Exploring mechanisms of metastasis suppression in metastatic melanoma

By

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Abstract

Melanoma is responsible for 76% of deaths from skin cancer, making it the deadliest form of commonly diagnosed skin cancer. The deadly nature of melanoma is due to its tendency towards rapid metastasis early in tumor progression. Metastasis is the process of cells exiting the primary tumor and forming secondary tumors in other parts of the body. Metastasis accounts for as much as 90% of morbidity and mortality associated with cancer. Therapeutically targeting and treating melanoma metastases is a challenging clinical goal, as metastatic cells are heterogeneous and can be morphologically and genetically distinct from the primary tumor. This dissertation examines two distinct approaches towards preventing or treating disseminated melanoma metastases: 1) Re-introduction of metastasis suppressor protein fragments to prevent metastatic colonization, and 2) Treating disseminated metastases with a targeted small molecule treatment. By examining two discrete approaches of treating metastatic melanoma, this work sheds light on the clinical viability of using metastasis suppressors or metastasis-targeting drugs in patients with metastatic melanoma.

To examine strategies for metastasis suppression through metastasis suppressor proteins, we examined fragments of KISS1. Expression of KISS1 inhibits metastatic colonization at secondary sites, rendering disseminated cells dormant. KISS1 must be secreted outside of the cell to suppress metastasis, where furin cleaves KISS1 into kisspeptins at three dibasic sites. This cleavage liberates an internal kisspeptin, Kisspeptin-54 (KP54, K^{67} to F^{121}), which is amidated and can bind a $G_{q/11}$-coupled protein receptor KISS1R. The mechanism of action for KISS1 metastasis suppression has long been assumed to be KP54 interacting with KISS1R. However, expression of KISS1R is not necessary for KISS1 metastasis suppression, and the extracellular processing of KISS1 hints at an alternative hypothesis: a different kisspeptin may be responsible for

suppressing metastasis. To test this hypothesis, all possible kisspeptins (KISS1 Manufactured Peptides, or KMP) were generated based on known dibasic cleavage sites (M^{1}–Q^{145}; M^{1}–R^{56}; M^{1}–R^{67}; M^{1}–R^{124}; R^{56}–R^{66}; R^{67}-F^{121}; R^{56}–F^{121}; R^{56}–Q^{145}; R^{67}–Q^{145}; R^{124}–Q^{145}) and were used in an experimental metastasis assay to characterize their abilities to suppress metastasis. We found that while KP54 suppressed metastasis, additional KMP lacking the KISS1R binding site (LRF-NH_2) were able to completely suppress metastasis ($p<0.05$). In particular, one kisspeptin (KMP2, M^{1} – R^{56}) suppressed metastatic traits *in vitro* as well as completely suppressing metastasis *in vivo*. To identify the signaling pathways used by KMP2 to suppress metastasis, a genome wide CRISPR/Cas9 screen was performed in KMP2-expressing B16-F10 melanoma cells. As a whole, these data suggest that metastasis suppression by KISS1 is not necessarily contingent on KISS1R activation, and also supports investigation into additional receptors.

To investigate the efficacy of targeting metastases with small molecules, we also investigated the impact of ML246 (AKA metarrestin). Metarrestin was discovered by a high throughput assay for molecules which disassemble the perinucleolar compartment (PNC). Perinucleolar compartments are structures composed of RNA and RNA binding proteins near the nucleolus. These structures are enriched in metastatic cells and are druggable targets which target metastases and not normal epithelium. We examined the impact of metarrestin treatment on orthotopic tumor growth, microscopic metastasis formation, and macroscopic metastasis formation. We found that metarrestin treatment had no significant impact on metastatic outgrowth, but suppressed intradermal tumor growth. Based on these data, we can infer that PNC-positive metastases may be too small a population to effectively target in this model of metastatic melanoma. This treatment paradigm may be more effective in conjunction with a more potent approach to metastasis suppression.

Overall, the work in this dissertation identified a potent metastasis suppressing peptide fragment of KISS1, KMP2, and described the efficacy of metarrestin treatment of disseminated melanoma metastases. The metastasis suppression induced by KMP2 expression was far more potent than the effects of metarrestin treatment, suggesting that treatment deliveries and targets are critical considerations in the development of anti-metastatic therapeutics.

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Dedication

Dedicated to my husband, Brice, who has always believed in me.

