A375 cells were sensitive to each inhibitor tested (i.e., had a statistically significant decrease in metabolic activity) whereas the other metastatic cell line, WM239A, was not affected by inhibitor treatment (Fig. 5.2a). Additionally, the WM115 and WM239A cells did not exhibit a statistically significant increase in MMP activity in the presence of any inhibitor tested. Even within one cell line there were differences in responses to treatment. A375 cells exhibited an increase in MMP activity in two out of the four inhibitors, and WM35 cells increased proteolytic activity in response to three of the inhibitors. Although we targeted the same pathway, we saw different results, perhaps due to off target effects, or because the drugs targeted different members of the same pathway. Although not used in the clinic, Sorafenib provides a contrast to PLX4032 and highlights distinct effects RAF inhibitors impose on
melanoma cells in vitro. The differences that we observed in MMP activity, morphology, and migration due to PLX4032 and Sorafenib underscore the need to assess the effect of drug treatment on multiple cell functions in addition to viability. An advantage of the in situ sensor peptide assay in this study is that it can be adapted to address many different aspects, not limited to MMP activity. Although here we only studied two cell functions, MMP activity and metabolic activity, this assay would lend itself to inclusion of additional fluorescent spectrums to study more cell functions, such as apoptosis, concurrently.

With better culture systems and methods to monitor a variety of cell functions, one can begin to understand what factors affect a drug’s success before animal models or clinical trials. Here, only melanoma cells were tested, but these results may translate to other solid tumors; ~15% of tumors carry Ras pathway mutations, with several, such as colorectal and ovarian cancer, containing specific BRAF mutations [18, 229]. By functionalizing hydrogels with different fluorescent sensors, drugs can simultaneously be evaluated for both proteolytic activity and cell viability in order to provide a quicker, more comprehensive study of how a drug affects cells (e.g., by a plate reader-compatible assay). An in situ measure of MMP activity now becomes a material property and therefore avoids biological variance that may result from transfections. As a result, a functionalized hydrogel system can be applied to nearly any cell type or can even be used to encapsulate small explanted sample tissues. Ultimately, by developing a more physiologically relevant and functionalized 3D hydrogel system, this approach can be a suitable intermediary between traditional 2D screening techniques and animal studies. Preclinical studies are costly and time-consuming, and, unfortunately, the majority of promising drug candidates eventually fail to reach FDA approval. The development of better preclinical screening models, especially those that allow in situ measurements of important cellular responses in three dimensions, will aid in identifying successful drug candidates earlier, and thereby save time and money on drug development and animal studies.
5.6 Conclusions

Here, we highlight the importance of MMP activity, which has often been considered an important mediator of the metastatic cascade. While we cannot conclude that increased motility as a result of PLX4032-induced MMP activity could explain the delayed relapse of melanoma patients, the results presented here raise important questions. Typically, drugs are evaluated based on specificity and cytotoxicity; however, their effects on MMP activity are often not studied. We show the potential importance of studying the effects of drugs on proteolytic activity in 3D microenvironments and the implications it may have in tumor responses and patient treatment.

5.7 Materials and Methods

All cell lines were generous gifts from Professor Natalie Ahn (University of Colorado Boulder, Department of Chemistry and Biochemistry). MMP activity/metabolic activity assays and zymography experiments were prepared and performed on encapsulated cells as previously described 24 h after drug treatment [184]. Migration and immunostaining experiments were prepared and analyzed similarly to previous studies [139, 186]. Experiments were performed at least three independent times with two replicates per condition. Statistical significance was determined when \( p < 0.05 \). Please refer to SI Materials and Methods for detailed methods.

5.8 Acknowledgements

The authors would like to thank Professor Natalie Ahn (Univ. of Colorado, Dept. of Chemistry and Biochemistry) for generously providing the melanoma cell lines used in this study and Kyle Kyburz for the Matlab code to analyze cell migration. This study was supported in part by the National Institutes of Health (R01 CA132633 and EB018505) and the Howard Hughes Medical Institute. This work was done in close collaboration with Dr. Jennifer Leight as well as Caitlin Jones and Austin Lin.
5.9 Supplemental Information

5.10 SI Materials and Methods

5.10.1 Materials

Four-arm 20kDa PEG hydroxyl (JenKem Technology USA) was functionalized with 5-norbornene-2-carboxylic acid (Sigma) as previously described [6]. The crosslinking MMP-degradable peptide (KCGPQG\IWGQCK) and the pendant adhesion peptide (CRGDS) were purchased from American Peptide Company, Inc. The photoinitiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate was synthesized as described previously [133]. PLX4032, Sorafenib, AZD6244, and CI-1040 were purchased from Chemietek, reconstituted at a stock concentration of 10 mM in DMSO and used at 1 µM in all experiments.

5.10.2 Cell culture

All cell lines (WM35, WM115, WM239A, A375) were generous gifts from Professor Natalie Ahn (University of Colorado, Department of Chemistry and Biochemistry). Cells were cultured on tissue culture-treated polystyrene plates in RPMI 1640 (Life Technologies) with 10% (vol/vol) fetal bovine serum (FBS, Life Technologies). Experiments were carried out with 1% FBS. Cell lines were passaged no more than 15-20 times after thawing.

5.10.3 Cell encapsulation

Cell encapsulations were performed as described previously [184]. Briefly, a single cell suspension of 2 x 10^6 cells/mL (for metabolic activity, zymography, and immunostaining experiments) and 2 x 10^5 cells/mL (for migration experiments) were encapsulated within a PEG-peptide macromer precursor solution. PEG-norbornene (3 mM) was crosslinked with the bicysteine MMP-degradable peptide (25% off stoichiometry, 4.5 mM peptide) in the presence of 1.7 mM LAP. RGD was included at 1 mM and for MMP activity studies the sensor peptide was incorporated at 0.1 mM.
50µL of the cell/macromer solution was pipetted into a 6-mm-diameter ring mold, polymerized under ~5 mW/cm² UV light (XX Series) centered around 365 nm for 3 minutes.

