2016-03-01

Forward Chemical Genetics Drug Screen Yields Novel Proteases and Proteolytic Inhibitors of HGF–induced Epithelial–Mesenchymal Transition

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements of the degree of

Master of Science

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March 2016

ABSTRACT
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Hepatocyte Growth Factor (HGF)-induced Epithelial-Mesenchymal Transition (EMT) is a complex cellular pathway that causes epithelial cell scattering by breaking cell-cell contacts, eliminating apical-basal polarity, and replacing epithelial markers and characteristics with mesenchymal markers. Early EMT events include a brief period of cell spreading, followed by cell compaction and cell-cell contact breaks. A forward chemical genetics drug screen of 50,000 unique compounds measuring HGF-induced cell scattering identified 26 novel EMT inhibitors, including 2 proteolytic inhibitors. Here, we show that B5500-4, one of the EMT inhibitors from the screen, blocks HGF-induced EMT by a predicted blocking of the protease furin, in addition to secondarily blocking Beta-Secretase (BACE).

We also show that MMP-12 and MMP-9 are required for HGF-induced EMT to progress. MMP-12 is required for cell contraction, and its inhibition produces a continuous cell spreading phenotype.

We also demonstrate that both furin and BACE activity are required for HGF-induced EMT to proceed, but that they are involved in separate pathways. We show that BACE inhibition leads to a failure of cell spreading in early EMT, and that EphA2 is a member of this pathway. We also demonstrate that it is likely BACE2, and not BACE1 that is responsible for early cell spreading. Furin is also required for HGF-induced cell scattering, but does not play a role in the cell spreading process. These findings highlight the importance of proteolytic activity at the earliest stages of HGF-induced EMT.

Key Words: EMT, cell spreading, cell compaction, HGF, BACE, EphA2, Furin, MMP-9, MMP-12

ACKNOWLEDGEMENTS

I would like to deeply, deeply thank my research advisor, Dr. Marc Hansen. In the spring of 2010, I entered his office to learn more about his lab and research. While he provided answers to my questions, we ended up talking more about hockey, Minnesota, and movies. That meeting—while at the time seeming so inconsequential—set me on the path to devoting myself to the study and science of physiology and cell biology, to pursuing this master's degree, and to ultimately altering my life and career goals for the better. I am forever grateful to Dr. Hansen for his mentorship, guidance, friendship, and patience.

I also acknowledge the pillar of support and love that my family has been to me, not just through my time as a graduate student, but through my whole life. To my sisters and brother, my grandparents, my two nephews, and especially to my loving parents, I say thank you. Thank you for helping me to become who I am today.

I would like to thank the other members of my graduate committee, Dr. Juan Arroyo and Dr. Joshua Andersen, for their guidance and help through my graduate student journey. I have always felt kindness and support from each of you as I have stepped and stumbled my way through my degree. Thank you.

I also thank Jessica Jensen and Melissa McNeil for their help with cell culture and live cell imaging. They were invaluable assets to me. Also to Dr. Watt and Catalina Rodriguez, who were instrumental in providing molecular docking data on B5500-4. Thanks are also in order for Frost Biologic, who provided key data to the characterization of B5500-4.

I express my deepest thanks and gratitude to the Department of Physiology and Developmental Biology for funding me through TA and RA opportunities. This work would not have been possible otherwise.

I would also like to take a moment to thank Connie Provost for her constant guidance and help through the entire graduate school process, from applying, to scheduling, to filling out forms and paperwork, and to getting me towards graduating. Thank you so much for your constant prodding to get me in the right direction. It was always a pleasure to work with you.

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CHAPTER 1: Introduction and Literature Review

An Overview of the Biological Uses of Epithelial-Mesenchymal Transition

The development of most organ systems requires restructuring and reorganization of

embryonic cells into functional tissues through multiple stages of Epithelial-mesenchymal

transition (EMT) and mesenchymal-epithelial transition (MET) (Birchmeier 1996). Groups of

individual cells assemble into epithelial tissues by forming cell-cell contacts with their neighbors

and establishing an apical-basal polarity, which occurs through the sequential organization of

adherens junctions, tight junctions, and desmosomes (Barasch 2001). Additionally, gap

junctions between cells enable communication throughout the epithelial cell layer (Defamie

2014). Separation between epithelial sheets and adjacent tissues is maintained by the

formation of a basal lamina, a layer of extracellular matrix upon which epithelial sheets sit

(Barasch 2001). Mesenchymal cells, on the other hand, maintain minimal cell-cell contacts,

invade through and within the extracellular matrix, and are typically found throughout

connective tissues below the epithelial layer and its basal lamina (Defamie 2014).

The transformation from epithelial to mesenchymal phenotype is a complex cellular

event characterized by the loss of cell-cell junctions and apical-basal polarity, and the

acquisition of mesenchymal characteristics and migratory abilities (Stoker 1985, Weidner 1991,

Bhargava 1992, Ridley 1995). Epithelial cells are induced to undergo EMT, or 'scatter' in the

case of cultured cells, by a variety of signaling and environmental factors, including HGF, VEGF,

EGF, TGF-beta, Wnt, Notch, tissue wounding, and hypoxia (Ridley 1995, Morabito 2001, Kim

2002, Timmerman 2004, Saika 2003, Boyer 1992). EMT is induced by these different factors at

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some of the earliest stages of tissue organization during embryonic development (Kalcheim 2015).

Three distinct stages of EMT and MET are required for determining the final cell-fates of many specialized tissues, and are referred to as Primary, Secondary, and Tertiary EMT. The first instances of EMT within the developing embryo are known as Primary EMT, and occur in most multicellular organismal development (Kalcheim 2015). Examples include EMT events during mammalian implantation, during gastrulation in metazoans, and during neural crest development in vertebrates. The formation of the parietal endoderm is one of the first EMT events in embryonic development. Cells from the inner cell mass break cell-cell contacts, migrate through the primitive endoderm, and relocate along the inner blastocyst walls to form the parietal endoderm (Kalcheim 2015). Mesoderm formation also requires EMT. In this process cells invade through the primary streak and reorganize into the mesodermal tissue. EMT is also observed throughout later development. Somites undergo EMT during the vertebral development of the embryo. Several rounds of EMT and MET occur during heart valve development (Barasch 2001).

The Role of EMT in Disease Progression

While epithelial cell scattering is required for embryonic development, tissue maintenance, and wound healing, its inappropriate activation contributes pathologically to fibrosis, Crohn's disease, cataract formation, and cancer metastasis (Bataille 2008, Kalluri 2003, de Longh 2005, Ruiz 1996).

Cancer and some other disease types misuse EMT to form dangerous and life-threatening phenotypes. Cancer cells often hijack EMT pathways, leading to increased cell

scattering and mesenchymal characteristics. Cancer cells undergoing EMT produce proteolytic enzymes that degrade the basement membrane, allowing for individual cells to locally invade and migrate to distant tissues. Mesenchymal-epithelial transition then occurs, resulting in the formation of distant metastases (Polyak 2009).   Accompanying EMT during cancer progression are a variety of changes to cellular behavior, including resistance to apoptosis, enhanced survival, genome instability, and resistance to chemotherapy, all of which affect disease progression and prognosis (Blick 2008, Kalluri 2009, Polyak 2009). It is particularly important to figure out the best methods of inhibiting the EMT program from progressing in cancer, as metastatic cancer is much more difficult to treat than non-metastatic cancer.

The Role of HGF and the c-Met Pathway in Driving the EMT Program

Hepatocyte Growth Factor (HGF) and its target, the c-Met receptor tyrosine kinase are of particular interest in cancer research. HGF-induced EMT initiates a complex and specific program referred to as invasive growth, and is most often characterized by the dissociation of cell-cell junctions via decreased membrane-localized E-cadherin, and increased N-cadherin expression. Downstream activation of the RAS, PI3K, STAT, Notch, and beta-catenin signaling pathways are required for HGF-induced EMT to progress (O'Brien 2004, Marshall 1995, Graziani 1991, Boccaccio 1998, Monga 2002). The switch from epithelial to mesenchymal states is driven by activation of key transcription factors SNAIL, SLUG, TWIST, and ZEB1/2 (Rodriguez-Paredes 2011). In carcinomas, aberrant activation of the c-Met pathway leads to more metastatic behavior in cancers and to a poorer prognosis in multiple cancer types (Baschnagel 2014, Yoshinao 2000, Masuya 2004).

Epigenetic changes can also regulate EMT progression. Cell scattering can be initiated when mesenchymal genes and their promoter sites undergo H3 acetylation or H3K4 trimethylation, or when there is hypermethylation of their repressor sites (Rodriguez-Paredes 2011, Ehrlich 2009, Ehrlich 2002). The miRNA-200 and miRNA-205 families have specifically been linked to the regulation of the EMT program, which in turn are regulated by histone modification (Fullgrabe 2011). These and many other epigenetic factors have shown pronounced effects in the regulation of the cell scattering program.

In addition to intracellular signaling cascades and regulatory networks, a variety of extracellular environmental cues contribute to the HGF-induced cell scattering event. Cell-substrate stiffness can directly influence the ability of epithelial cells to undergo HGF-induced EMT (Hoj 2014); increasing the pliability of the surface substratum reduces cell-substrate tension, which in turn reduces HGF-induced cell scattering (Hoj 2014). Increases in cell-substrate tension also leads to increased nuclear localization of YAP/TAZ, which act as mechanotransduction sensors and mediators that respond to tension changes in the cellular microenvironment (Sirio 2011).

Other signaling pathways can also interact with and affect the progression of HGF-induced cell scattering. TGF-beta can enhance HGF-induced EMT, and has a chemotaxic effect on scattering cells. VEGF and TGF-beta act competitively to enhance HGF, and VEGF will neutralize the additive scattering of TGF-beta (Chung 2011). The crosstalk that exists between major signaling pathways and the c-Met pathway serves to underscore the complexity of the scattering pathway, and emphasizes the need to further study this cellular event in greater detail.

The Role of Proteases in EMT and HGF-pathway Programs

Among the earliest changes in cell behavior during EMT initiation includes proteolytic activation and deactivation (Tervonen 2015, Craig 2015, Ha 2015, Gray 2014). Cell surface serine protease Hepsin activity increases downregulates HGF activator inhibitor type 1 (HAI-1), increasing HGF-pathway activation and EMT progression (Tervonen 2015). Chemical inhibition of matrix metalloproteinases 2/9 by Sorafineb inhibits c-Met and MEK/ERK pathways, leading to decreased migration and invasion of hepatocellular carcinoma cells (Ha 2015). Additionally, matrix metalloproteinases 3, 7, and 8 have been implicated in EMT progression in patients with idiopathic pulmonary fibrosis (Craig 2015). Additionally, type-II transmembrane serine protease matriptase inhibition leads to a failure of HGF-dependent EMT in MDCK cells (Gray 2014).

Increasing numbers of proteases are being found to regulate EMT progression; however, the roles of many of these proteases have yet to be fully understood (Tervonen 2015). Additionally, many additional proteases may yet hold undiscovered roles in c-Met pathway and EMT regulation.

A High-Throughput EMT Assay Yields Novel EMT Inhibitors

While traditional research methods have provided valuable insights into signaling pathways and their mechanics, a forward chemical genetics screen provides a fresh perspective of the c-Met pathway by allowing for the simultaneous discovery of novel molecular components and new drug inhibitors of HGF-induced EMT. Pharmacologically inhibiting the scattering phenotype not only provides the opportunity to further understand the molecular components of the pathways involved, but can also reveal the therapeutic potential and clinical relevance of the novel inhibitors in the treatment of several human diseases,

including and especially cancer metastasis (Kubinyi 2002, Russel 2004, Carlson 2012, Cornelius 2011) .

The forward chemical genetic screen stands as a unique method for identifying novel molecular components in a pathway, and provides several key advantages and disadvantages. In this approach, molecules from large chemical libraries are assessed for biological activity in a high-throughput and high content assay system that measures the biological output of some cellular process or pathway. Compounds identified in screening are expected to target cellular machinery required for the cellular process or pathway of interest. The drawback to this method is that once a drug candidate is identified, further work must be done to identify the molecular target of the compound.