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Chapter I: Introduction

Cancer and metastasis

Cancer, is steadily gaining ground as a worldwide epidemic. An estimated 1 in 7 deaths worldwide is attributed to cancer, and over 60% of these deaths occur in low- and middle-income countries which lack adequate healthcare resources and infrastructure (Siegel, Miller, & Jemal, 2016). In the United States, cancer is the leading cause of death in 21 states, which is largely due to decreases in death from other causes (Siegel et al., 2016). Despite prevention and early detection technologies becoming more widespread, cancer promises to burden the global healthcare system as the population ages. By the year 2030, an estimated 21.7 million new cancer cases and 13 million cancer deaths are expected to occur. To stem the tide of cancer, a better understanding of the disease itself is necessary.

Cancer is defined as the abnormal behavior of a cell which can result in uncontrolled growth or death. Cancer is generally thought to arise from a combination of internal (e.g. inherited genetic mutations, inflammation, hormones, immune conditions, etc.) and environmental factors (smoking, pollution, exposure to carcinogens, UV radiation, etc.). Of all cancer diagnoses in the United States, the top three diagnosed cancers are breast cancer (female), prostate cancer, and lung/bronchus (Siegel et al., 2016). Better early detection methods in breast cancer and prostate cancer (mammograms and prostate exams, respectively) have helped to detect these cancers early to reduce mortality. Lung cancer continues to be a prominently diagnosed cancer due to population smoking trends (Kochanek, Murphy, Xu, & Tejada-Vera, 2016). Globally, cancer incidence and deaths continue to climb despite better attempts to prevent and detect it early.

Most types of cancer are not inherently deadly. Cancers which are detected early have a much lower mortality rate than more progressed tumors. It is later in the process of tumor

progression, when a cancer metastasizes, that cancer becomes deadly. Metastasis, the multi-step process of cells exiting the primary tumor to form secondary tumors, is the cause for as much as 90% of the morbidity and mortality associated with cancer. Metastatic cancers become deadly when secondary tumors disrupt tissue function throughout the body. If metastasis could be prevented, managed, or delayed, cancer could be managed more like a chronic disease. Wholly preventing or curing cancer on a population-scale is an unlikely goal, but finding a way to stop, prevent, or treat metastasis as a chronic condition is a more attainable clinical goal.

Metastasis can occur in three general ways. Cells can metastasize through the bloodstream, the lymphatic system, or across body or organ cavities. Advances in biomarker technology, circulating tumor cell detection, cell-free DNA detection, and tumor deep sequencing have shed light on which cancers are likely to metastasize, but the need to more precisely identify patients with metastatic tumors remains. Patients with more aggressively metastatic cancer types require a more aggressive treatment as opposed to patients with a localized, non-metastatic tumor type. Identifying biomarkers or other signs of metastatic propensity would improve treatment accuracy and precision for cancer patients.

The metastatic cascade

The process of metastasis is complex and involves a series of interdependent steps, which are cumulatively referred to as the metastatic cascade. Failure to complete even a single step in the metastatic cascade results in cell death. While tumors can shed upwards of one million cells into the bloodstream per day, very few of these cells successfully complete the metastatic cascade (Weiss, 1990). These sequential steps selectively enrich for a population of highly plastic, invasive, metastatic cells with the ability to survive in a new microenvironment.