5.10.4 Metabolic activity

alamarBlue Cell Viability Reagent (Life Technologies) was added to culture media at 1:10 (vol/vol) and incubated at 37°C, 5% CO₂ for 6 h. Fluorescence measurements were made with a Synergy H1 microplate reader (BioTek) at 560 nm excitation/590 nm emission.

5.10.5 MMP activity

MMP activity was measured as described [184]. Briefly, MMP-sensitive peptides (GGPQG|1WGQK|Dde-AEEAc-C) (AEEAc purchased from Anaspec) were synthesized using solid phase peptide synthesis on a Tribute Protein Synthesizer (Protein Technologies) with a Rink Amide MBHA resin (Novabiochem). Fmoc protected amino acids were purchased from Chem-Impex. Dabcyl (Anaspec) and fluorescein (Life Technologies) coupling, peptide cleavage, and purification were performed as described [184]. Fluorescence measurements of MMP activity were conducted at 24 h (494 nm excitation /521 nm emission) with a 7x7 matrix area scan using a Synergy H1 microplate reader (BioTek). The average was calculated for the entire matrix, and background (MMP peptide functionalized gels with no encapsulated cells) was subtracted.

5.10.6 Zymography

Zymography was performed as described [184]. Briefly, A375 cells were encapsulated (2 x 10⁶ cells/mL) and incubated for 2 h in 1% FBS RPMI media. The media was then replaced with serum free RPMI. Conditioned media was collected after 48 h of culture, frozen, and lyophilized. Samples were reconstituted in Laemmli sample buffer [230], and equal volumes were loaded into a 10% zymogram gel (Life Technologies) with Precision Plus Dual Color Protein Standard (Bio-Rad). The zymography assay was performed as described [230]. Zymogram gels were scanned to
digital images (Epson, Perfection 4490 Scanner), converted to grayscale, inverted, and quantified using ImageJ software (NIH).

5.10.7 Immunostaining

Immunostaining samples were prepared by encapsulating A375 cells similarly to metabolic activity/MMP activity gels, without the fluorogenic peptide sensor. Samples were treated with PLX4032 for 24 h and then fixed and stained for paxillin (clone Y113, Millipore), F-actin (TRITC-phalloidin, Sigma), and DAPI (Life Technologies). Briefly, cells were fixed at room temperature for 30 min in 1:10 buffered formalin, washed twice with PBS/Tween20 (0.05% vol/vol; PBST) for 15 min. Samples were permeabilized for 30 min at room temperature with PBS/Triton-X (0.01% vol/vol), washed twice in PBST, blocked in 5% BSA in PBST for 1 h, and then incubated with paxillin antibody (1:400) at 4°C overnight. Samples were rinsed twice with PBST for 30 minutes, then incubated with the secondary antibody (1:200), TRITC-phalloidin, and DAPI overnight at 4°C. Samples were washed twice with PBST for 30 min each and then imaged on an LSM 710 confocal microscope (Zeiss). Cell morphology was analyzed using samples prepared in a similar way, but rather than fixing samples, they were stained with calcein (Life Technologies) to visualize overall cell shape and quantified via ImageJ (NIH) for aspect ratio.

5.10.8 Migration

Cells were encapsulated within soft hydrogels (E~200 Pa, 45% off stoichiometry, 2.7 mM crosslinker peptide) that were reacted to thiolated coverslips within a rubber mold [184]. A small drop (6 µL) of macromer solution was polymerized under the coverslip to adhere the coverslip to the bottom of the well plate. Cells were allowed to recover overnight in 1% serum media, and then the media was changed to either contain DMSO (control), PLX4032, or Sorafenib. The samples were then placed on a live-cell microscope (Nikon; TE2000) with a cell chamber (InVivo Scientific). Images were taken every 20 min at three different areas of each gel over the course of three d. A 450 µm z-stack in the center portion of the hydrogel with 15 µm step sizes was obtained
at every acquisition position. Z-stacks were then compressed into z-projections and the x-y cell movement was tracked in MetaMorph (Molecular Devices), and the x-y positions of each cell was then analyzed using Matlab [139] to calculate maximum displacement and cell speed.

5.10.9 Data analysis and statistics

Experiments were performed at least three independent times with two replicates per condition. Data was analyzed with Graphpad Prism 5 software, using a students t-test (Fig. 5.3) or a one-way ANOVA with Tukey's multiple comparison posttests (Figs. 5.2 and 5.4). Statistical significance was determined when $p < 0.05$.

5.11 SI Figures

Figure 5.S1: Verification of metabolic activity as a suitable measure for cell number. Both the WM35 and A375 cells were assessed to determine whether metabolic activity scaled with DNA content within each sample. Metabolic activity was measured 24 h after drug treatment. Gels were then degraded using collagenase, and the cell pellet was lysed to measure the DNA content. Metabolic activity was normalized to DNA content and found to scale with each other (i.e., the ratio between the two metrics was approximately 1 in each condition tested). Mean ± SD, n = 2, *$p < 0.05$ by one-way ANOVA and Tukey posttests.
Figure 5.S2: Quantification of metabolic activity and MMP activity. (a) Metabolic activity as measured by alamarBlue for each cell type and inhibitor. Bars represent percent metabolic activity normalized to the control. (b) MMP activity as measured by fluorescent MMP sensor peptide for each cell type and inhibitor. Mean ± SEM, n = 5, *p < 0.05, **p < 0.01, ***p < 0.001 compared to control (DMSO) by one-way ANOVA and Tukey posttests.