In a prior publication, we reported a drug screen of 50,000 distinct chemical compounds in a high throughput assay testing inhibition of cell scattering and reported those with known biological activity, which highlights how this approach works (Langford 2012). In our screening assay, MDCK cells are seeded on a 96-well trans-well plate and induced to undergo EMT with HGF. The screen yielded 20 biologically relevant small chemical inhibitors of HGF-induced cell scattering out of the compound library.   One of these 20 chemical scaffolds is a neuronal calcium channel blocker (Langford 2012). Subsequent experiments designed to understand why a calcium channel blocker would prevent EMT revealed that a calcium influx follows HGF stimulation and is required for progression of EMT.

CHAPTER 2: Results

HGF Causes Cell Surface Area Spreading, then Contraction in MDCK Cells

The cellular events associated with HGF-induced epithelial scattering of MDCK cells have been carefully characterized previously (Sperry 2010). Here we repeat the results of Sperry et al. (Sperry 2010) by seeding MDCK cells into collagen-coated imaging dishes. After 24-48 hours, small colonies of MDCK cells were picked for brightfield live cell imaging before initiation of imaging. To observe epithelial scattering, cultures were stimulated with HGF immediately prior to initiation of imaging. For each experiment, we performed imaging on a minimum of 15 colonies in order that we might observe reproducible effects throughout each culture. Further, experiments were reproduced 3 times to highlight any differences between independent experiments. Thus, for each experimental condition, we analyzed at least 3x colonies of MDCK cells. Our results show that MDCK cell colonies stimulated with HGF undergo cell spreading, which occurs within the first two hours after HGF treatment (Fig. 2.1A). To quantitatively define this cell spreading, we measured to amount of the culture dish covered by colonies of MDCK cells during imaging and normalized this area to the number of cells in each colony. This quantitation shows that MDCK cell contact with the collagen substrate increases by some 44% within the first two hours (Fig. 2.1A, C). Following the two hour cell spreading phase, HGF-induced MDCK cell colonies begin a period of contraction that lasts the following 6 hours. During contraction, cells initiate migration and rupture cell-cell adhesions. According to our quantitation of cell area during imaging, MDCK cell surface area returns to levels observed prior to responding to HGF stimulation and may contract even further, particularly as cell-cell contacts break. Unstimulated MDCK cells show limited changes in cell spreading, not

contractile activity, limited migration, and only slow repositioning within the colony (Fig. 2.1B). While we do see unstimulated colonies grow in size, there is no net gain in cell area as most of the change in size is accounted for by cell division.

Identification and Chemical Optimization of B5500

From the 50,000 chemical compound pilot screen, 20 biologically active EMT blockers were identified, including one nicotinamide derivative, N-(5-iodo-6-methyl-2-pyridinyl)-3-nitrobenzamide (B5500) (Fig. 2.2A). Of these, 11 bear an unknown mechanism of action.

We reasoned that molecules of high interest would affect cellular machinery required for EMT in multiple model systems, so we sought to assess which molecules might affect EMT in another system unrelated to MDCK cells. Further, the ability to use human cell lines, as opposed to canine MDCK cells, would allow us to employ a number of assay systems that rely on human cell lines and that could provide key information about the molecular target of our compounds. We therefore assessed compounds that are active in our screening assay for the activity in blocking invasive behavior of the trophoblast cell line Sw71. Screening these 11 compounds through a cell invasion assay of SW71 human trophoblast cells in a collagen matrix again demonstrated their ability to block EMT from progressing, demonstrating a molecular mechanism that is present in multiple organism and tissue types (in this case, canine kidney and human trophoblast cell lines) (Fig. 2.2C). Of the 11 EMT inhibitors tested in the SW71 invasion assay, 7 compounds showing statistically significant reduction of invasion, including B5500. 10 uM B5500 inhibited SW71 invasion by 33% of the DMSO control trial.

We sought to determine which of these molecules would be the most likely to reveal novel biology about epithelial scattering. One issue with our overall approach is that

microtubules are required for epithelial scattering of MDCK cells in response to HGF. Since microtubule-targeting agents are found with high frequency in phenotypic assays, we sought to avoid working with compounds that inhibit HGF-induced EMT by targeting the microtubule cytoskeleton. In order to understand which molecules target microtubules, we assessed compounds from our original screen in proliferation assays using the NCI-60 panel of cancer cell lines. Since microtubules are required for cell division, compounds bearing microtubule targeting activity would inhibit cell proliferation of the majority of cell lines in this panel. Inhibition of a subset of cell lines would instead suggest the targeting of a protein required for the growth of some cells, revealing a potential mechanism of action for the compound under question. We found that only B5500 had no general effects on cell growth in these proliferation assays, meaning it is the only compound that certainly works independently of a microtubule-based mechanism.

We sought to optimize the specific activity of B5500 in arresting HGF-induced epithelial scattering. We generated 49 chemical derivatives of B5500 and assessed them in our original screening assay, yielding three analogs with increased activity (Fig. 2.2D). For each of these new derivatives, the activity in affecting scattering or migration was compared to anti-proliferative activity. B5500-25 and B5500-26 show potent cell growth inhibition that accompanies their activity in the HGF-induced EMT assay. Another compound, N-(5-bromo-6-methyl-2-pyridinyl)-pyrimidine-5-carboxamide (B5500-4) (Fig. 2.2B), affects cell proliferation only at high concentrations, but retains significant activity in blocking HGF-induced EMT. This compound likely affects HGF-induced EMT completely independently of microtubules. Since it is

more potent than B5500, we sought to use this molecule for our subsequent target

identification experiments.

In order to confirm that B5500-4 affects scattering behavior of human cells and to

determine its potency, we measured its activity in invasion assays using SW71 cells at

decreasing concentrations. We found that B5500-4 does in fact block EMT and invasion in this

system and does so with an IC-50 of ~27 nM (Fig. 2.2B).

B5500-4 Target Identification

We initially sought to identify a target pathway for B5500 and B5500-4 by assessing the

readout of compound treatment in a series of cell culture systems in which inflammatory

biomarker expression is analyzed. This so-called BIO-Map system consists of primary human

cells that are grown in culture alone or in combination, then activated to generate an

inflammatory response. In total, 12 different culture models were used and expression of a

total of >70 inflammatory markers were analyzed. The value of this system is that inhibition of a

signaling pathway will give a predictable readout that will be comparable to that generated

with inhibitors of known pathway components. As such, the BIO-Map system is a powerful

approach to identifying the target of a small molecule inhibitor of unknown mechanism, at least

at the pathway level. However, no significant changes in the expression of any inflammatory

markers were associated with B5500-4 treatment in this system (Fig. 2.3), suggesting that this

molecule does not target signal transduction machinery.

Additionally, B5500 was assessed for its activity in blocking the activity of protein

kinases with a panel of 230 kinases. B5500 was not active against any kinases tested (Fig. 2.4).

This compound is not a kinase inhibitor, as we might expect from the lack of a discernable readout in the BIO-Map system.

To determine what effects B5500-4 had on more classic EMT markers, we performed western blots on e-Cadherin (E-cad), SNAIL, and SLUG in several human cancer cell lines (A549, Psn-1, and Panc-1). A549, a human lung cancer cell line, maintains many epithelial characteristics, including high expression of E-cad, and low expression of mesenchymal markers SNAIL and SLUG. Interestingly, the B5500-4-treated cell lines show no statistically significant change in E-cad, SNAIL, or SLUG protein levels in cancer cell lines A549, Psn-1, and Panc-1 (Fig. 2.5A). This finding suggests that B5500-4 does not act by increasing epithelial characteristics within cells (A549), nor by decreasing mesenchymal characteristics (Psn-1, Panc-1). This does not rule out, however, that B5500-4 could act via preventing the transition from epithelial to mesenchymal states.

In order to identify any changes in gene expression caused by B5500-4, we performed a qPCR assay of 384 genes known to be involved in the regulation of EMT, extracellular matrix, cytoskeleton (Fig. 2.6A-D). SW71 cell samples were induced with B5500-4 or DMSO (control) for 24 hours, and cDNA samples were produced from harvested mRNA extracts. The cDNA samples were run through the PCR plates, and the differential expression of various genes in cells treated with B5500-4 or DMSO compared. Several genes demonstrated large changes in expression with B5500-4 treatment, including and especially Col4A2 (1.05×10^4 fold change), MMP-12 (-7.37×10^6 fold change), and mTOR (-2.22×10^3 fold change) (Fig. 2.6A-B).

The Functional Requirement of MMPs in HGF-induced Epithelial Cell Scattering

The changes in expression of MMP-12 and of extracellular matrix in cells treated with B5500-4 suggest a possible role for cell surface proteases in the HGF pathway. The chemical structure of B5500-4 is also suggestive of binding within a proteolytic binding pocket, as the central amide bond of the linker region closely resembles the peptide bind of an amino acid chain (Fig. 2.2B). We hypothesized that B5500-4 might be a protease inhibitor and specifically target proteases in the MMP family. MMPs have already been implicated in EMT progression and cancer metastasis, but the roles of MMP-12 and MMP-9 have yet to be fully characterized in the context of epithelial cell scattering, cell spreading, and cell contraction (Craig 2015, Ha 2015).

 In order to determine whether matrix metalloproteinases are required for HGF-induced EMT, inhibitors of proteases MMP-12 and MMP-9 were used to treat MDCK cells prior to induction of scattering with HGF.

Chemical inhibition of MMP-9 by Ilomastat of HGF-induced MDCK cells blocks EMT (Fig. 2.7C). Neither cell spreading nor cell compaction occurs (Fig. 2.7E), indicating an unknown role for MMP9 within the c-Met cell scattering program. It is possible that MMP9 is required for both cellular processes, or that its role precedes cell spreading and contraction.

MDCK cells treated with Marimastat (a blocker of MMP-12) fail to scatter after HGF stimulation (Fig. 2.7). While spreading is observed, it is drastically slowed. MDCK colonies treated with Marimistat take 8 hours to reach the extent of maximal spreading observed in control MDCK cells, which occurs in 2 hours (Fig. 2.7E). Additionally, no cell compaction is observed for the duration of the experiment. This suggests that MMP-12 must be required

for activation of the cell compaction program, but it remains unclear how MMP-12 inhibition alters the time-frame and rate of cell spreading.

BACE is Required for Epithelial Cell Spreading in Early HGF-induced EMT; Furin is Required for Epithelial Cell Scattering, but Not Y-secretase

We sought to test whether B5500 or B5500-4 are inhibitors of matrix metalloproteinases. Biochemical assays measuring the activity of different MMPs were performed in the presence of a series of concentration of B5500. We tested several MMP family members, including MMP 1-3, 7-10, and 12-14. None of these proteases demonstrated any reduction in activity at even the highest concentration (10uM) of B5500. Despite that MMPs are indeed required for HGF-induced epithelial scattering of MDCK cells, B5500 does not prevent scattering by targeting MMPs.

In order to test whether the molecular target(s) of B5500-4 included any other proteases during EMT, a collaborator conducted molecular modeling experiment to measure B5500-4 binding affinity cell surface proteases (furin and beta-secretase (BACE). In both cases, B5500-4 was expected to bind the active site of these proteases. We therefore determined to test the role of these proteases in HGF-induced epithelial scattering.

MDCK cells were seeded onto collagen-coated imaging plates, treated with appropriate inhibitor, stimulated to undergo epithelial scattering with HGF treatment, and subjected to live cell imaging (Fig. 2.8). Darunavir (furin chemical inhibitor) treatment resulted in a failure of epithelial cell scattering, suggesting a role for furin in the HGF pathway (Fig. 2.8A). Neither cell spreading nor cell contraction occurs in furin-inhibited MDCK cells during HGF stimulation, especially when compared with HGF-control MDCK cells (Fig. 2.1).

Chemical inhibition of Y-secretase proved to be cytotoxic; cell die 6 hours after treatment (Fig. 2.8B), suggesting an important survival role for Y-secretase, one that acts independently of the c-Met pathway.

MDCK cells treated with BACE inhibitor fail to undergo normal HGF-induced scattering. These cells showed undergo a cell compaction without undergoing any cell spreading. No effect of BACE inhibitor is observed in MDCK cells that have not been stimulated with HGF (Fig. 2.9A-B). The compaction observed in BACE-treated cells parallels cell compaction in control cells treated with HGF after spreading has occurred (Fig. 2.9B). These results suggest a role for BACE in the HGF pathway through regulating cell spreading, but not in cell compaction.