Figure 1

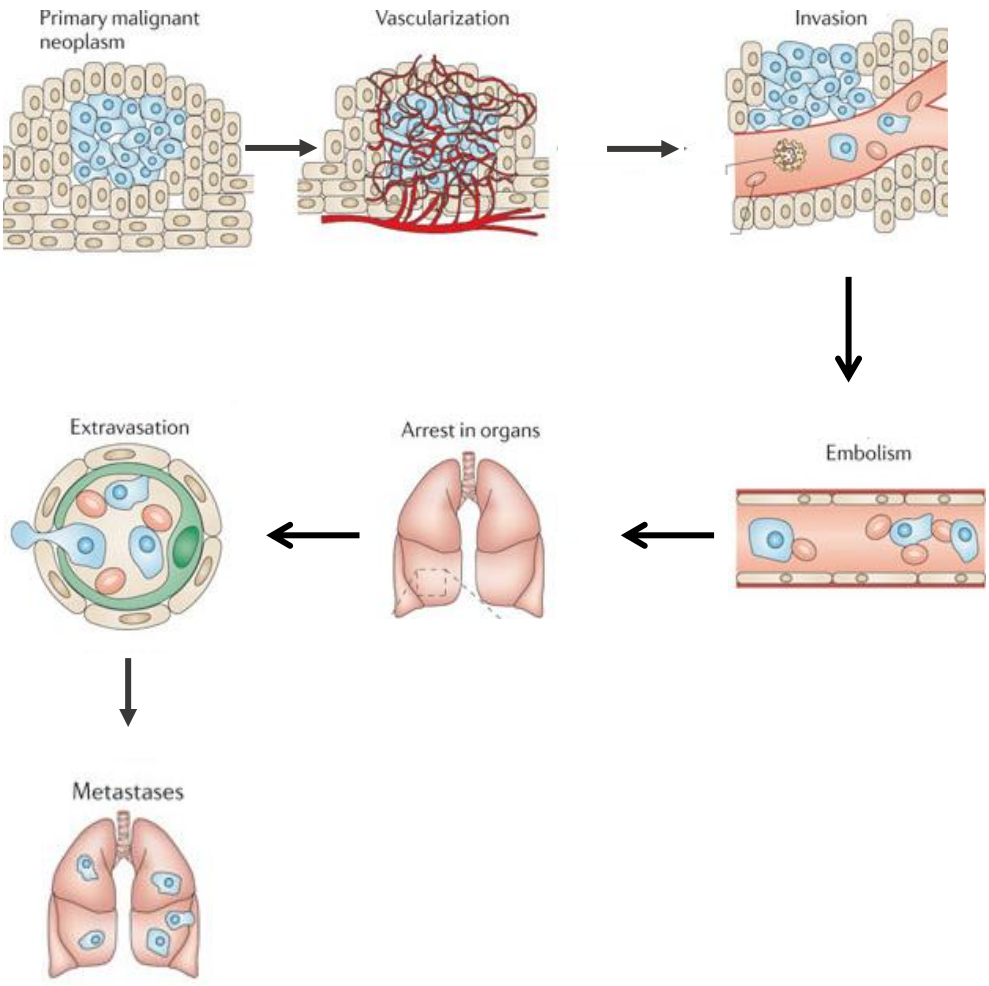

Primary malignant neoplasm
Vascularization
Invasion
Embolism
Arrest in organs
Extravasation
Metastases

Figure 1: The major steps of the metastatic cascade for solid tumors. Figure adapted from Francia et. al (2011)

Tumors begin to shed cells early during tumor progression, but metastases are more likely to be detected from more advanced tumors (Butler & Gullino, 1975). As tumors begin to grow and divide, they quickly outgrow the nutrient supply available in the stromal tissue. construction of novel vasculature around the tumor. Oxygen can diffuse 150-200 microns through a tissue, which results in the core of even small tumors quickly becoming hypoxic. Hypoxia, the state of tissues in a low oxygen environment, induces the stabilization of hypoxia inducible factors 1 and 2 (HIF1 and HIF2), which are transcription factors which target genes that allow cells to survive in times of nutritional depletion (Semenza, Roth, Fang, & Wang, 1994). These HIF proteins drive a transcriptional profile upregulating angiogenic factors such as VEGF, which is secreted into the microenvironment and stimulates blood vessel formation (Y. Liu, Cox, Morita, & Kourembanas, 1995; Mazure, Chen, Yeh, Laderoute, & Giaccia, 1996). The formation of a tumor-supporting vasculature allows tumors to shuttle nutrients in and waste products out. The vasculature formed by this process is noteworthy for its "leakiness," or the gaps in the endothelial wall. These gaps in the endothelium facilitate easier transition of nutrients or cells in and out of the vasculature, as well as allowing for easy formation of new vascular sprouts. These angiogenic activities facilitate further growth and invasion in the tumor.

With the nutritional support system generated from angiogenesis, tumors now can continue to grow and invade into the surrounding stromal tissue. As tumors grow into the stroma, they secrete enzymes such as collagenases and matrix metalloproteases that degrade the microenvironment. These enzymes work to clear additional space for tumor outgrowth. In concert with the secretion of proteolytic enzymes, tumors also secrete other factors to condition the microenvironment to preferentially support tumor outgrowth. Prominent secreted factors such as VEGF and CXCL-family chemokines create a chemoattractive gradient which recruits

endothelial cells and immune cells (Roodman, 2004). Tumors, which rely heavily on glycolytic metabolism, also secrete lactate as a glycolytic by-product. Lactate acidifies the tumor microenvironment, conditioning the surrounding stromal tissue which has been found to support tumor growth and metastasis (Song et al., 2015). Tumors secrete a proteolytic, inflammatory, acidic milieu to shape their surroundings in a manner which favors growth and metastasis.