Figure 5.S3: A375 cell migration metrics at 24, 48, and 72 hours. (a) Fraction of migrating cells as defined as having a displacement >15 µm. PLX4032 treatment caused a statistically significant increase in migrating cells compared to sorafenib at all time points and was higher than control samples. (b) Cell speed of migrating A375 cells. PLX4032 resulted in faster cell speeds at 24 and 48 hours after treatment. (c) Maximum displacement of migrating A375 cells. At 24 and 48 hours, A375 cells travelled farther with PLX4032 treatment compared to control or sorafenib samples. Mean ± SEM (± 95% CI for (b) and (c)) n = 3, *p <0.05 based on two-way ANOVA and bonferroni posttests.
Figure 5.S4: Migration of WM35 cells at 24, 48, and 72 h. (a) Fraction of migrating cells as defined as having a displacement >15 µm. In the WM35 cells, the difference in the fraction of migrating cells became more apparent over time, with the largest difference in motility at 72 h with PLX4032 treatment. (b) Cell speed of migrating WM35 cells. Single cell speeds were not different until 72 h despite more migrating cells at each time point. (c) Maximum displacement of migrating WM35 cells. The average farthest displacement was most apparent at 72 hours in the WM35 cells, with no statistical difference at 24 and 48 h between control and drug treated samples. Mean ± SEM (± 95% CI for (b) and (c)) n = 3, *p < 0.05 based on two-way ANOVA and bonferroni posttests.

Figure 5.S5: MMP inhibitor does not affect metabolic activity. To verify that MMP activity is not confer a protective effect to A375 cells, the broad spectrum MMP inhibitor was used (10 µM) in conjunction with and without PLX4032 (1 µM) for 24 h. A decrease in MMP activity was observed with GM 6001 treatment, but there was no change in metabolic activity in the presence or absence of GM 6001. Mean ± SEM, n = 4, *p < 0.001 two-way ANOVA and bonferroni posttests. There is no statistically significant difference in metabolic activity between the DMSO conditions (white bars) with and without GM 6001.
Figure 5.S6: A375 cells encapsulated within PEG hydrogels and treated with DMSO control. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 µm z-stack in the center of the hydrogel. Cells under control conditions do not exhibit high rates of motility. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 µm.

Figure 5.S7: A375 cells encapsulated within PEG hydrogels treated with 1µM PLX4032. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 µm z-stack in the center of the hydrogel. Cells cultured with PLX4032 exhibit increased migration speed and displacement compared to control or Sorafenib conditions. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 µm.
Figure 5.S8: A375 cells encapsulated within PEG hydrogels treated with 1µM Sorafenib. Images were acquired every 20 min and compiled into time-lapse movies for an 8-h window centered around 24 h after treatment. The images shown in the movie are projections of a 450 µm z-stack in the center of the hydrogel. Cells cultured with Sorafenib do not exhibit high rates of motility. The elapsed time from the start of the movie is shown as hour: minutes. Scale bar, 100 µm.
Chapter 6

Conclusions and Recommendations

Melanoma is a highly drug resistant form of skin cancer, but the cause of its robustness is not fully understood. The cellular microenvironment is a complex and important factor that can affect cell function (Fig. 6.1) [5, 116, 159], but its importance is not well understood in the context of melanoma drug responsiveness. The melanoma tumor environment consists of melanocytes that transform into melanoma cells, keratinocytes that make up the majority of cells in the epidermal layer, and fibroblasts that deposit most of the extracellular matrix and paracrine signaling to the epithelia. In an effort to understand the influence of native environment \textit{in vitro}, matrix elasticity, for example, has been shown to influence mammary epithelial cell morphology and signaling [7]. Additionally, whether the cells are cultured on tissue culture-treated polystyrene (TCPS) or as multicellular aggregates can affect their sensitivity to clinically available drugs [102]. Throughout this thesis, we sought to better understand the role of the local microenvironment on melanoma cell function and whether it altered responses to clinically relevant drugs.

To answer the question of whether the microenvironment can influence melanoma cell function, the effect of matrix elasticity was assessed first. From there, we asked whether 2D versus 3D cell culture was an important component of recapitulating more \textit{in vivo}-like responses to PLX4032 treatment, namely decreased efficacy. Many studies compare TCPS culture to multicellular spheroids within a collagen matrix [19, 22, 40], but several factors change between these two culture conditions. For example, TCPS is orders of magnitude stiffer than any soft tissue in the body; cell-cell contacts are present in spheroids though not necessarily in 2D monolayer culture;
collagen is a naturally derived materials and can sequester growth factors, suffer from batch-to-batch variability, and less control over bulk properties than synthetic systems [86, 87, 94, 160]. Therefore, we aimed to determine whether dimensionality was important to drug responsiveness, specifically whether a soft 2D substrate was sufficient to elicit a more drug resistant response similar to what is observed in 3D culture compare to traditional TCPS culture. To make a more direct comparison, we cultured cells on TCPS, on top of hydrogels, and also as single cell suspensions encapsulated within a hydrogel of the same modulus. Lastly, we asked whether 3D culture caused a change in proteolytic activity because in a 3D environment cells are now surrounded by a matrix, which is more physiologically relevant, and would require matrix degradation if the cells were to spread or move.

First, the role of matrix elasticity was investigated on melanoma drug responsiveness in two different human cell lines. The WM35 radial growth phase (RGP) cells and the metastatic A375 cells were tested. In order to probe the role of elasticity, PEG-peptide hydrogels were designed to alter the crosslinking density such that the modulus of the hydrogels was varied between 0.6,
1.6, and 13.1 kPa. This range should encompass a physiologically relevant range of stiffnesses; the exact modulus of healthy and malignant skin is not well documented and it varies greatly from patient-to-patient. Interestingly, the WM35 cells showed stiffness-dependent apoptotic rates and focal adhesion size and formation, while the metastatic A375 cells had similar levels of apoptosis and cell-matrix interactions regardless of elasticity. Focal adhesion size was measured, and they were found to be smaller and fewer on softer substrates in the WM35 cells. The metastatic A375 cells had similar-sized and numbers of focal adhesions based on paxillin staining. The WM35 cells exhibited increased apoptosis as the gels were made softer, but the A375 cells had similar levels cell death irrespective of stiffness. Based on these two major findings, we hypothesized that stiffness-induced focal adhesion formation was leading to changes in survival signaling in the WM35 cells, while the A375 cells were less dependent on the underlying substrate in order to receive survival cues.