Summary of Novel Proteases Involved in HGF-pathway

An illustration provides a graphical summary of the roles of the tested proteases within the c-Met pathway (Fig. 2.10). When induced with HGF, MDCK cells begin a cell spreading phase, which requires BACE. After 2 hours of spreading, the MDCK cells undergo a cell contraction phase, which requires MMP12. Regulation of both of these processes likely requires MMP9 and furin, but not Y-secretase, which activity is likely required for cell survival.

CHAPTER 3: Discussion

Cellular Mechanics of Early EMT

Consistent with earlier reports, we have shown that MDCK cells undergo HGF-induced epithelial cell scattering in a two-phase process, beginning with an early phase of cell spreading and followed by a later cell contraction phase. It is during the cell contraction phase that cell-cell contacts are broken and cells fully separate from their neighbors, allowing for formerly epithelial cells to migrate and invade as individual, solitary mesenchymal cells.

Whether cell-cell contacts become weakened during early epithelial scattering, such as during the early cell spreading phase, remains an open question. The current thinking of the field, though in flux, is that forces coming from migration or other actomyosin-based contractile processes are sufficient to rupture fully cell-cell junctions without any prior changes to the molecular architecture of the junction. In support of this, it appears that reduction in and restoration of cellular contractility is enough to rupture cell-cell contacts (Hoj 2014), indicating that contractile forces alone are enough to rupture cell-cell junctions. However, our prior work show that exogenous expression of a 'constitutively active' fragment of the cytoskeleton-membrane linker protein, zyxin, results in an inability of cells to detach from each other during epithelial scattering and results in cells that attempt to migrate while maintaining long tethers to other cells (Sperry 2011). A resolution to this controversy is that contractile forces are likely sufficient to drive cell-cell detachment during HGF-induced epithelial scattering, but that this must be preceded by a relaxation of actomyosin-based tensin forces that results in uncoupling of cadherin cell-cell adhesion receptors from the actin binding protein, alpha-catenin, which

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form cadherin-catenin-actin complexes that link cell contacts to the actin cytoskeleton only when under tension.

Our focus here has been to study and characterize the mechanics of this two phase process. A powerful approach to understanding complex cellular processes in tissue culture models, particularly for identifying cellular components required for the process under question, is to employ unbiased chemical screening. We follow up on a high content phenotypic screening approach that led us to a molecule with activity that is highly specific for blocking HGF-induced epithelial scattering and not cell division processes (a sign of microtubule targeting).

B5500-4 Characteristics and Mechanism

We optimized the original molecule identified in screening to increase the specificity for inhibition of HGF-induced epithelial scattering over anti-proliferative activity, yielding the molecule B5500-4. This molecule inhibits HGF-induced epithelial scattering in MDCK cells in the original screening assay and blocks invasion of trophoblast cells with high potency. Interestingly, it produces no effect on the expression of classic EMT markers, such an E- and N-cadherin, SLUG, and SNAIL, when used to treat mesenchymal cancer cells. While we have not looked at whether B5500-4 can prevent expression of mesenchymal markers in cells undergoing epithelial scattering, our results suggest that inhibition of the target of our compound cannot induce a reversion to an epithelial phenotype among cells that are already mesenchymal.

Defining the mechanism of action of B5500 and B5500-4 has proven to be a major challenge, as is often the case for molecules identified in phenotypic high content screens.

Assessment for activity in blocking the activity of a large panel of 230 kinases shows that these compounds are not kinase inhibitors. We then turned to another high content screen where inhibition of different signaling pathways has specific and recognizable readout patterns, the so-called BioMAP screen. Even here the results are negative and suggest that the target of these compounds is not a signaling molecule.

Clues to the identity of the molecular target were first obtained in assessing changes in expression patterns resulting from B5500-4 treatment using a PCR array system, where the most affected genes are an extracellular matrix protein and a matrix metalloproteinase. In fact, recent findings from this lab have shown that characteristics of the extracellular matrix, including its substrate stiffness, can have dramatic effects on the ability of MDCK cells to migrate while undergoing HGF induction (Hoj 2014) and support a role for matrix remodeling enzymes like MMPs in HGF-induced epithelial scattering.

MMP Function in the Mechanics of HGF-induced Epithelial Scattering

The MMP proteins MMP-12 and MMP-9 had both been shown in previous studies to be involved in epithelial cell scattering, though not directly in response to HGF [40]. While our qPCR assay indicates that MMP-12 might be a target of B5500-4 and is thus required for epithelial cell scattering, we also assessed other MMP proteins for which there are readily available inhibitors. Our time-lapse microscopy of epithelial scattering of MDCK cells in the presence of Marimastat and Ilomastat, chemical inhibitors of MMP-12 and MMP-9, respectively, demonstrates that MMP-9 and -12 are both required for epithelial cell scattering in MDCK cells. More interesting is that MMP-12 inhibition blocked the cell contraction phase without affecting the cell spreading phase. MMP-9, in contrast, inhibited both cell spreading

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and cell contraction phases of the cell scattering program, suggesting a molecular target upstream and required for both programs in the scattering pathway.

Furin and BACE in HGF-induced Epithelial Cell Scattering

While MMP-9 and -12 were shown to be required for epithelial scattering, biochemical assays reveal that B5500 is not an inhibitor of matrix metalloproteinases. Clues as to its mechanism came from docking studies performed by a collaborator in silico and suggested that B5500 and B5500-4 bind the active sites of the surface proteases furin and BACE. To our knowledge, a role for either protease in HGF-induced epithelial scattering has not been reported. BACE is produced as an inactive proenzyme protease and requires proteolytic processing to become an active BACE protease (Creemers 2001). Interestingly, it is furin that has been reported to mediate BACE processing (Creemers 2001). Treating MDCK cells with inhibitors of these proteases and examining the impact of epithelial scattering reveals that both are required, though the effect of inhibition of each protease is distinct. And since furin is one of the major proteases to convert pro-BACE to BACE, it was assumed that both would be involved in the same pathway. The findings that BACE inhibition causes an HGF-dependent cell contraction response in MDCK cells, whereas furin does not, went against our hypothesis. We had hypothesized that the inhibition of either protease would produce a similar cellular effect, and yet it was the opposite that proved true. It became apparent that while furin may be responsible for pro-BACE cleavage, this certainly wasn't the method of BACE activation involved in the c-Met pathway.

CHAPTER 4: Methods and Protocols

Immunofluorescence Assays

Showing alterations to actin rearrangement via Frost-100 were demonstrated using immunofluorescence assays. MDCK cells plated for 24-hours onto collagen-coated coverslips were induced with HGF and either B5500-4, B5500, or DMSO (control). After a 24 hour incubation period, cells were fixed via paraformaldehyde, and stained with phalloidin, anti-BACE, or anti-EphA2 for subsequent imaging.

EMT Migration Assays

EMT assays were performed to quantify the anti-scattering effects of B5500 and B5500-4. MDCK cells were plated onto trans-well plates and left to incubate for 24 hours. At 24 hours, the cells were induced with both HGF and either B5500-4, B5500, or DMSO (control) at a variety of concentrations (10 uM, 1 uM, 0.1 uM, 0.01 uM) and given an additional 24 hours to migrate through the membrane. Cells were fixed via paraformaldehyde, and stained with crystal violet stain. Fixed cells on the upper membrane surface were wiped away with cotton swabbing, leaving only migrated cells on the bottom of the transwell filter membrane. The wells were imaged and quantified using the SpotDenso tool on the Fluorchem Analysis program.

SW71 Cell Invasion Assay

The invasion assay measures the amount of cellular invasion colonies of cells undergo, which in part relies on the ability of the cells to undergo EMT. Matrigel solution was poured into a bottom well of a transmembrane system, on top of a thin-film membrane and allowed time to congeal. SW71 cells were seeded into the plate's top chamber and starved of Fetal Bovine Serum (FBS). A FBS gradient was created by adding a 5% FBS solution in the bottom

well. The FBS acted as a chemoattractant to the FBS-starved cells, initiating invasion down through the matrigel. B5500-4 and B5500 were added at increasing concentrations (10 uM, 1 uM, 0.1 uM, 0.01 uM) to the top well at the same time as the introduction of the FBS gradient. A 24 hour incubation period was allowed for the cells to invade. Cells were then fixed and stained with a Dissociation solution, and the bottom membrane was extracted and used to generate a cell count for the invaded cells.

Time-lapse Live-cell Microscopy

MDCK cells seeded onto collagen-coated Delta-T movie dishes for 24 hours will be induced with Frost-100 at 10 uM, followed shortly by HGF. Single-frame images will be taken at multiple time points (every 2 minutes for 13 hours) and stitched together into a time-lapse movie using Slidebook software. Comparing Frost-100 induced MDCK cells to DMSO control MDCK cells will reveal the effects that Frost-100 have on inhibiting migratory behavior.

Western Blot

Western blot of classic EMT protein markers--E-cadherin, N-cadherin, Slug, and Snail-- were performed to quantify the changes to the epithelial state of the target cells induced with Frost-100. MDCK cells were seeded for 24 hours and subsequently induced with HGF and increasing concentrations of B5500-4 or B5500 (10 uM, 1 uM, 0.1 uM, 0.01 uM). At 24 hours after induction, cells were lysed using a cell lysis buffer containing 2-mercaptoethanol and protease inhibitors, and the cytosolic contents were collected and isolated via centrifugation. A Bradford assay was performed on each of the cell lysate samples to equalize relative protein concentrations, which was run through an SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was incubated with either anti-e-cadherin antibodies, anti-n-

cadherin antibodies, anti-Slug antibodies, or anti-Snail primary antibodies followed by secondary antibodies. Final blot images were then developed using the enhanced chemiluminescence (ECL) detection system. The same membranes were also developed in an anti-β-actin primary antibody (mouse), followed by anti-mouse secondary antibodies in a subsequent blot to provide an internal control. Quantification of the blots were obtained using the spot-denso analysis tool on the developed X-ray films as a part of the Fluorchem SP imaging system.

qPCR Array

Human trophoblast SW71 cells were cultured for 24 hours and then induced with 5 uM B5500-4 or with DMSO (control). After an additional 24 hour incubation time, the cells were harvested and the RNA extracted using a Qiagen RNA extraction kit. The RNA samples were equalized via Nanodrop spectrophotometry, and reverse-transcribed into cDNA using the Invitrogen Superscript II Reverse Transcriptase kit. The cDNA was added to SYBR Mastermix, aliquotted into the PCR array plate, and run through a Qiagen thermocycler for 50 cycles, according to kit specifications. The Frost-100 and DMSO cDNA samples were compared to each other in terms of number of cycles needed to reach an arbitrary threshold, which assigned each trial a raw score, and gave an indication to the relative amounts of RNA from each sample and the fold-change between the two samples. Individual genes with the highest fold-change in expression were repeated in multiplicates to confirm the change in expression.

GLISA Assay

A specific type of ELISA assay—the GLISA assay—was performed to measure ATP-bound proteins of interest, showing the effects of B5500-4 on the levels of the specific proteins in their activation state. SW71 cells were seeded for 24 hours and subsequently induced with B5500-4 at a variety of concentrations (10 uM, 1 uM, 0.1 uM, 0.01 uM) for an additional 24 hours. Cells were harvested with a cell lysis buffer containing 2-mercaptoethanol and protease inhibitors, centrifuged, and snap frozen in liquid nitrogen. Equalized protein samples--measured via Bradford assay—were then added to each well containing the GLISA substrates. After incubation for 1 hour, lysate contents were discarded, and primary antibody solution was added and allowed to incubate for 30 minutes. Primary antibody solution was discarded and replaced with a secondary antibody solution, and incubated for 1 hour. The wells were rinsed and fixing reagent was added and allowed to incubate for 15 minutes. The freezing reagent was added and the wells are imaged and quantified using spectrophotometry.