Invasive tumors can eventually shed cells which migrate out of the immediate tumor microenvironment, invade the basement membrane, and intravasate into the vasculature or lymphatic system. This step of metastasis, cells exiting the primary tumor, can begin early in tumor progression, but is more highly associated with larger tumors. To exit the tumor, cells must physically migrate away from the tumor and squeeze between or through epithelial or endothelial cells (Liotta, 1986; Wolf et al., 2003). To migrate, metastatic cells must dynamically reorganize their actin cytoskeleton to facilitate movement (Brinkley et al., 1980; Volk, Geiger, & Raz, 1984). Cells form focal adhesions by phosphorylation of focal adhesion kinase (FAK) and integrin binding to adhere to anchors in the microenvironment as they travel through the stroma (Huttenlocher et al., 1998; Ruoslahti, 1994). Migratory cells will extend projections, form adhesions, and then use the actin-myosin cytoskeleton to pull the rest of the cell through the tissue.

There are two primary mechanisms of invasion: individual cell migration and collective cell migration (Friedl, Locker, Sahai, & Segall, 2012). Individual cell migration is characterized by single cells invading the surrounding tissue and escaping the tumor as individual units. Collective cell migration, however, describes a phenomena where a cluster of cells collectively invades surrounding tissue while retaining cellular junctions (Friedl et al., 2012). Carcinomas, a cancer type originating from epithelial cells, tend to invade through collective cell migration

(Friedl et al., 2012). Sarcomas, cancers which arise from mesenchymal cells such as melanocytes, muscle cells, bone, and neurons, tend to instead invade using individual cell migration (Wolf et al., 2003). These cells lack cellular junctions and invade the surrounding tissue as single cells. Either mechanism of invasion can result in cells escaping the tumor, invading surrounding tissue, and crossing the basement membrane. From here, cells can intravasate into the bloodstream or the lymphatic system.

Within the bloodstream or lymphatic system, metastatic cells encounter a new host of challenges. These migratory cells must evade immune detection from circulating and tissue-associated immune cells. One mechanism to avoid immune detection is metastatic cells can coat themselves with platelets to avoid immune surveillance. Within this platelet-tumor cell aggregate, the cells are held together with integrin bridges, which deters detection by natural killer cells (Tesfamariam, 2016). Once they enter the circulatory system, metastatic cells must also withstand the immense pressure of vascular shear forces, which can tear most epithelial cells apart (Wirtz, Konstantopoulos, & Searson, 2011). Resistance to mechanical stress is recognized as a key characteristic for successful metastatic cells both in the bloodstream and in the stroma (Kumar & Weaver, 2009). Within the circulation, metastatic cells must also resist anoikis, an apoptosis program initiated by a lack of anchorage. Many metastatic cells overcome this by overexpressing mitochondrial anti-apoptotic proteins such as Bcl-2, Bcl-XL, and Mcl-1 and/or downregulating pro-apoptotic proteins such as Bax and the caspaces (Patel, Camacho, Shiozawa, Pienta, & Taichman, 2011). Resistance to mechanical stressors in the vasculature and evasion of the immune system are major bottlenecks to the metastatic cascade.

Even metastatic cells do not linger indefinitely in the circulatory or lymphatic systems. The cells require a stationary setting to form full-fledged metastases. Early metastases are often

identified in lymph nodes proximal to the primary tumor (Greenblatt, 1933). However, the determination of metastatic sites is not exclusively proximity-based. Melanomas can metastasize to different sites in the skin, and bladder cancer cells can metastasize across the bladder to another site on the bladder. However, many metastases target the lung, bone, and brain (Babaian, Johnson, Llamas, & Ayala, 1980). The exact factors which contribute to the metastatic site are not fully understood, however the affinity of certain cancers to metastasize to these sites suggests that these microenvironments are conducive to metastatic outgrowth. In 1889, Paget noted the predisposition of certain cancers to metastasize to certain sites (Paget, 1889). This site-specific metastasis was described as the interaction of the correct "seed" (cancer cell) reaching a "congenial soil," or a tissue where disseminated cells could survive and thrive. The discovery of tissue-specific addressins, molecules which lymphocytes use to target specific tissues supports that metastatic cells could also selectively express surface markers to target metastatic sites (Kieran & Longenecker, 1983; Nakache, Berg, Streeter, & Butcher, 1989; Ruoslahti, 1994). Once these cells reach the appropriate secondary site, they are primed to enter the final phase of the metastatic cascade.