As follow-up work, the ERK and AKT survival pathways (Fig. 6.2) were explored (Chapter 7) though the results were inconclusive. AKT levels were higher in the A375 cells, but ERK was generally lower than the WM35 cells. Interestingly, pAKT and total AKT levels were changing with stiffness in the A375 cells, indicating that these cells do sense and respond to the underlying substrate. Despite a slight decrease in AKT signaling on soft gels, however, the levels of FAK were approximately the same on all hydrogels in the A375 cells. Therefore we were able to conclude that while cell-matrix interactions appeared unchanged with substrate elasticity, the A375 are able to sense and translate rigidity in a way that survival signaling is altered. The WM35 cells, on the other hand, exhibited less AKT activity compared to A375 cells but also showed some dependence on elasticity due mostly to the change in the pAKT. Perhaps the WM35 cells have increased susceptibility to PLX4032 because they have lower expression levels of AKT.

Future experiments could further analyze the expression levels of AKT to understand what level of regulation of AKT is altered. From the studies in the Appendix chapter, we were unable to draw a conclusive correlation between stiffness-induced FAK phosphorylation and AKT activity, but more thorough studies could be performed to confirm that hypothesis. We suggest exploring
Figure 6.2: ERK and AKT signaling pathways important to cell survival. Focal adhesion formation and growth factor binding can cause increased signaling through the ERK and AKT pathways. Both pathways promote proliferation and suppress apoptosis, among other functions. ERK is also a known regulator of MMPs.

the regulation and expression of pro- and anti-apoptotic proteins that are important to cell survival. Expression of proteins important to the apoptotic pathway could be monitored by western blot or by PCR. Initial studies evaluating several anti-apoptotic proteins in the A375 cells were inconclusive though not exhaustive. Additionally, the adhesive peptide could changed to mimic collagen [135, 138], which is a major component of the dermis, or elastin [137]. Peptides other than fibronectin mimics may be beneficial because it has been shown that fibronectin but not collagen protects melanoma cells from anoikis by increasing AKT expression [62], and AKT aids in survival against BRAF inhibition [63]. Perhaps we achieved more drug resistant responses due to the protective effect of RGDS, and we might see increased susceptibility with another extracellular matrix (ECM) mimic.
Next, we sought to understand whether 2D versus 3D culture platforms could alter the drug responsiveness of human melanoma cells. Again, the WM35 and A375 cells were tested in hydrogels with a modulus of 0.4 kPa in both 2D and 3D systems along with TCPS as a reference point. Interestingly, the WM35 cells were found to be more sensitive to PLX4032 when cultured on top of or encapsulated within hydrogels compared to TCPS. In line with these findings, previous studies have shown that WM35 cells continue to display sensitivity to BRAF or MEK treatment in collagen-embedded spheroids [22, 39]. In comparison, we observed that the metastatic A375 cells were overall less responsive to PLX4032. In fact, 3D samples exhibited statistically decreased apoptosis compared to TCPS. This phenomenon of less sensitivity in 3D has been observed with other metastatic melanoma cell lines previously [38, 39], and has often been attributed to the nature of 3D cell culture. Based on our findings, we suggest that in metastatic melanoma, 3D cell culture is sufficient to elicit it more drug resistant responses to pharmacological inhibition compared to 2D substrates, but whether it is due to cell-matrix interactions covering the entire surface of a cell or due to a more spherical cell shape that confers this resistance, is not well understood.

We propose that further experiments could be performed to vary the RGDS concentration within the encapsulated cell samples to understand whether cell-matrix interactions are important to decreased drug sensitivity. Perhaps a gel formulation that does not allow cell-mediated degradation (via the d-isoform of the isoleucine residue in the MMP cleavable peptide, or use a PEG-dithiol) or hydrolytic degradation (use PEG-amine, which is not subject to hydrolysis) could be used, so that the cell is frozen in a rounded shape. These studies could elucidate whether cell adhesion or cell morphology that results from a 3D culture platform is important to recapitulate metastatic melanoma survival. Additionally, the adhesion peptide could be changed in order to avoid the use of RGDS, as discussed above. Peptides that mimic cell-cell interactions could also be used to further understand if, and which types of, intercellular interactions are important; for example, E- versus N-cadherin peptide mimics could be tested to assess if either one confers more drug resistance to melanoma cells. Another study could build off of the direct comparison of single cells to multicellular spheroids that was studied to further determine if spheroids result in different re-
sponses compared to single cells. The heterogeneity of a tumor spheroid makes analysis of results from single cells versus spheroids difficult, but a more thorough study comparing the two culture conditions might elucidate important factors to melanoma survival.

Lastly, we studied the effects of BRAF or MEK inhibition on proteolytic activity on melanoma cells. The study utilized a fluorescent MMP sensor peptide in order to quickly assess the effects of drug treatment on MMP activity on four different melanoma cell lines due to four different inhibitors. Interestingly, we observed several conditions where MMP activity increased; we had hypothesized that it would decrease because the ERK signaling pathway regulates MMPs. Through this study, we found heterogeneous responses to drug treatment, whether we analyzed how a single drug affected the four cell types or how a single cell line responded to the four inhibitors. For example, the RGP WM35 cells exhibited statistically significant increases in MMP activity and significant decreases in metabolic activity to PLX4032, AZD6244, and CI-1040. The WM35 cells were not responsive to Sorafenib as evidenced by almost no change in metabolic activity or MMP activity. The metastatic WM239A cells, on the other hand, were not sensitive to any of the inhibitors tested with only slight decreases in metabolic activity and very modest changes in proteolytic activity. Because we observed this unexpected result of increased MMP activity in response to drug treatment in several conditions, we sought to understand whether this led to a functional change in cell behavior. Cell morphology was analyzed, which showed an increase in elongation as well as an increase in cell motility (as measured by fraction of migrating cells, speed, and maximum displacement). For the migration studies, PLX4032 and Sorafenib, both of which can inhibit RAF, were studied. And while PLX4032 induced increases in migration metrics, Sorafenib did not. We hypothesize this may be due to the fact that Sorafenib can inhibit other proteins besides BRAF (including CRAF and PDGFR) [221], and so this may prevent melanoma cells from compensating for a decrease in ERK signaling by not allowing the cells to utilize CRAF to bypass BRAF.