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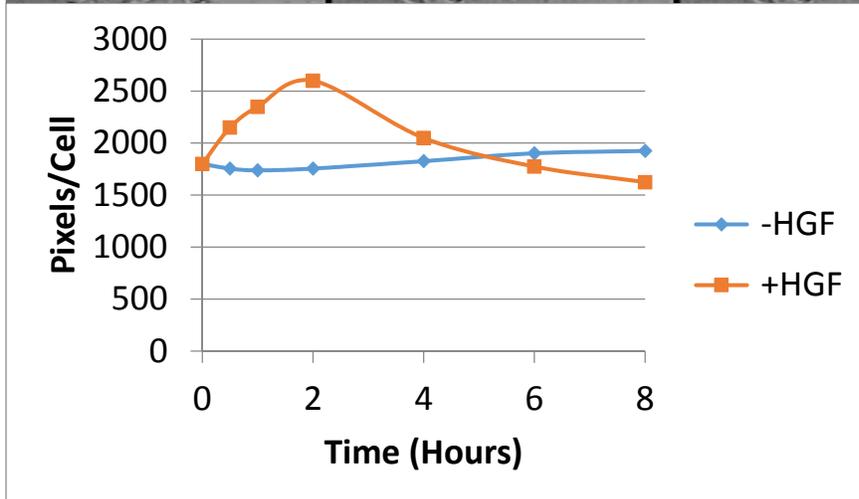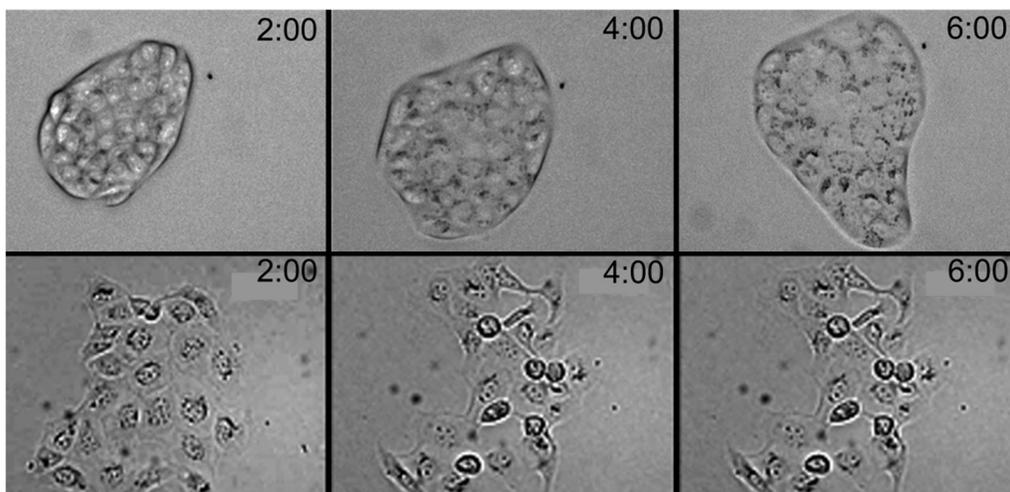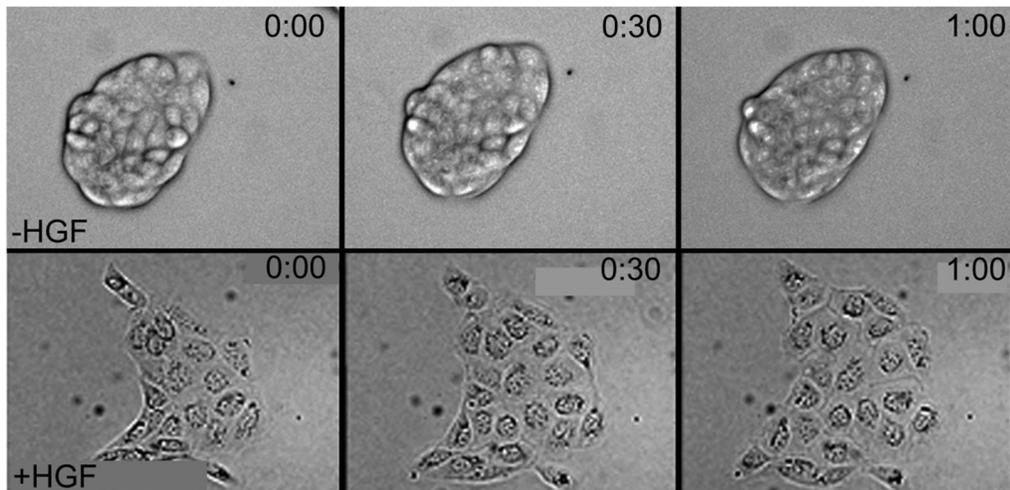
Figure 2.1: HGF-induced EMT Includes Phase of Cell Spreading, Followed by Phase of Cell Contraction. (A) MDCK cell time-lapse microscopy of +/-HGF treatment reveals cell scattering events. (B) Graph of cell surface area over time. HGF-treated cells increase surface area by 44% within first two hours, followed by cell contraction phase, when cells break cell-cell contacts and begin scattering.
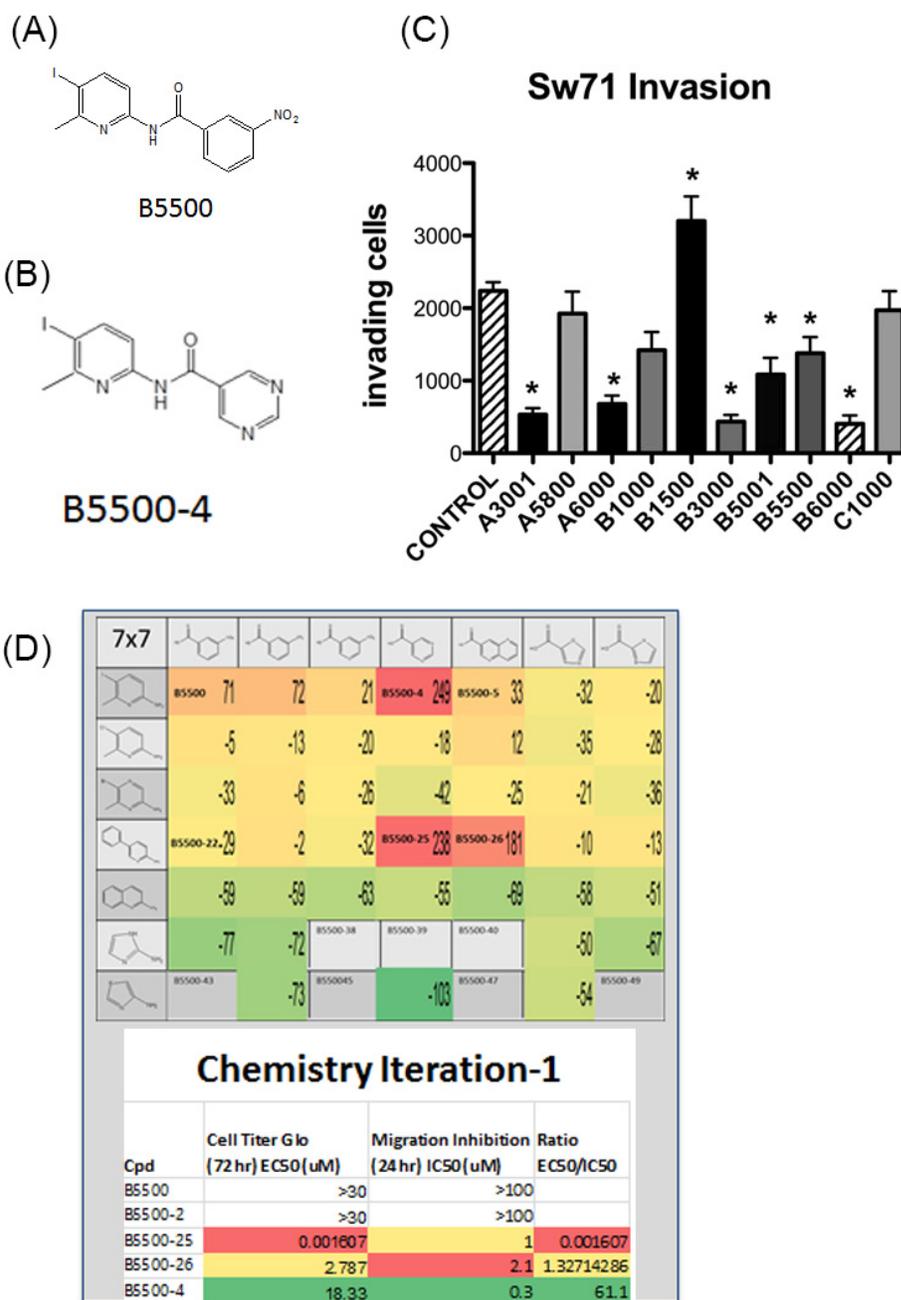
Figure 2.2: Characterization and Optimization of B5500. (A) Chemical structure of B5500. (B) Chemical structure of B5500-4. (C) SW71 cell invasion assay. SW71 cells were induced to invade through 3D collagen matrix onto a transwell membrane, towards 10% FBS chemoattractant. Tested compounds had final concentration of 10 uM. (D) Structure-activity relationship assay of B5500 and chemical derivatives. Left-side and right-side variations in the table axes represent chemical compound changes made to the B5500 scaffold. 49 derivatives were tested at a final concentration of 10 uM. Cell growth inhibition and migration inhibition of most potent compounds included in table.

Chemistry Iteration-1

Cpd	Cell Titer Glo (72 hr) EC50 (uM)	Migration Inhibition (24 hr) IC50 (uM)	Ratio EC50/IC50
B5500	>30	>100	
B5500-2	>30	>100	
B5500-25	0.001607	1	0.001607
B5500-26	2.787	2.1	1.32714286
B5500-4	18.33	0.3	61.1

Figure 2.3: Inflammation Assay Reveals No Significant Change in Inflammatory Marker Expression. 12 cell lines were induced with B5500-4 at several concentrations (10 uM, 3.33 uM, 1.11 uM, 37 nM) for 24 hrs and expression of a listed of inflammatory markers were measured. Markers revealed change beyond allowed standard error (gray zones) were noted to yield extremely small and insignificant changes from B5500-4 treatment.