Upon reaching these sites, metastatic cells either lodge themselves in a capillary or adhere to the vessel wall and extravasate. After lodging in the capillary or extravasating into the secondary tissue, the cells then colonize the tissue and divide to form full-fledged metastases. This change in environment requires a change in cellular behavior. While migrating, and traveling through the lymph or circulatory systems, metastatic cells do not tend to divide. However, upon reaching a secondary site, metastatic cells require a change in behavior to divide and form macroscopic metastases. This switch from a migratory, mesenchymal phenotype back to an epithelial phenotype is often referred to as MET, or mesenchymal to epithelial transition

(Auersperg et al., 1999). This phenomenon is observed in normal physiology during development, and cancer cells recapitulate this behavior at the last phase of the metastatic cascade. The metastatic cells can then switch to a dividing, tumor-like phenotype to successfully form metastases.

However, metastases do not necessarily form macroscopic metastases. Once metastatic cells have seeded a secondary tissue, they can lie dormant, sometimes for months or years (Alexander, 1983; Celia-Terrassa & Kang, 2016; Romero, Garrido, & Garcia-Lora, 2014; Stein-Werblowsky, 1978; Sugarbaker, Ketcham, & Cohen, 1971). Metastatic dormancy is generally attributed to the adaptation of disseminated tumor cells to an unfamiliar microenvironment (Giancotti, 2013). A key characteristics of single-cell metastatic dormancy is cell cycle arrest, often due to mitogenic signaling from host tissue (Osisami & Keller, 2013). Dormant metastatic cells can linger in quiescence or recover and resume division.

The metastatic cascade is complex and incredibly inefficient. For metastases to form, each of these steps must be successfully completed. Even though tumors can shed upwards of one million cells per day, patients do not present with millions of metastases. Yet despite this lack of efficiency, metastasis still occurs and is the main cause of cancer-associated mortality. Metastases which survive the metastatic cascade have evaded the immune system, withstood shear forces of the bloodstream, selected and conditioned a new tissue, and created their own metastatic niche. The survivors of the metastatic cascade are cells, later tumors, which are uniquely suited to thrive in a new environment.

Clinically targeting metastasis

Clinically targeting metastasis remains an elusive goal. Metastases are challenging to target, as they often have different characteristics from their primary tumors. To complete the steps of the

metastatic cascade and become metastases, metastatic cells must behave differently from tumor cells. Therefore, to therapeutically target metastases, it is necessary to utilize targeted therapies to detect and treat metastases. Targeted anti-metastatic therapies generally consist of neutralizing antibodies and small molecule kinase inhibitors. While these therapies can be specifically targeted based on the genetic composition of the primary tumor, these therapies are not the complete answer to the problem of metastasis. Tumor heterogeneity notwithstanding, it is established that metastases have a higher accumulation of mutations than primary tumor cells (Cifone & Fidler, 1981; Tlsty, Margolin, & Lum, 1989). Because of this inherent genomic instability, targeting multiple metastases from the same primary tumor can have varying outcomes. A mutation found in the primary tumor could be absent in metastases, rendering targeted therapies without a target. Some therapies can be lackluster in most patients but demonstrate a striking response in a small subpopulation. Overall, by utilizing the characteristics that allow metastases to be successful in the first place, metastatic cells also evade treatment and therapy.