Future work could analyze what causes the increase in MMP activity despite decreased ERK signaling. We did not analyze protein expression levels - only activity, and so further work could be performed to understand at what level of regulation the MMP activity is changing. MMP activity
could change through increased cleavage of pro-MMPs to the active forms, increased production of MMPs as evidenced by mRNA levels, or decreased expression of TIMPs in the presence of PLX4032. A previous study linked increased invasion into collagen gels via the STAT3 pathway [168], however, we did not observe a correlation between MMP activity and STAT3 (unpublished data). Because collagen is a fibrillar hydrogel, cells may utilize different modes of migration; in our PEG-peptide hydrogels, cells are required to degrade the matrix in order to migrate, whereas in collagen gels the cells can move along the fibers and deform the network [187, 201].

Longer-term studies could build on the method of in situ MMP activity via a fluorogenic peptide [184] to study the role of other proteases important to the metastatic cascade. Other enzymes that can degrade the local matrix and have been implicated in melanoma metastasis (e.g., cathepsins and urokinase-type plasminogen activator, uPA) [231, 232] can be studied by using a peptide sequence meant to mimic these proteins. Melanoma cell lines from different stages of progression can then be cultured in the presence of the different fluorogenic peptide sequences to understand how proteolytic activity changes with melanoma progression. We propose using a combination of peptide sequences/probes in order to visualize matrix degradation due to different proteases during cell migration (Fig. 6.3). By using several sequences at once, we can quantitatively measure this enzymatic activity as well as understand the spatiotemporal distribution and use of the proteases by melanoma cells. In addition, properties such as the adhesive ligand mimic, the concentration of the adhesive ligand, and matrix elasticity can all be altered to better understand what type of microenvironment is most conducive to melanoma metastasis and migration.

This thesis research took a minimalist approach to studying specific cell-matrix and cell-cell interactions and how they may influence melanoma drug responsiveness in an effort to deconvolute complicated biological systems. With the knowledge gained through this research, we propose to increase the complexity of the in vitro culture system by utilizing a co-culture platform to understand the potential influence of other cell types on melanoma growth, invasiveness, and drug responsiveness. Spheroid cultures used in this thesis were formed from a single cell type and exhibited increased invasion in the presence of PLX4032. Previous preliminary studies have shown
Figure 6.3: Simultaneously visualizing cell migration and proteolytic activity. (A) Cells are typically rounded after encapsulation and fluorescence should be low due to little degradation of the fluorogenic sensor peptides. (B) As cells migrate through the hydrogel matrix, the peptide sensors will be cleaved and therefore increase in fluorescence that can be quantified either by a standard plate reader or potentially through microscopy. This will aid in localization as well quantification of enzymatic activity. Image courtesy of Jennifer Leight and Kristi Anseth from NIH R21 (#1551854) grant proposal.

that melanoma clusters grown in proximity to fibroblasts exhibited migration toward the healthy fibroblasts without any external stimulus, namely drug treatment (Fig. 6.4). From this observation, the influence of stromal fibroblasts can be understood in the context of melanoma metastasis or whether fibroblasts confer any protective effects from drug treatment. This experimental setup can also be combined with the different protease-sensitive fluorogenic peptide sensors described above to understand the mechanisms through which leading single cells and the subsequent follower cells use to migrate through a matrix. Future work could then address the role of healthy and cancer-associated fibroblasts in melanoma invasion and drug sensitivity. Preliminary experiments showed that healthy fibroblasts may suppress multicellular melanoma spheroid growth via paracrine signaling (unpublished data). As a follow-up to this observation, melanoma spheroids from different stages of progression could be cultured both in the presence and absence of healthy or activated fibroblasts. Perhaps healthy fibroblasts, but not cancer-associated fibroblasts, suppress growth and proteolytic activity of melanoma cells. Drug sensitivity could also be studied in this co-culture system in order to better recapitulate the \textit{in vivo} environment.

Lastly, to gain further clinical relevance, these hydrogel culture platforms could be utilized
Fibroblast-laden collagen with melanoma clusters

Figure 6.4: Co-culture of WM239A metastatic melanoma cell clusters cultured in the presence of fibroblasts. (a) Co-culture migration experimental setup. Healthy fibroblasts (red) were encapsulated within a collagen ring that surrounds the PEG hydrogel containing WM239A melanoma clusters (green). (b) Time lapse images of single cell migration away from a melanoma cluster. In the center of the images is a melanoma cluster encapsulated within a PEG hydrogel. A single cell broke away and was subsequently followed by other cells from the aggregate. The fibroblasts were located just beyond the scale bar in these images. Images were obtained every 20 minutes but sequential images from every 4 hours are shown. Scale bar, 40 µm.

To study xenograft samples derived from patient biopsies. We previously obtained xenograft samples (human patient-derived samples that were then implanted into a mouse before being surgically removed again) from Professor Natalie Ahn. Based on previous work, melanoma xenograft cells can survive within our hydrogel matrices and exhibit an invasive phenotype (Fig. 6.5), a behavior that is not observed on traditional TCPS cell culture conditions. One possible direction is to study the drug responsiveness of patient-derived samples to move towards a drug screening platform. A means by which to test drug efficacy on patient-derived samples could aid in finding the best treatment for each individual personalized medicine [233] is based on the knowledge that typically only a subset of patients will exhibit significant results in response to a given anticancer therapy [234]. And while the field of personalized medicine is not completely realized, a culture platform that allows for simultaneous evaluation of *ex vivo* drug sensitivity as well as proteolytic profiles would contribute greatly. Patient samples could be encapsulated within hydrogels containing the protease-sensitive peptide sensors to determine the enzymatic activity of an individual's tumor and screen for drug efficacy. From this culture platform, ideally, physicians and scientists could understand the metastatic potential and best treatment options available for each unique case.