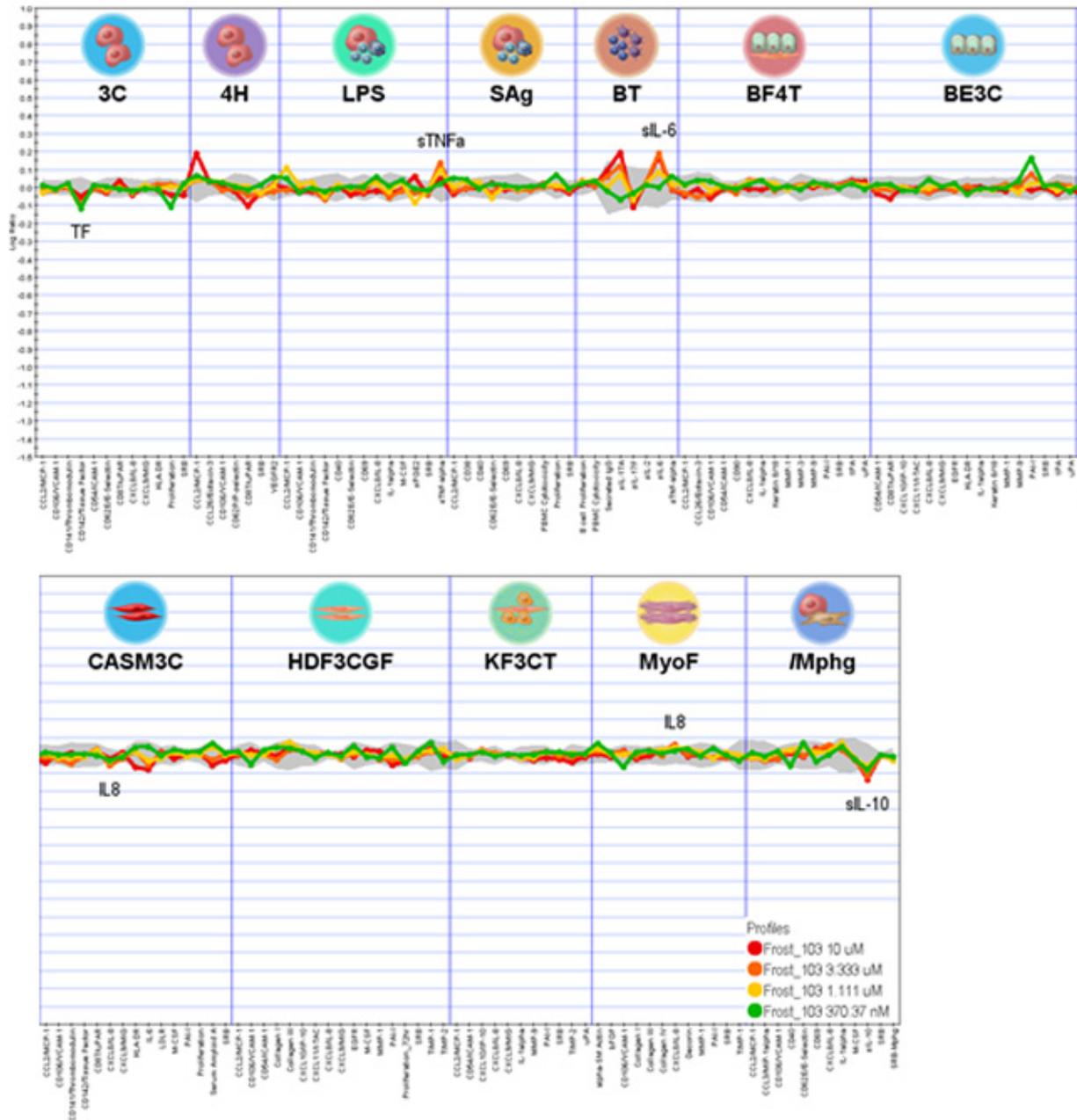| Kinase	B5500 Data 1	B5500 Data 2	Kinase	B5500 Data 1	B5500 Data 2	Kinase	B5500 Data 1	B5500 Data 2
ABL1	97.65	99.50	CDK9/cyclinK	94.69	100.15	ERK1	86.61	91.88
ABL2/ARG	93.50	90.77	CDK9/cyclinT1	94.16	87.98	ERK2/MAPK1/P42MAPK	90.70	91.81
ACK1/TNK2	108.99	102.86	CHK1	97.77	98.03	FAK/PTK2	94.49	94.43
AKT1 (dPH, S473D)	94.40	90.01	CHK2	105.69	101.02	FER	96.77	96.84
AKT2/PKBb	98.55	89.04	CK1a1	99.32	98.82	FES/FPS	87.18	89.08
AKT3	96.51	92.20	CK1d	98.49	99.17	FGFR1	86.86	90.94
ALK	89.33	96.39	CK1e	82.07	73.89	FGFR2	85.46	88.35
ALK1/ACVRL1	101.44	96.99	CK1g1/CSNK1G1	96.79	101.28	FGFR3	80.20	83.45
ALK2/AVCR1	101.40	105.30	CK1g2	90.23	95.32	FGFR4	101.81	105.41
ALK4/ACVR1B	98.28	102.84	CK1g3/CSNK1G3	99.27	95.15	FGR	97.67	92.42
ALK5/TGFB-R1	96.09	102.90	CK2a	98.21	101.02	FLT1	99.20	102.61
ARAF	98.86	94.03	CK2a2	86.59	93.30	FLT3	97.80	91.23
ARK5	94.76	97.76	c-Kit	110.80	110.04	FLT4/VEGFR3	98.31	102.38
ASK1/MAP3K5	98.75	101.07	CLK1	101.37	102.44	FMS	91.96	101.84
Aurora A	99.11	98.31	CLK2	105.37	99.25	FRK/PTK5	99.78	95.41
Aurora B	101.38	95.45	CLK3	102.03	103.11	FYN	87.74	91.54
Aurora C/AURKC	83.05	74.92	CLK4	97.73	97.20	GCK/MAP4K2	95.89	95.79
AXL	110.42	104.53	c-MER	95.67	94.59	GRK2	101.36	96.07
BLK	100.43	101.14	c-MET	105.06	99.98	GRK3/ADRBK2	100.88	96.32
BMX	101.98	96.86	COT1/MAP3K8	84.60	87.52	GRK4	105.90	110.81
bRAF	101.19	100.97	CSK	103.20	103.13	GRK5	101.61	96.96
BRK	80.38	93.88	c-Src	98.52	100.88	GRK6	107.66	100.80
BRSK1	89.34	77.88	CTK/MATK/HYL	106.65	103.31	GRK7	91.96	96.22
BRSK2	99.12	95.65	DAPK1	90.49	82.24	GSK3a	95.53	105.38
BTK	99.88	93.39	DAPK2	102.18	103.95	GSK3b	98.43	95.64
CAMK1a	97.80	99.52	DCAMKL2	102.19	103.23	HCK	102.04	96.70
CAMK1b	103.20	100.58	DDR2	96.87	94.80	HGK/MAP4K4	92.86	96.85
CAMK1d	99.17	105.06	DMPK	106.49	105.65	HIPK1	99.32	104.99
CAMK1g	94.13	93.61	DRAK1/STK17A	101.54	86.00	HIPK2	100.73	102.77
CAMK2a	80.28	106.91	DYRK1/DYRK1A	100.84	103.58	HIPK3	104.70	102.51
CAMK2b	93.05	94.16	DYRK1B	106.10	100.12	HIPK4	102.93	97.51
CAMK4	88.30	86.72	DYRK2	86.59	94.71	IGF-1R	89.21	84.77
CAMKIId	84.70	80.79	DYRK3	99.13	99.84	IKK alpha/CHUK	82.63	80.16
CAMKIIg	90.17	95.12	DYRK4	88.08	95.54	IKK beta	100.72	90.15
CAMKK1	101.97	100.31	EGFR	98.69	96.48	IR	106.83	105.69
CAMKK2	93.94	99.24	EPHA1	97.41	95.05	IRAK1	101.71	97.17
CDK1 / cyclinA	100.83	102.23	EPHA2	87.95	90.57	IRAK4	99.95	104.84
CDK1/cyclin B	95.13	95.49	EPHA3	86.94	83.56	IRR/INSRR	97.85	95.42
CDK2 / cyclin A	74.35	76.46	EPHA4	91.82	101.72	ITK	100.66	105.18
CDK2 / cyclin E	94.46	95.20	EPHA5	91.34	97.77	JAK1	103.85	104.31
CDK3/cyclin E	91.36	96.61	EPHA7	85.98	85.40	JAK2	99.99	100.73
CDK4/cyclin D1	86.16	84.17	EPHA8	92.20	95.67	JAK2 (V617F)	83.30	88.15
CDK4/cyclin D3	103.83	94.22	EPHB1	97.07	97.38	JAK3	103.70	96.86
CDK5/p25	100.39	96.82	EPHB2	102.72	100.14	JNK1a1	102.21	103.52
CDK5/p35	74.72	63.90	EPHB3	94.14	95.92	JNK2a2	104.78	102.98
CDK6/cyclin D1	92.89	94.76	EPHB4	94.78	95.92	JNK3	97.11	102.06
CDK6/cyclin D3	92.27	93.60	ErbB2/HER2	91.94	98.81	KDR/VEGFR2	91.86	95.81
CDK7/cyclinH/MNAT1	103.81	110.78	ErbB4/HER4	94.85	99.13	KHS/MAP4K5	85.38	88.90

Figure 2.4: Kinase Screen of B5500 Does Not Block Kinases in Kinase Screen. B5500 was tested for effects on activity levels of a total of 230 kinases. Raw scores were generated from screen data, with 100 indicating a 100% normal level of kinase activity. B5500 does not significantly alter any of the 230 kinases screened.

Kinase	B5500 Data 1	B5500 Data 2	Kinase	B5500 Data 1	B5500 Data 2	Kinase	B5500 Data 1	B5500 Data 2
LCK	81.83	83.92	P38b	98.30	101.39	RIPK5	101.65	102.50
LIMK1	97.88	97.78	P38d/MAPK13	93.99	101.67	ROCK1	88.86	95.51
LKB1	113.17	113.00	P38g/MAPK12	105.49	88.01	ROCK2/ROKa	89.93	96.58
LOK/STK10	98.00	97.69	p70S6K	96.77	101.24	RON/MST1R	100.17	97.90
LRRK2	101.30	92.98	p70S6Kb /RPS6KB2	103.54	107.66	ROS/ROS1	88.59	92.53
LYN	86.52	93.47	PAK1	103.55	104.14	RSK1	97.19	95.08
LYN B	101.45	98.40	PAK2	85.80	86.84	RSK2	89.39	88.15
MAPKAPK2	97.71	94.97	PAK3	101.50	92.13	RSK3	97.18	97.45
MAPKAPK3	97.38	100.19	PAK4	93.44	101.28	RSK4/RPS6KA6	95.01	97.96
MAPKAPK5/PRAK	101.04	83.30	PAK5	82.54	80.16	SGK1 (d1-59, S422D)	93.44	99.12
MARK1	105.14	99.80	PAK6	103.05	102.90	SGK2	97.82	100.40
MARK2	101.41	101.93	PASK	101.81	94.33	SGK3/SGKL	100.65	101.92
MARK3	93.24	93.89	PBK/TOPK	98.43	96.65	SIK2/SNF1LK2/QIK	131.98	136.34
MARK4	101.78	100.16	PDGFRa	82.10	89.54	SLK/STK2	89.12	73.40
MEK1	98.00	100.83	PDGFRb	81.16	81.21	SNARK/NUAK2	100.41	102.26
MEK2	103.77	107.98	PDK1/PDPK1	99.71	98.75	SRMS	101.20	102.37
MEKK2	104.48	109.34	PHKg1	95.59	100.86	SRPK1	88.43	88.21
MEKK3	102.66	96.03	PHKg2	91.19	88.64	SRPK2	75.77	76.86
MELK	84.86	90.00	PIM1	95.99	95.78	STK16	96.98	89.70
MINK/MINK1	100.12	100.88	PIM2	90.08	88.17	STK22D	105.66	95.16
MKK6	105.04	109.63	PIM3	85.58	87.62	STK25/YSK1	91.52	98.49
MLCK/MYLK	89.80	91.15	PKA	99.13	95.23	STK33	95.74	98.54
MLCK2/MYLK2	102.59	92.43	PKC mu	101.46	107.15	SYK	109.28	104.87
MLK1/MAP3K9	93.68	103.89	PKC nu	104.21	99.77	TAK1/TAB1/MAP3K7	98.39	98.21
MLK2/MAP3K10	106.45	107.61	PKCa	91.15	86.12	TAOK1	97.40	97.06
MLK3/MAP3K11	103.35	100.65	PKCbl	93.65	96.22	TAOK2/TAO1	89.05	98.23
MNK2	103.87	103.38	PKCbll	99.70	99.00	TAOK3/JIK	102.61	109.22
MRCKa/CDC42BPA	90.10	94.67	PKCd	86.73	90.26	TBK1	115.71	115.69
MRCKb/CDC42BPB	103.68	100.27	PKCepsilon	90.41	91.34	TEC	94.11	89.33
MSK1/RPS6KA5	82.26	88.07	PKCeta	103.09	104.26	TGFbR2	100.67	101.60
MSK2/RPS6KA4	98.59	98.30	PKCg	99.79	96.48	TIE2/TEK	97.74	95.91
MSSK1/STK23	93.70	95.12	PKCiota	94.66	99.13	TRKA/NTRK1	92.70	81.77
MST1/STK4	107.35	98.63	PKCtheta	98.05	98.13	TRKB/NTRK2	76.12	80.32
MST2/STK3	114.53	100.49	PKCzeta	90.90	96.24	TRKC	94.26	93.30
MST3/STK24	104.11	106.30	PKD2 / PRKD2	97.57	94.90	TSSK2	95.74	95.19
MST4	99.97	102.59	PKG1 alpha	94.02	99.85	TTK	99.58	103.98
MUSK	104.65	97.81	PKG1b	102.42	105.94	TXK	109.17	109.38
NEK1	104.12	104.40	PKG2/PRKG2	81.31	83.32	TYK1/LTK	115.43	113.29
NEK11	94.66	101.78	PKN1/PRK1	98.17	97.67	TYK2	98.26	100.27
NEK2	108.46	100.26	PKN2/PRK2	99.55	95.55	TYRO3/SKY	88.18	90.03
NEK3	148.21	132.42	PLK1	100.69	103.62	VRK1	100.49	92.03
NEK4	104.12	101.75	PLK2	107.23	98.78	Wee1	92.31	92.97
NEK6	155.50	138.84	PLK3	102.27	100.91	WNK2	101.64	107.53
NEK7	100.61	99.50	PRKX	96.48	99.87	WNK3	97.89	107.83
NEK9	97.81	102.11	PYK2/PTK2B/FAK2	83.92	80.63	YES	99.95	99.54
NIK/MAP3K14	83.20	96.29	RAF1	97.88	102.25	ZAK	99.42	105.23
NLK	90.72	98.61	RET	81.22	84.09	ZAP70	100.91	97.89
P38a	101.44	94.15	RIPK2	106.84	108.32	ZIPK/DAPK3	97.25	104.80