Melanoma

Melanoma deserves recognition as a cancer which is unique in its predisposition to metastasis as well as its steadily increasing incidence since 1950 (Balch, 1992). Melanomas arise from melanocytes, cells from the basal layer of the epidermis which produce melanin in response to UV exposure. Melanin, which comes in forms of eumelanin and pheomelanin, is thought to be an oxidant of UV radiation. Most melanomas arise from a mutational profile traced back to a signature indicative of UV damage, a C $\rightarrow$ T mutation at dipyrimidine sites (Brash et al., 1991). These mutations can accumulate in tumor suppressor genes such as p53 or PTEN, as well as oncogenic drivers such as BRAF or NRAS and give rise to a transformed melanocyte (Brash et

al., 1991; Pierceall, Mukhopadhyay, Goldberg, & Ananthaswamy, 1991). Early in melanoma tumor progression, melanomas undergo an epidermal horizontal (or radial) growth phase where the tumor cells remain in the epidermis and simply grow out horizontally through the epidermal stroma. The radial phase is then followed by the vertical growth phase, characterized by melanoma cells breaking through the epidermis and into the dermal layer. Melanomas in the vertical phase of tumor progression are most likely to metastasize, but all melanomas are presumed to be aggressive due to the developmental origins of melanocytes.

Melanocytes are mesenchymal cells which arise from the neural crest during embryonic development. During development, melanocytes travel along the neural crest to their destination in the embryo. Postnatally, melanocytes are not transcriptionally programmed to migrate or travel from their locations. However, in response to DNA damage or other transformative events, melanocytes regain their mesenchymal phenotypes and migrate, invade, and intravasate early during melanoma tumor progression. Melanomas are considered the most metastatic and deadly skin cancer due to this aggressively metastatic nature.

Metastasis suppressors

One method to help distinguish metastatic tumors from less aggressive tumors is the presence of metastasis suppressors. Metastasis suppressors are a growing family of endogenously expressed proteins and RNA that block one or more steps of the metastatic cascade. When expressed by a tumor, metastasis suppressors do not have a substantial impact on primary tumor growth (growth inhibition of <50% is the generally accepted metric). The first metastasis suppressor, Nm23-H1, was discovered in 1988 as a metastasis suppressing gene with the ability to suppress metastasis in murine K-1735 melanoma cells (Steeg, Bevilacqua, Kopper, et al., 1988; Steeg, Bevilacqua, Pozzatti, Liotta, & Sobel, 1988). Since this discovery, the field has expanded to include several

Nm23 family members as well as the more than 30 additional members of the validated metastasis suppressor family. These metastasis suppressors have potential to be used as a marker of prognosis or a measure of aggressiveness in certain tumor types. There even exists the opportunity to use metastasis suppressors as a form of clinical intervention.

Metastasis suppressors all have normal physiological roles, but many suppressors only suppress metastasis in a specific tumor type. As cancer develops, the expression of metastasis suppressors is often silenced or deleted during tumor progression. This silencing can be accomplished through epigenetic regulation (e.g. methylation), or mutation and silencing due to genomic instability (e.g. mutation or chromosomal deletion) (Shirasaki, Takata, Hatta, & Takehara, 2001; Steeg, Ouatas, Halverson, Palmieri, & Salerno, 2003). Most metastasis suppressors have an effect at multiple steps of the metastatic cascade, while a smaller number selectively inhibit a specific step of the metastatic cascade (Bohl, Harihar, Denning, Sharma, & Welch, 2014). The ultimate outcome is that tumors expressing metastasis suppressors tend to have less incidence of metastasis and overall improved prognosis. Metastasis suppressors represent a potent, natural defense against metastasis.

The idea of utilizing metastasis suppressors clinically has been discussed since the field was established. Many metastasis suppressors have been clinically observed in relation to patient prognosis and have prognostic or therapeutic potential (Bohl et al., 2014; Nash & Welch, 2006). A loftier goal than predicting prognosis would be to use metastasis suppressors as a tool to prevent or treat metastases using metastasis suppressors. To clinically utilize metastasis suppressors, there are two general approaches: 1) target pathways used by metastasis suppressors with small molecules to recapitulate metastasis suppressor signaling, or 2) re-introduce metastasis suppressors, via exogenous administration or gene therapy, back into metastatic

lesions. The problems with targeting metastasis suppressor signaling are the lack of knowledge the field has gained on the exact mechanisms of these molecules. While the primary functions of many metastasis suppressors are understood in normal physiology, several metastasis suppressor mechanisms of action remain unelucidated. Embarking on a treatment strategy without the full understanding of a mechanism is problematic and unrealistic. There are also inherent challenges associated with treatment via metastasis suppressor. Gene therapy directed at metastases requires the ability to selectively target metastases. Exogenous administration of metastasis suppressors would require a cell-penetration mechanism (such as TAT) for intracellular metastasis suppressors. Extracellular metastasis suppressors would require stabilizing and potentially metastasis-targeting components to ensure delivery. While the goal of clinically harnessing metastasis suppressors remains alive, this goal requires a selective set of circumstances to work. A likely metastasis suppressor candidate for therapeutic development would be a secreted or extracellular metastasis suppressor.