In summary, we have studied the effects of matricellular signaling on melanoma drug re-
Figure 6.5: Xenograft sample from a human patient and grown in a mouse. This xenograft sample was encapsulated within a PEG hydrogel and allowed to grow for approximately 24 hours. Time lapse images show outgrowth from the main body of the sample at approximately 8-hour intervals. Scale bar, 100 µm. Images courtesy of Michael Schwartz.

Sponsiveness using synthetic hydrogels. Prior to these studies, it was unknown whether matrix rigidity could alter melanoma drug responsiveness or that a soft hydrogel for 2D cell culture can be sufficient to mimic 3D culture results. Additionally, we found that treatment with a clinically available drug (PLX4032) can cause an unwanted side effect of increased proteolytic activity, which correlates to increased migration in vitro. While we cannot, at this time, conclude that the high rate of patient relapse might be due, at least in part, to the increased migratory potential of
PLX4032-treated melanoma cells, we hope other researchers will take note. Overall, the use of synthetic hydrogels has allowed us to study specific contributions of the local microenvironment on melanoma cell function. We hope that this can be used by other researchers in order to answer additional fundamental biological questions about melanoma and perhaps be utilized to study and eventually overcome melanoma drug resistance.
Bibliography


131


Chapter 7

Appendix

7.1 Introduction

Melanoma is a form of aggressive skin cancer with a 5-year survival rate of less than 15% for metastatic cases [10]. This is due in large part to the highly drug resistant nature of metastatic melanoma, where the mechanisms of drug resistance are not well understood [13, 47]. PLX4032 is a specific mutant BRAF small molecule inhibitor [19] that showed excellent clinical efficacy in Phase I and Phase II clinical trials [2, 25, 68]. Despite the unprecedented tumor shrinkage, almost all patients eventually show PLX4032 resistance, typically within 6 months after starting treatment [26, 68]. One of the possible influences on melanoma drug responsiveness could arise from the local tumor microenvironment. For example, matrix elasticity in 2-dimensional cell culture can influence cell function [7, 116, 151], and 2D versus 3D culture can affect drug sensitivity by influencing factors such as availability, receptor signaling, and transport [38, 102, 158]. Previously, we showed that depending on the stage of melanoma progression, differential drug sensitivity was observed in response to changes in matrix rigidity [183]. We therefore asked what might contribute to this response in the two cell types tested and what mechanosensing machinery might be involved. The early stage radial growth phase (RGP) WM35 cells exhibited increased apoptosis and smaller focal adhesions on soft substrates; the metastatic A375 cells showed no change in focal adhesion size or levels of apoptosis within the range of stiffnesses tested. As a result, we attempted to determine the importance and roles of the survival signaling pathways ERK and AKT in melanoma survival. The results in this appendix documents and summarizes the key findings to
7.2 Results

7.2.1 Matrix elasticity regulates ERK and AKT signaling

In order to assess the effects of substrate stiffness on melanoma survival signaling, we used PEG-peptide hydrogels. These step-growth networks were formed via the photoinitiated thiol-ene reaction; norbornene-functionalized four-arm 20 kDa PEG (-ene) was reacted with cysteine-containing peptides (-thiol) to form the network. Specifically, the hydrogels were crosslinked with matrix metalloproteinase (MMP) degradable peptides (KCGPQG↓IWQGCK, down arrow denotes sight of cleavage) that are flanked by cysteines on either end. The fibronectin-derived CRGDS peptide was covalently incorporated as a pendant functional group to promote cell adhesion and spreading. The modulus of the hydrogels was varied by altering the crosslinking density, either by forming the gels off stoichiometry or by changing the concentration of PEG (Table 7.1). Three formulations were selected that were previously characterized and resulted in hydrogels with moduli of 13.1 kPa (stiff), 1.6 kPa (medium), and 0.6 kPa (soft) [183].

<table>
<thead>
<tr>
<th></th>
<th>[PEG] (mM)</th>
<th>[Crosslinker] (mM)</th>
<th>[Pendant RGD] (mM)</th>
<th>Modulus E (kPa)</th>
<th>Crosslink Density Px (mM)</th>
<th>Equilibrium Mass Swelling Ratio q</th>
<th>% Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stiff</td>
<td>7.5</td>
<td>14.25</td>
<td>1.5</td>
<td>13.1 ± 0.4</td>
<td>5.17 ± 0.40</td>
<td>21 ± 1</td>
<td>95.3 ± 0.2</td>
</tr>
<tr>
<td>Medium</td>
<td>3</td>
<td>4.5</td>
<td>1.5</td>
<td>1.6 ± 0.1</td>
<td>0.83 ± 0.09</td>
<td>45 ± 1</td>
<td>97.7 ± 0.1</td>
</tr>
<tr>
<td>Soft</td>
<td>3</td>
<td>3.9</td>
<td>1.5</td>
<td>0.6 ± 0.1</td>
<td>0.36 ± 0.05</td>
<td>62 ± 1</td>
<td>98.4 ± 0.1</td>
</tr>
</tbody>
</table>

Cells were seeded on top of these hydrogels and on TCPS, and western blots were performed to assess ERK (Fig. 7.1d) and AKT (Fig. 7.2d) levels. The WM35 cells expressed lower phospho ERK (pERK) (Fig. 7.1a) and total ERK (Fig. 7.1b) on any hydrogel surface compared to TCPS, which resulted in almost no change in ERK activity (pERK/total ERK) across all substrates (Fig. 7.1c). The A375 cells, on the other hand, had overall lower levels of pERK, which had a general
downward trend as the gels were made softer (Fig. 7.1a). Levels of total ERK in the A375 cells had a similar decreasing trend as pERK levels (Fig. 7.1b), and resulted in overall similar ERK activity on all substrates that was also lower than the WM35 cells (Fig. 7.1c).