Figure 2.4 (continued): Kinase Screen of B5500 Does Not Block Kinases in Kinase Screen. B5500 was tested for effects on activity levels of a total of 230 kinases. Raw scores were generated from screen data, with 100 indicating a 100% normal level of kinase activity. B5500 does not significantly alter any of the 230 kinases screened.
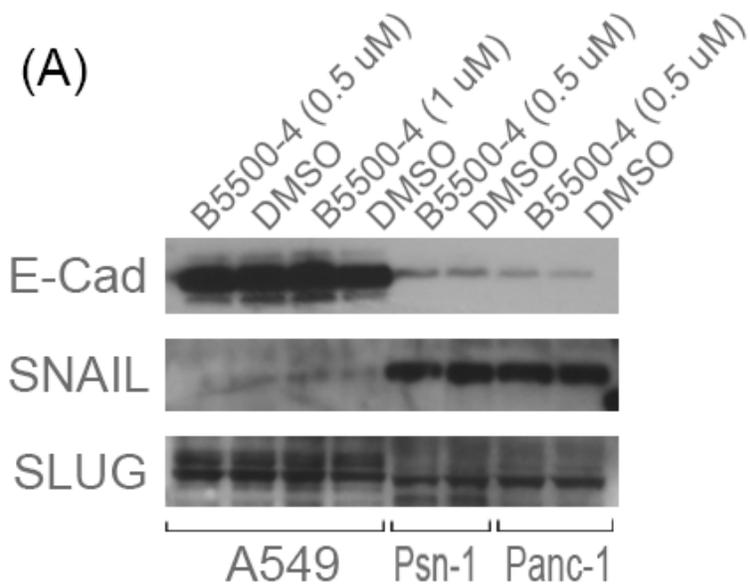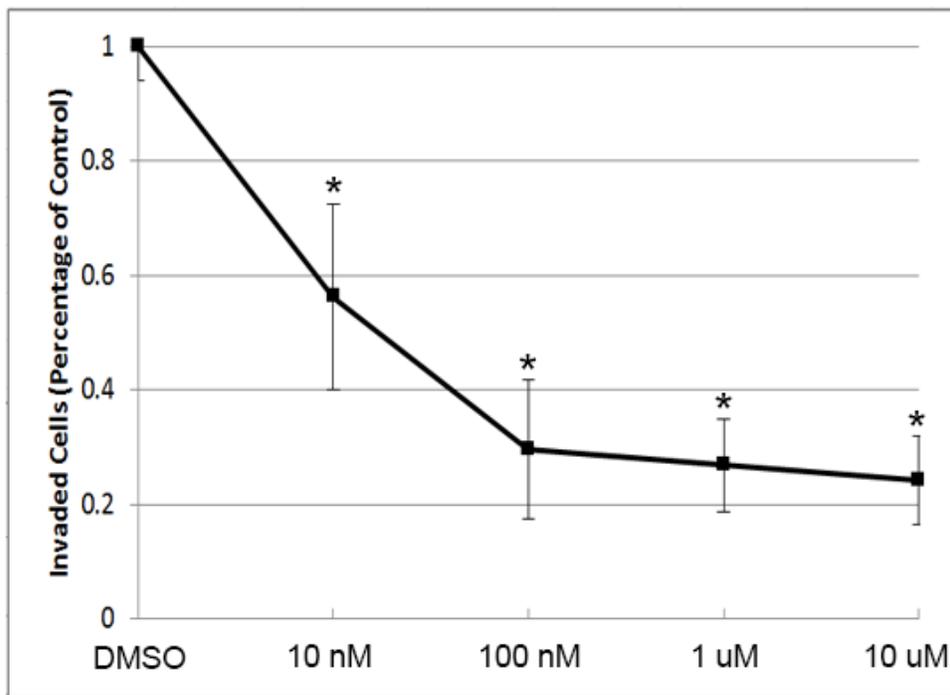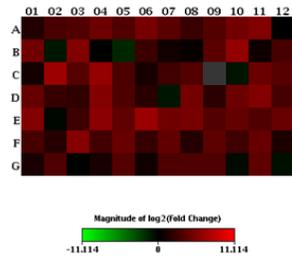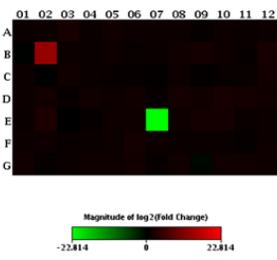
Figure 2.5: B5500-4 Does Not Alter Levels of Classic EMT Markers. (A) Western blot of EMT markers in human cancer cell lines with addition of B5500-4 or DMSO. A549, Psn-1 and Panc-1 cells lines were tested for changes in expression in e-Cadherin, SLUG, and Snail. (B) SW71 cell invasion assay of B5500-4. Dosage curve yielded IC-50 of ~27 nM.
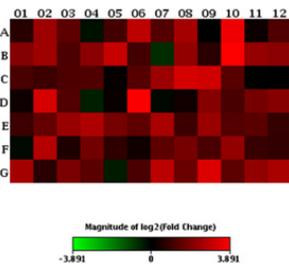
(A) Visualization of log2(Fold Change)

Magnitude of log2(Fold Change): -11.114 | 0 | 11.114

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AKT1 2.75 OKAY	AKT1S1 8.92 OKAY	AKT2 11.53 OKAY	AKT3 25.73 OKAY	CAB39 11.02 OKAY	CAB39L 35.99 OKAY	CDC42 15.43 OKAY	CHUK 5.35 OKAY	DDIT4 12.02 OKAY	DDIT4L 32.26 OKAY	DEPTOR 52.30 OKAY	EIF4B 1.01 OKAY
B	EIF4E 29.32 OKAY	EIF4EBP1 -1.90 OKAY	EIF4EBP2 52.24 A	FKBP1A -1.09 OKAY	FKBP8 -3.20 OKAY	GSK3B 5.80 OKAY	HIF1A 1.63 OKAY	HRAS 1.28 OKAY	HSPA4 15.53 OKAY	IGF1 85.04 A	IGFBP3 1.54 OKAY	IKBKB 8.40 OKAY
C	ILK 1.79 OKAY	INS 116.60 A	INSR 13.81 OKAY	IRS1 92.19 OKAY	MAPK1 9.83 OKAY	MAPK3 2.10 OKAY	MAPKAP1 7.08 OKAY	MLST8 11.56 OKAY	MTOR -2216.10 A	MYO1C -1.85 OKAY	PDPK1 24.57 OKAY	PIK3C3 13.61 OKAY
D	PIK3CA 20.13 OKAY	PIK3CB 5.13 OKAY	PIK3CD 4.21 OKAY	PIK3CG 53.58 A	PLD1 10.76 OKAY	PLD2 3.26 OKAY	PPP2CA -1.91 OKAY	PPP2R2B 33.44 OKAY	PPP2R4 3.85 OKAY	PRKAA1 26.53 OKAY	PRKAA2 53.05 OKAY	PRKAB1 8.10 OKAY
E	PRKAB2 53.44 OKAY	PRKAG1 -1.22 OKAY	PRKAG2 5.98 OKAY	PRKAG3 67.37 OKAY	PRKCA 18.55 OKAY	PRKCB 110.40 A	PRKCE 28.93 OKAY	PRKCG 36.76 A	PTEN 12.13 OKAY	RHEB 20.11 OKAY	RHOA 11.46 OKAY	RICTOR 22.86 OKAY
F	RPS6 8.20 OKAY	RPS6KA1 3.12 OKAY	RPS6KA2 57.38 OKAY	RPS6KA5 7.27 OKAY	RPS6KB1 23.72 OKAY	RPS6KB2 4.89 OKAY	RPTOR 18.28 OKAY	RRAGA 3.03 OKAY	RRAGB 16.08 OKAY	RRAGC 6.46 OKAY	RRAGD 22.31 OKAY	SGK1 2.38 OKAY
G	STK11 2.18 OKAY	STRADB 10.25 OKAY	TELO2 -1.04 OKAY	TP53 2.07 OKAY	TSC1 12.62 OKAY	TSC2 1.61 OKAY	ULK1 9.77 OKAY	ULK2 10.45 OKAY	VEGFA 10.48 OKAY	VEGFB -1.39 OKAY	VEGFC 17.06 OKAY	YWHAQ -1.65 OKAY

(B) Visualization of log2(Fold Change)

Magnitude of log2(Fold Change): -22.814 | 0 | 22.814

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ADAMTS1 1.86 OKAY	ADAMTS13 2.13 OKAY	ADAMTS8 3.88 OKAY	CD44 1.82 OKAY	CDH1 2.90 OKAY	CLEC3B 2.02 OKAY	CNTN1 2.01 OKAY	COL11A1 1.98 OKAY	COL12A1 1.52 OKAY	COL14A1 2.19 OKAY	COL15A1 1.87 OKAY	COL16A1 2.00 OKAY
B	COL1A1 -1.07 OKAY	COL4A2 10337.80 OKAY	COL5A1 2.02 OKAY	COL6A1 1.91 OKAY	COL6A2 2.27 OKAY	COL7A1 1.64 OKAY	COL8A1 1.91 OKAY	CTGF 2.85 OKAY	CTNNA1 3.44 OKAY	CTNNB1 2.45 OKAY	CTNND1 1.54 OKAY	CTNND2 3.98 OKAY
C	ECM1 1.98 OKAY	FN1 1.02 OKAY	HAS1 2.69 OKAY	ICAM1 1.86 OKAY	ITGA1 1.67 OKAY	ITGA2 1.45 OKAY	ITGA3 1.29 OKAY	ITGA4 2.03 OKAY	ITGA5 1.33 OKAY	ITGA6 1.57 OKAY	ITGA7 1.60 OKAY	ITGA8 3.22 OKAY
D	ITGAL 2.05 OKAY	ITGAM 2.95 OKAY	ITGAV 1.60 OKAY	ITGB1 2.77 OKAY	ITGB2 2.24 OKAY	ITGB3 2.57 OKAY	ITGB4 1.71 OKAY	ITGB5 3.39 OKAY	KAL1 2.86 OKAY	LAMA1 3.80 OKAY	LAMA2 3.47 OKAY	LAMA3 2.60 OKAY
E	LAMB1 1.97 OKAY	LAMB3 5.81 OKAY	LAMC1 1.02 OKAY	MMP1 1.51 OKAY	MMP10 2.40 OKAY	MMP11 2.34 OKAY	MMP12 -7374760.71 OKAY	MMP13 2.25 OKAY	MMP14 3.63 OKAY	MMP15 3.67 OKAY	MMP16 1.92 OKAY	MMP2 2.17 OKAY
F	MMP3 2.06 OKAY	MMP7 2.31 OKAY	MMP8 1.70 OKAY	MMP9 1.76 OKAY	NCAM1 2.22 OKAY	PECAM1 3.40 OKAY	SELE 2.46 OKAY	SELL 1.90 OKAY	SELP 1.81 OKAY	SGCE 1.95 OKAY	SPARC 1.44 OKAY	SPG7 2.06 OKAY
G	SPP1	TGFBI	THBS1	THBS2	THBS3	TIMP1	TIMP2	TIMP3	TNC	VCAM1	VCAN	VTN

(C) Visualization of log2(Fold Change)