KISS1: Metastasis suppressor

After observing that metastatic melanomas frequently present with deletions of chromosome 6, it was observed that re-introduction of a normal human chromosome 6 into metastatic melanoma cells suppressed metastasis by 95% (Miele et al., 1996; Welch et al., 1994). This discovery suggested that there were metastasis suppressing genes present on chromosome 6. To identify the responsible gene(s) of chromosome 6 involved in metastasis suppression, subtractive hybridization of cDNAs resulting from re-introduction of different regions of chromosome 6 was performed. The finding from this study was KISS1, a 1.0 kB transcript only expressed in metastasis-suppressed melanomas with re-introduction of chromosome 6 (Lee et al., 1996; Lee & Welch, 1997a). An analysis of normal melanocytes and radial growth phase melanomas

identified endogenous KISS1 expression, but this detection of expression was lost in vertical growth phase melanomas or metastatic lesions (Lee et al., 1996). Cumulatively, these data led to the conclusion that KISS1 was a gene responsible for metastasis suppression, and that it was likely located on chromosome 6.

In 1998, the chromosomal localization of KiSS-1 was found to be on chromosome 1q32, not chromosome (West, Vojta, Welch, & Weissman, 1998). The necessary element on chromosome 6 was later identified as CRSP3, an upstream regulator of KiSS-1 expression (Goldberg et al., 2003). Expression of CRSP3 on chromosome 6 was found to be inversely correlated with metastatic potential and TXNIP expression, a thioredoxin-binding protein also found on chromosome 1 (Shirasaki et al., 2001). TXNIP is a downstream target of CRSP3. Expression of TXNIP is positively correlated with expression of KISS1 and metastasis suppression, and is lost when CRSP3 is depleted. These data identified why the loss of chromosome 6 in melanoma led to increased metastatic potential, and shed light on KiSS-1 upstream regulation. These findings were not limited to melanoma, however. Not long after its discovery, KiSS-1 was also found to be a potent metastasis suppressor in breast cancer cells, which opened the door to the possibility for KiSS-1 being a metastasis suppressor gene in several cancer types (Lee & Welch, 1997b). The multi-functionality of KiSS-1 as a metastasis suppressor gene suggested a potential clinical significance for it, either as a biomarker or as a basis for a therapeutic.

The understanding of how the KiSS-1 metastasis suppressor gene functioned was a mystery for several years after its discovery. Analyses of the KiSS-1 cDNA transcript found that KiSS-1 encoded a 145-amino acid peptide with a 19-amino acid secretion sequence (Lee et al., 1996). The peptide sequence also predicted a SH3 binding sequence, prohormone convertase processing sites, and a putative protein kinase C phosphorylation site (Lee et al., 1996). These hints buried

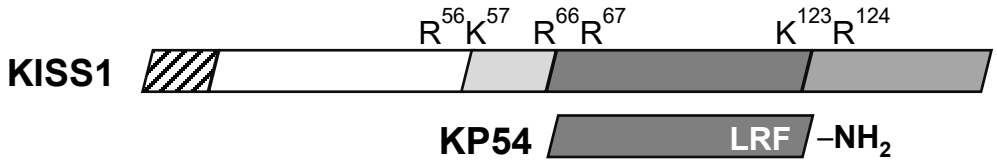
in KiSS-1's peptide sequence, hereafter KISS1, did not shed light on how the peptide itself suppressed metastasis. In 2001, three groups independently investigating orphaned G-protein coupled receptors identified that an internal fragment of KISS1 was the ligand for a rat G-protein coupled receptor denoted by three names: GPR54, AXOR12, and hOT7T175 (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). This internal fragment of KISS1, metastin or later KP54, was an internal 54-amino acid peptide nestled between two dibasic cleavage sites (Ohtaki et al., 2001). These groups definitively demonstrated that KP54, and specifically its 10 C-terminal amino acids, was the ligand to GPR54/AXOR12/h0T7T175, which is now commonly known as KISS1R.