In addition, the WM35 cells had a general decrease in phospho (S473) AKT (pAKT) levels as the gels were made softer, while total AKT remained approximately unchanged (Fig. 7.2). As a result, the RGP WM35 cells exhibited a slight decrease in AKT activity (pAKT/total AKT) as the modulus of the substrate was made softer (Fig. 7.2c). The metastatic A375 cells had a general downward trend in pAKT, as well as total AKT levels as the gels were made softer (Fig. 7.2a, b). Interestingly, the A375 cells had overall higher AKT activity compared to the WM35 cells and had a slight decrease in activity on the soft hydrogels compared to TCPS and gels with stiff and medium moduli (Fig. 7.2c).
7.2.2 Matrix elasticity effects on FAK activity

Because both the ERK and AKT signaling pathways are, in part, regulated by focal adhesion formation, we next characterized the levels of phospho (Y397) FAK (pFAK) as a function of matrix elasticity via western blot (Fig. 7.3d). We have previously shown that focal adhesion size changes for the WM35 cells, but not the A375s when seeded on gels of varying stiffnesses [183], so we sought to understand whether or not there was a relationship between focal adhesion size and FAK activity levels. In both cell lines, pFAK levels were low on all hydrogel substrates compared to TCPS (Fig. 7.3a). The total levels of FAK remained relatively constant regardless of substrate rigidity in the WM35 cells, while the A375 cells exhibited a slight dependence with decreasing levels observed on lower modulus substrates (Fig. 7.3b). FAK activity (pFAK/total FAK) was much lower on hydrogels compared to TCPS in both cell types (Fig. 7.3c).

Figure 7.2: AKT activity of WM35 and A375 cells seeded on top of TCPS or hydrogels. Western blots were performed to assess levels of pAKT (a), total AKT (b) or AKT activity (c) in both cell types. Image of a western blot (d). n = 6, *p < 0.05 for conditions within each cell type, #p < 0.05 for indicated stiffness compared to WM35 cells.
7.2.3 FAK regulates AKT and influences melanoma drug responsiveness

To test whether FAK can affect AKT signaling, which is implicated in melanoma survival, we used a FAK inhibitor to prevent phosphorylation of FAK at Y397. Using the inhibitor PF573228, we confirmed that it decreased pAKT and pFAK levels after 4 hours of inhibitor treatment in the WM35 cells on TCPS (Fig. 7.4a). The inhibitor was also potent enough to nearly ablate signaling through pERK as well. The A375 cells were also assessed, and while P573228 did decrease pAKT slightly, it was not as potent as in the WM35 cells (Fig. 7.4a). The PI3K inhibitor LY294002 was also used as a positive control to inhibit signaling through AKT. LY294002 decreased ERK signaling in both cell lines and did not appear to decrease pFAK levels.

Because PF573228 decreased AKT signaling, which is an important survival pathway, we determined whether pFAK inhibition affected melanoma cells’ responsiveness to PLX4032 treatment (Fig. 7.4b). PLX4032 was either administered alone, with PF573228, or with LY294002. Interestingly, LY294002 and PF573228 alone cause significant decreases in metabolic activity in
Figure 7.4: Testing whether PF573228 or LY294002 inhibited AKT and affected PLX4032 sensitivity. (a) Western blots showing that pAKT was affected by PF573228 in both cell types, exhibiting decreased levels of expression. pERK and pFAK were also evaluated and affected by PF573228 or LY294002 for 4 hours on TCPS. n = 2 (b) Metabolic activity was measured after 48 hours of PLX4032 treatment. n = 3, *p < 0.05, **p < 0.01.
both cell lines compared to the control. PLX4032 treatment alone caused an even further decrease in metabolic activity in the WM35 cells, but not in the A375 cells. In both the WM35 and A375 cells, drug treatment in combination with either the pFAK or PI3K inhibitors caused another decrease in metabolic activity compared to PLX4032 treatment alone. Because the WM35 cells had exhibited a larger decrease in AKT signaling with PF573228 treatment and were previously shown to be more sensitive to PLX4032 treatment [183], we also measured the levels of apoptosis (Fig. 7.5). PLX4032 treatment alone caused the largest increase in apoptosis, while the combination of pFAK or PI3K inhibitors and PLX4032 did not lead to any synergistic effects, and instead, caspase activity was lower than with PLX4032 treatment alone.

![Figure 7.5: Apoptosis as measured by caspase 3 activity in the presence of PF573228 or LY294002 in response to PLX4032 treatment. n = 2.](image)

### 7.3 Discussion

We observed differential responses to matrix elasticity between two melanoma cell types (i.e., radial growth phase WM35 and metastatic A375 cells) based on ERK and AKT activity. The RGP cells exhibited decreased levels of pAKT and lower AKT activity, but overall higher ERK activity on hydrogels compared to the metastatic A375 cells. In an attempt to determine
whether FAK had any influence on AKT activity, we inhibited FAK phosphorylation with a small molecule inhibitor and assessed its effects on PLX4032 drug responsiveness. The WM35 cells showed increased sensitivity to PLX4032 based on metabolic activity (Fig. 4b), but this severe drug response was not reflected in levels of apoptosis (Fig. 7.5). As a result, we hypothesize that the decrease in metabolic activity may be due to a strong anti-proliferative effect on the WM35 cells, rather than causing an increase in apoptosis. Additionally, because ERK activity is higher in the WM35 cells, we propose that the WM35 cells may be more reliant on the ERK pathway than the A375 cells. This might explain why the WM35 cells generally have more sensitivity to PLX4032 treatment in 2D, but also in 3D.