Magnitude of log2(Fold Change): -3.891 | 0 | 3.891

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	AHNAK 1.52 OKAY	AKT1 5.34 OKAY	BMP1 2.68 OKAY	BMP2 -1.19 OKAY	BMP7 2.07 OKAY	CALD1 7.39 OKAY	CAMK2N1 2.43 OKAY	CAV2 5.77 OKAY	CDH1 1.01 OKAY	CDH2 12.67 OKAY	COL1A2 1.15 OKAY	COL3A1 2.26 OKAY
B	COL5A2 4.15 OKAY	CTNNB1 5.39 OKAY	DSC2 2.28 A	DSP 4.43 OKAY	EGFR 8.22 OKAY	ERBB3 2.05 OKAY	ESR1 -1.58 OKAY	F11R 4.77 OKAY	FGFBP1 1.56 OKAY	FN1 14.84 OKAY	FOXC2 4.89 OKAY	FZD7 4.35 OKAY
C	GNG11 2.17 OKAY	GSC 1.90 OKAY	GSK3B 2.29 OKAY	IGFBP4 2.31 OKAY	IL1RN 1.07 OKAY	ILK 2.49 OKAY	ITGA5 5.26 OKAY	ITGAV 9.60 OKAY	ITGB1 2.51 OKAY	JAG1 2.51 OKAY	KRT14 -1.03 OKAY	KRT19 1.01 OKAY
D	KRT7 1.18 OKAY	MAP1B 9.60 OKAY	MMP2 2.38 OKAY	MMP3 -1.37 OKAY	MMP9 1.11 OKAY	MSN 14.26 OKAY	MST1R -1.08 OKAY	NODAL 1.21 OKAY	NOTCH1 4.18 OKAY	NUDT13 2.19 OKAY	OCLN 3.52 OKAY	PDGFRB 4.20 OKAY
E	PLEK2 1.92 OKAY	DESI1 2.85 OKAY	PTK2 5.08 OKAY	PTP4A1 6.47 OKAY	RAC1 3.51 OKAY	RGS2 2.69 OKAY	SERPINE1 6.03 OKAY	GEMIN2 1.87 OKAY	SMAD2 5.15 OKAY	SNAI1 2.43 OKAY	SNAI2 2.59 OKAY	SNAI3 1.74 OKAY
F	SOX10 -1.11 OKAY	SPARC 6.91 OKAY	SPP1 1.37 OKAY	STAT3 3.37 OKAY	STEAP1 1.62 OKAY	TCF3 1.19 OKAY	TCF4 2.66 OKAY	TFPI2 3.46 OKAY	TGFB1 2.19 OKAY	TGFB2 4.78 OKAY	TGFB3 1.82 OKAY	TIMP1 1.69 OKAY
G	TMEFF1 5.73 OKAY	TMEM132A 1.57 OKAY	TSPAN13 3.67 OKAY	TWIST1 2.60 OKAY	VCAN -1.34 OKAY	VIM 2.20 OKAY	VPS13A 7.23 A	WNT11 3.09 OKAY	WNT5A 10.43 OKAY	WNT5B 2.68 OKAY	ZEB1 4.80 OKAY	ZEB2 6.00 OKAY

(D) Visualization of log2(Fold Change)

Magnitude of log2(Fold Change): -1.329 | 0 | 1.329

Layout	01	02	03	04	05	06	07	08	09	10	11	12
A	ACTR2 -1.82 C	ACTR3 -1.82 C	ARAP1 -1.82 C	ARFIP2 1.97 OKAY	ARHGAP6 1.38 OKAY	ARHGDIB 1.27 OKAY	ARHGEF11 1.23 OKAY	ARPC1B 1.25 OKAY	ARPC2 1.16 OKAY	ARPC3 1.71 OKAY	ARPC4 1.11 OKAY	ARPC5 -1.17 OKAY
B	AURKA 1.24 OKAY	AURKB 1.33 OKAY	AURKC 1.08 OKAY	BAIAP2 1.15 OKAY	CALD1 1.12 OKAY	CALM1 1.25 OKAY	CASK 1.43 OKAY	CCNA1 1.36 OKAY	CCNB2 1.10 OKAY	CDC42 1.54 OKAY	CDC42BPA 1.23 OKAY	CDC42EP2 1.15 OKAY
C	CDC42EP3 1.43 OKAY	CDK5 1.48 OKAY	CDK5R1 1.39 OKAY	CFL1 -1.00 OKAY	CIT 1.04 OKAY	CLASP1 1.93 OKAY	CLASP2 1.02 OKAY	CLIP1 1.77 OKAY	CLIP2 1.39 OKAY	CRK 1.43 OKAY	CTTN 1.26 OKAY	CYFIP1 1.23 OKAY
D	CYFIP2 1.32 OKAY	DIAPH1 2.51 OKAY	DSTN 1.36 OKAY	EZR 1.03 OKAY	FNBP1L 1.22 OKAY	FSCN2 1.14 OKAY	GSN 1.32 OKAY	IQGAP1 1.34 OKAY	IQGAP2 1.72 OKAY	LIMK1 -1.01 OKAY	LIMK2 1.18 OKAY	LLGL1 1.55 OKAY
E	MACF1 1.66 OKAY	MAP3K11 1.32 OKAY	MAP4 2.29 OKAY	MAPK13 1.12 OKAY	MAPRE1 1.55 OKAY	MAPRE2 1.82 OKAY	MAPT -1.01 OKAY	MARK2 1.37 OKAY	MID1 1.48 OKAY	MSN 1.23 OKAY	MYLK 1.18 OKAY	MYLK2 1.44 OKAY
F	NCK1 1.66 OKAY	NCK2 1.64 OKAY	PAK1 1.34 OKAY	PAK4 1.49 OKAY	PFN2 1.34 OKAY	PHLDB2 1.12 OKAY	PIKFYVE 1.32 OKAY	PPP1R12A 1.40 OKAY	PPP1R12B 1.32 OKAY	PPP3CA 1.77 OKAY	PPP3CB 1.48 OKAY	RAC1 1.73 OKAY
G	RACGAP1 1.72 OKAY	RDX 1.41 OKAY	RHOA 1.24 OKAY	ROCK1 1.39 OKAY	SSH1 1.75 OKAY	SSH2 1.38 OKAY	STMN1 1.39 OKAY	TIAM1 1.99 OKAY	VASP 1.59 OKAY	WAS 1.21 OKAY	WASF1 1.47 OKAY	WASL 1.46 OKAY