The metastatic A375 cells, on the other hand, had overall higher levels of AKT (both pAKT and activity), which has been shown to protect melanoma cells from anoikis and BRAF knock-down [62, 63]. Increased levels of AKT signaling have been previously shown to exist in BRAF treatment-resistant melanoma cell lines [37, 63]. Interestingly, the levels of total AKT and total ERK decrease along with the phospho levels as the substrate elasticity decreases. This result has been reported before [119, 235], though it has not been thoroughly explored. We hypothesize that perhaps the A375 cells become less reliant on AKT when cultured on soft substrates as indicated by the decrease in AKT expression on soft hydrogels compared to TCPS or stiff gels. While we tested FAK to assess whether its role was important to melanoma survival, we noted a similar effect on metabolic activity in both the A375 and WM35 cell lines. Based on previous work analyzing focal adhesion size as a function of matrix rigidity [183], we did not hypothesize that FAK directly regulates or correlates with ERK or AKT in the A375 cells.

It is unclear at what level of regulation AKT is altered on soft hydrogels. While this is an interesting observation, more work would be necessary to understand through what mechanism total AKT levels decrease due to substrate elasticity, and why this does not result in increased levels of cell death with drug treatment. Potentially, other survival pathways may be able to compensate for the decreased levels of AKT in the A375 cells [45]. Future work might include studying the regulation of AKT in A375 cells and whether there is a relationship to FAK activity. The
A375 cells also have lower levels of ERK activity, and so it is possible the A375 cells have more anti-apoptotic proteins in place to aid in cell survival, independent of AKT or ERK. Based on a preliminary study to look at anti-apoptotic proteins, those particular proteins do not appear to be up-regulated (data not shown). We hypothesize that other pro-apoptotic proteins may be down-regulated instead. A more comprehensive overview of survival pathways (including pro- and anti-apoptotic proteins) would need to be analyzed in order to understand what confers A375 cells the ability to survive with less ERK than the WM35 cells and what might cause an increase in AKT despite its independence from cell-matrix interactions mediated by FAK.

7.4 Materials and Methods

7.4.1 Materials and reagents

Unless otherwise noted, all chemicals were purchased from Sigma and all cell culture reagents were purchased from Life Technologies. Four-arm PEG hydroxyl (20 kDa) was purchased from JenKem Technologies USA and all peptides were purchased from American Peptide Company: MMP crosslinker KCGPQGIWGQCK and adhesion peptide CRGDS. The FAK inhibitor PF573228 was purchased from Tocris; PLX4032 was purchased from Chemietek; and LY294002 was purchased from Cell Signaling. All inhibitors were dissolved in DMSO at a stock concentration of 10 mM. PLX4032 and PF573228 were used at 1 µM and LY294002 was used at 10 µM.

7.4.2 Cell culture

The WM35 and A375 cell lines were generous gifts from Professor Natalie Ahn (University of Colorado, Department of Chemistry and Biochemistry). Cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS). All experiments were carried out in 1% serum conditions.
7.4.3 Hydrogel formation

PEG-norbornene was functionalized as previously described [6]. Hydrogels were formed using the formulations listed in Table 7.1 and 0.05 wt% of the photoinitiator LAP, synthesized as previously described [133]. Macromer solutions were prepared, and then a 25 µL drop of solution was placed on a Sigmacote-coated glass slide and a thiolated 18 mm coverslip was placed on top of the drop. Coverslips were functionalized with a thiol-containing silane as previously described [183]. Hydrogels were placed under a UV light (XX Series) centered around 365 nm light, ~5 mW/cm² for 3 minutes. The coverslip and glass slide were separated and the gels were allowed to swell overnight. Gels were sterilized at room temperature in 5% isopropanol for 1 hour, rinsed twice with PBS, and then seeded with cells at 1 x 10⁵ cells/cm².

7.4.4 Western blotting

All antibodies were purchased from Cell Signaling unless otherwise noted. Five hydrogels of each stiffness and cell type (or 5 wells of cells plated on TCPS) were prepared. The replicate gels for each stiffness were combined by inverting the cell-seeded hydrogel on top of a 100 µL solution of lysis buffer (1X RIPA buffer, PhosStop and Complete Protease Inhibitor Cocktail). Protein concentrations were determined by microBCA (Pierce Technologies) and then at least 10 µg were loaded into 10-well 4-12% Bis Tris NuPage polyacrylamide gels. Proteins were transferred to a PVDF membrane (Bio-Rad Technologies) and probed for pERK (1:1000), ERK (1:1000), pAKT (1:1000), AKT (1:1000), pFAK (1:1000, Life Technologies), FAK (1:500, Santa Cruz), and GAPDH (1:1000). Donkey anti-mouse or donkey anti-rabbit conjugated with HRP were purchased from Jackson Immunoresearch and used at 1:5000. Membranes were imaged on an ImageQuant LAS 4000 (GE) imager using the DuraWest Super Signal HRP substrate (Pierce Technologies).
7.4.5  Metabolic activity

Cells were allowed to adhere overnight in 48-well plates, and then pretreated with either DMSO (control), PF573228, or LY294002 for 4 hours, and then new solutions containing those solutions with and without PLX4032 were added to samples. Cells were incubated with inhibitors for 2 days, then AlamarBlue was added approximately 4 hours before collection. Samples were read on a plate reader (485/530 ex/em) (Biotek Synergy H1).

7.4.6  Apoptosis

Samples were prepared similarly to metabolic activity experiments. Apoptosis was detected using the EnzCheck Caspase 3 Kit #2. When collecting samples, media was collected from the samples to collect any floating cells. This was combined with trypsinized cells that may have been left in the wells. These cells were spun down for 4 minutes at 1100 RPM, rinsed with PBS, and then the cell pellet was lysed using the provided lysis buffer. Samples were put through a freeze-thaw cycle to ensure complete lysis. The company protocol was followed. In addition, samples were normalized to the amount of DNA in each sample. The amount of DNA was quantified by taking 10 µL of the caspase 3 lysate and using the Quant-it PicoGreen assay kit.

7.4.7  Statistical analysis

All experiments were run at least 3 times, except apoptosis experiments, which were run twice. Each experiment had at least 2 technical replicates. Statistical tests were run in Graphpad Prism 5 with two-way ANOVAs and bonferroni posttests. Statistical significance was determined as p < 0.05.