Figure 2.6: qPCR Array Results Indicate Fold-increase of Select Genes. Genes related to (A) EMT, (B) ECM (C) cytoskeleton, and (D) mTOR pathway were tested. mRNA from SW71 cells induced with either DMSO or B5500-4 (10 uM) for 24 hrs was extracted, converted to cDNA, and run through qPCR assay. Genes of significant change include Col4A2 (1.05×10^4 fold change), MMP12 (-7.37×10^6 fold change), and mTOR (-2.22×10^3 fold change).
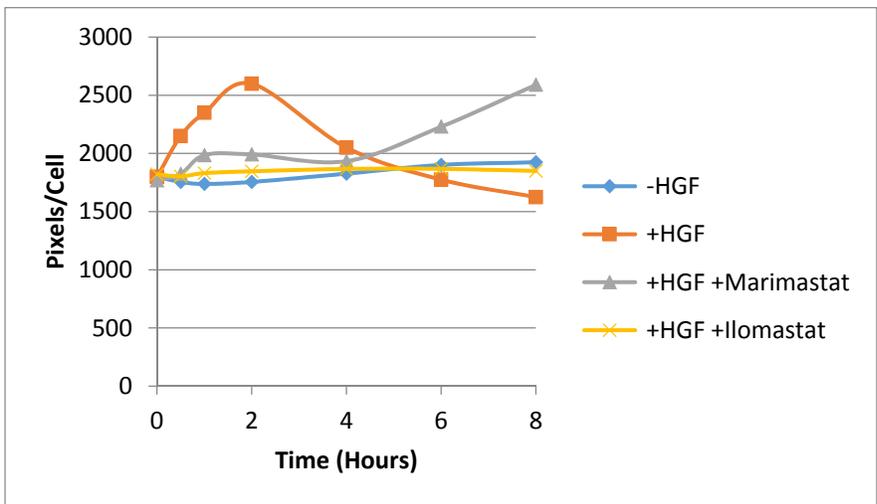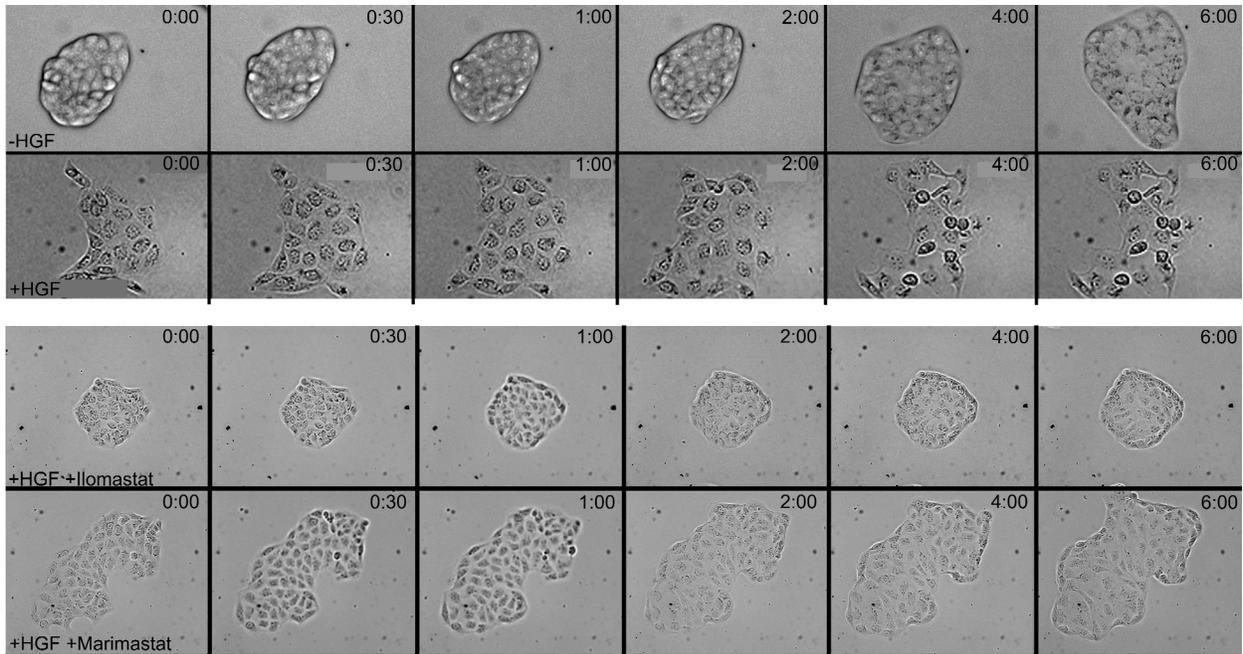
Figure 2.7: MMP-9 is Required for HGF-induced Epithelial Cell Scattering, and MMP-12 is Required for HGF-induced Cell Contraction Phase of Epithelial Cell Scattering. (A) MDCK time-lapse microscopy with HGF and Ilomastat (MMP-9) reveals failure of HGF-induced cell scattering. (B) MDCK time-lapse microscopy with HGF and Marimastat (MMP-12) shows MMP-12 role in HGF-induced cell contraction as a part of the cell scattering program. (C) MDCK cell surface area changes over time.
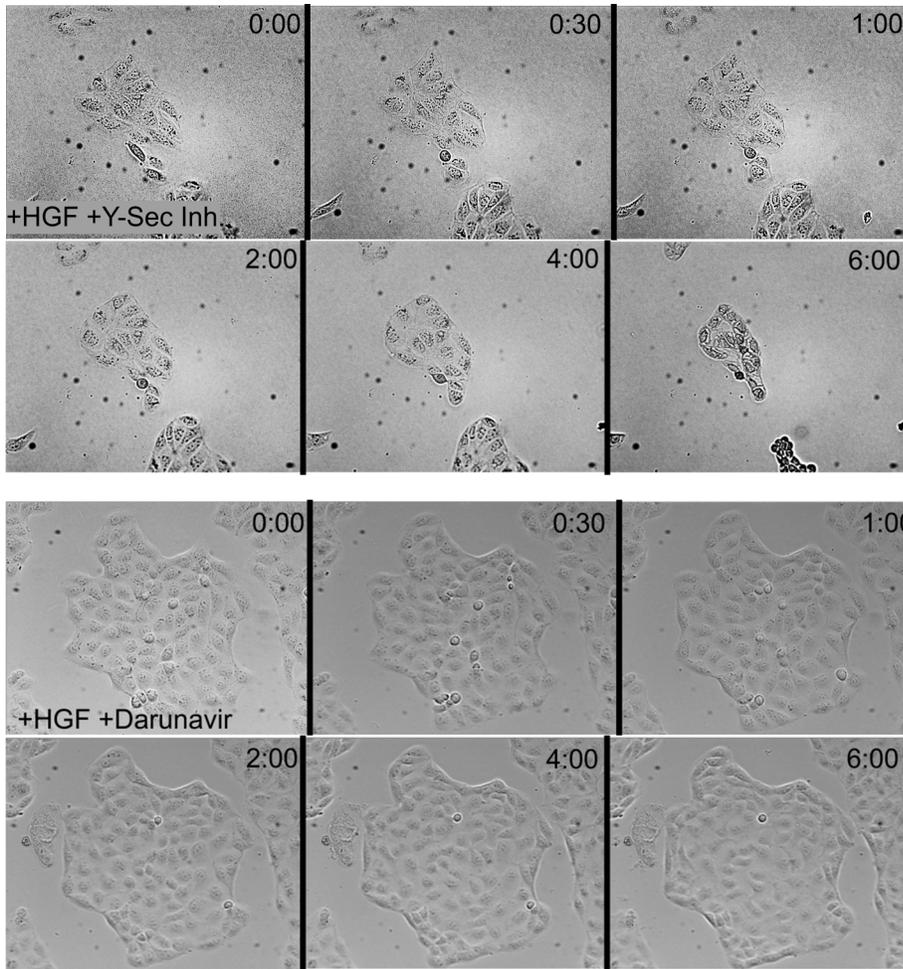
Figure 2.8: Furin Inhibition Blocks HGF-induced EMT; Y-Sec Inhibition is Cytotoxic. (A) MDCK cell time-lapse microscopy with HGF and Y-Secretase inhibitor reveals quick cell death by as early as 6 hours after treatment. (B) MDCK cell time-lapse microscopy with HGF and Darunavir (furin inhibitor) reveals failure of epithelial cell scattering.
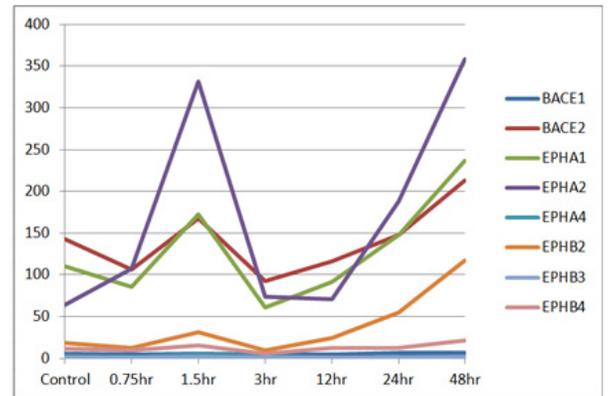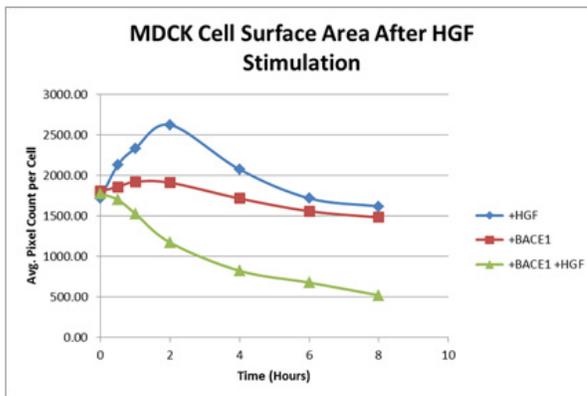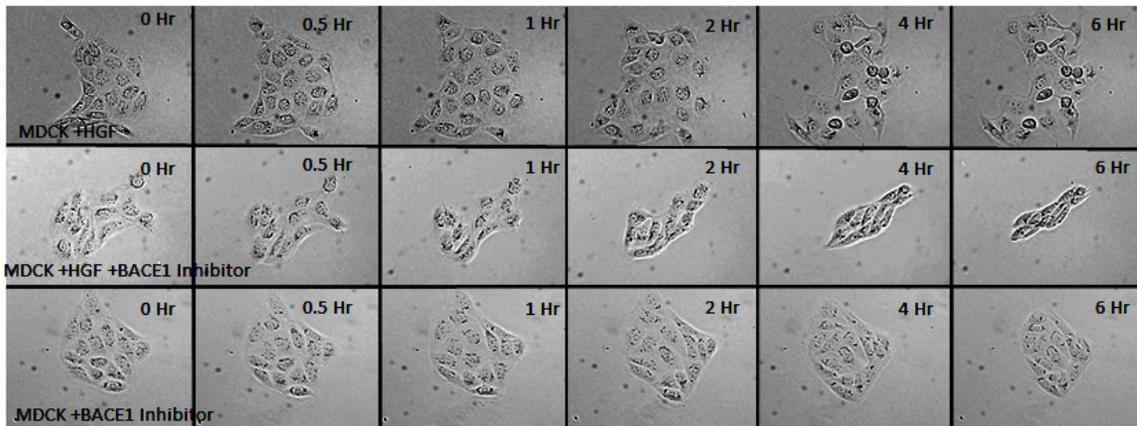
Figure 2.9: BACE is Required for Early EMT Cell Spreading. (A) Time-lapse live cell microscopy of MDCK cells were filmed over 6 hrs after induced with HGF and/or BACE inhibitor. (B) MDCK cell surface area chart illustrates the change in cell spreading/cell compaction during HGF induction. (C) Changes in gene expression of Eph family genes and BACE family genes at different time points after HGF induction.
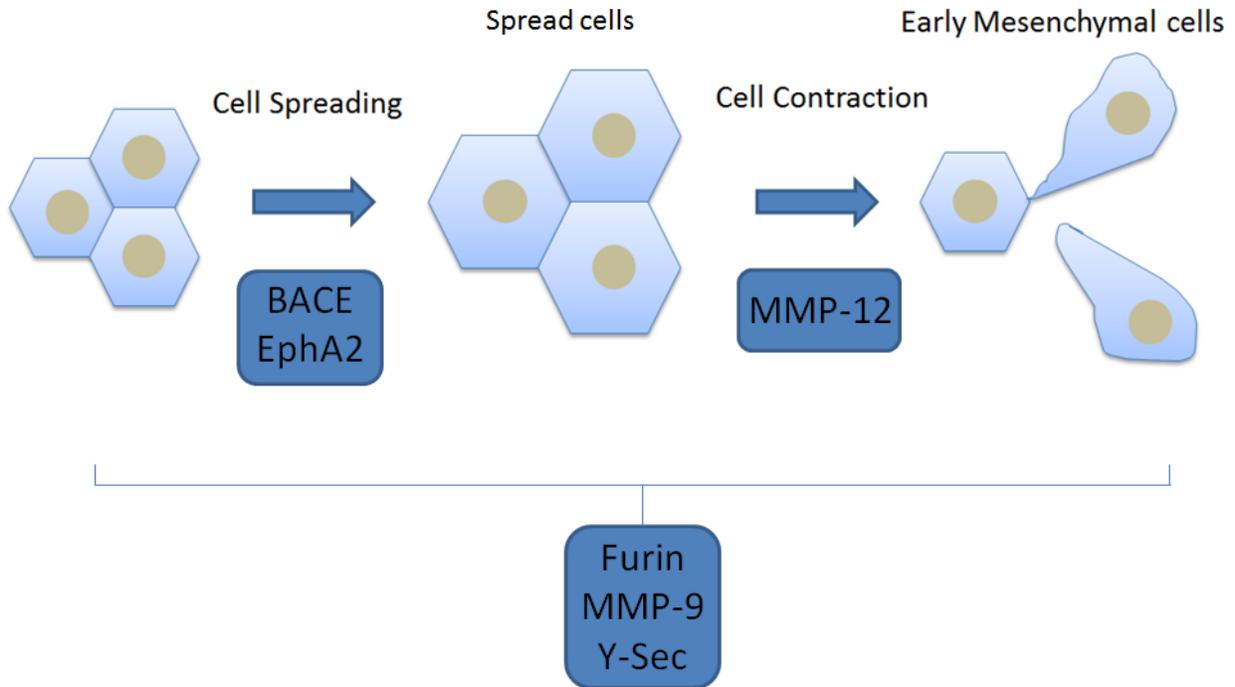
Figure 2.10: Model for Proteolytic Activity during Early EMT Events. A two-phase characterization of epithelial cell scattering includes cell spreading followed by cell contraction, which is when cell-cell contacts are broken. BACE activity is required for cell spreading to progress, while MMP-12 activity is required for cell contraction. Furin and MMP-9 are both required for cell scattering to progress, blocking both spreading and contraction phases. Y-Sec activity is required for cell survival and may not necessarily be involved in the c-Met pathway.

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EDUCATION

Brigham Young University   Sep 2006-Dec 2014
BS in Physiology and Developmental Biology
BS in Business Management: General Management Emphasis   GPA: 3.3

MS in Physiology and Developmental Biology   GPA: 3.7

WORK EXPERIENCE

Undergraduate Researcher   Aug 2009- Aug 2010
McKay School of Education: Brigham Young University

        Mentored under Dr. Ellen Williams and Dr. Joseph Matthews. Used analytical software (NVivo & Minitab) to characterize properties of school districts as effective professional learning communities.

Teacher's Aide: Biology 100   Aug 2010-Apr 2012
Biology Department: College of Life Sciences: Brigham Young University

        Under Dr. Melinda Ostraff, regularly led class lectures, mentored peers, and evaluated student performance. Developed new lecture presentations incorporating new course material; lecture topics covered basic biological concepts, including cell theory, mitsosis/meiosis, Mendelian genetics, evolution, and ecology.

Undergraduate/Graduate Researcher   Apr 2010- present
PDBio Department: College of Life Sciences: Brigham Young University

        Mentored under Dr. Marc Hansen. Group leader over biochemistry experiments. Characterized cell-cell junction proteins in epithelia under dynamic conditions, and molecular targets of small chemical inhibitors of Epithelial-Mesenchymal Transition (EMT) and Cancer Metastasis. Findings published in the Journal of Biochemical and Biophysical Research Communications and Journal of Experimental Cell Research.

        Leader of Senior Research Capstone Project. Tested potential anti-cancer drug compounds through several well-established drug development tests, including NCI-60 growth assays, EMT assays, toxicity studies, and pharmacokinetics/pharmacodynamics studies.

Chemical compounds to be licensed to IMD for clinical trials testing and submission for FDA approval.

Master's Thesis work on characterizing mechanism of action for potential anti-metastatic compound (B5500-4). Studies and experiments include techniques in immunofluorescent microscopy, time-lapse microscopy, western blot, ELISA, invasion assays, EMT assays, qPCR, and RNA-seq.

Course Team Instructor: Principles of Biology (PDBio 120)　　　　　　Jan 2013-Apr 2013
PDBio Department: College of Life Sciences: Brigham Young University

Team-taught PDBio 120 course with Dr. Juan Arroyo. Taught every other class lecture, co-wrote weekly exam questions, graded course material, mentored students during office hours, and coordinated undergraduate TA responsibilities and work.

Course Instructor: Advanced Physiology Lab　　　　　　　　　　　　　Aug 2012-present
PDBio Department: College of Life Sciences: Brigham Young University

Led 3-hour weekly laboratory course in Advanced Physiology (PDBio 363). Taught mini-lecture at start of class, mentored students during class and office hours, graded lab reports, and held test reviews.

SERVICE

Hospital Volunteer　　　　　　　　　　　　　　　　　　　　　Sep 2010- Jan 2013
Utah Valley Regional Medical Center: ER Trauma Center

Transported patients, medical equipment, and supplies to and from the ER. Regularly interacted with patients, doctors, and other medical staff.

Missionary Service　　　　　　　　　　　　　　　　　　　　Aug 2007- Aug 2009
The Church of Jesus Christ of Latter-day Saints

Served a two year, non-profit mission in Michigan. Invited others to learn more about religious beliefs. Also provided service to individuals and community organizations. Led and supervised 20-30 peers.

AWARDS

Cancer Research Center Summer Fellowship　　　　　　　　　　　　　Apr 2011
Brigham Young University Cancer Research Center

Awarded research grant for independent research project on the study novel small chemical inhibitors of tumor metastases. Tested structure-activity relationship of potential anti-cancer drug derivatives.

FUNDING

PUBLICATIONS

Call GS, Hansen MDH, et al. A zyxin-nectin interaction facilitates zyxin localization to cell-cell
        adhesions. Biochemical and Biophysical Research Communications. 2011 Nov
        25;415(3):485-9. doi: 10.1016/j.bbrc.2011.10.099. Epub 2011 Oct 28.

Hoj JP, Hansen MDH, et al. Cellular contractility changes are sufficient to drive epithelial
        scattering. Experimental Cell Research. 2014 Apr 26. pii: S0014-4827(14)00169-4. doi:
        10.1016/j.yexcr.2014.04.011.

PRESENTATIONS AND ABSTRACTS

Schuler JT, and Hansen MDH. Characterizing small chemical inhibitors of c-Met-induced EMT.
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REFERENCES

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