KISS1 processing

In identifying KP54/metastin as the ligand to KISS1R, these groups consequently shed light on KISS1 processing. The secretion sequence and presumptive dibasic sites were indeed processing elements to KISS1. When KISS1 is translated, it is secreted outside of the cell through the Golgi apparatus (Harihar, Pounds, Iwakuma, Seidah, & Welch, 2014; Nash et al., 2007). Before secretion, gamma secretase cleaves the 19-amino acid secretion sequence and KISS1 is then shuttled outside of the cell. KISS1 must be secreted outside of the cell to suppress metastasis, which cements its role as an extracellular protein (Nash et al., 2007). Once outside of the cell, KISS1 is then cleaved by furin, a ubiquitously expressed prohormone convertase, at three dibasic sites (R/K-XX-K/R) into kisspeptins (Harihar et al., 2014). Interestingly, furin is expressed throughout the cell and even in the Golgi apparatus, yet it is only once KISS1 is outside of the cell that this processing occurs (Harihar et al., 2014). Fragments of KISS1 that have been identified from tissue all seem to originate from KP54. The peptides identified have primarily consisted of the C-terminal domain of KP54, the region responsible for KISS1R binding. These

Figure 2

A


R56K57 R66R67 K123R124
KISS1
KP54
LRF
–NH2

B

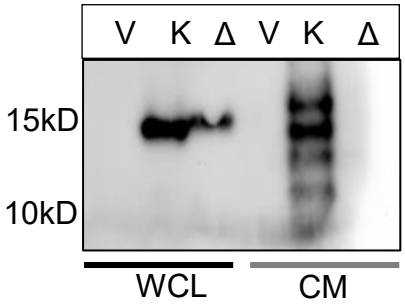

V K Δ V K Δ
15kD
10kD
WCL
CM

Figure 2: A, Diagram of KISS1, secretion sequence (diagonal lines) and cleavage sites. KP54 is depicted below with LRF-amide KISS1R binding motif. **B,** Western blot of whole cell lysate (WCL) and conditioned media (CM) immunoprecipitation from vector (V), KISS1 (K), and Δ SS-KISS1 (Δ), a non-secreted mutant. The banding pattern observed in conditioned media sample indicates the presence of kisspeptins.

peptides, named for their length, are KP-14, KP-13, and KP-10 (Bilban et al., 2004; Maguire et al., 2011; Mead, Maguire, Kuc, & Davenport, 2007). The smallest bioactive fragment of KP54 with the ability to bind KISS1R is KP10, the final 10 amino acids of KP54. KP10 binds KISS1R with a higher affinity than even KP54. These processing elements appear to be the normal, endogenous KISS1 processing elements necessary for KP54 liberation outside of the cell. Once furin cleaves KISS1, PAM amidates F^{121} and increases the affinity of KP54's C-terminal domain for KISS1R binding (Ohtaki et al., 2001). While the C-terminal amidation at F^{121} is optimal for receptor binding, the un-amidated KP54 is still able to bind KISS1R (Ohtaki et al., 2001). These processing events result in KISS1 and kisspeptins secreted outside of the cell and primed for bioactivity.

There is a biological system in place to negate KISS1 processing and subsequent signaling. The C-terminal LRF sequence of KP54 is required to bind KISS1R (Ohtaki et al., 2001). This LRF-amide binding motif is a common theme shared by the neuropeptide/neuropeptide receptor family. Without the LRF motif, KP54 is unable to bind to KISS1R (unpublished data). It was found that MMP9 can bind and cleave KISS1 between and Gly^{118} and Leu^{119} (Takino et al., 2003). This cleavage event prevents KP54 from binding KISS1R. Interestingly, it was also observed in this study that MMP2 and MMP9 can form a stable binding interaction with KISS1, seemingly contingent on KISS1's Cys^{53} and cysteine disulfide binding. This binding event was stable and seemed to only require association with the N-terminal domain of KISS1 (Takino et al., 2003). This study demonstrated an *in vivo* negative feedback to mitigate KISS1:KISS1R signaling.

KISS1R

KISS1R is a $G_{q/11}$-protein coupled receptor heavily distributed through the central nervous system and reproductive axis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). The activation of KISS1R leads to canonical $G_{q/11}$ downstream signaling. KISS1R binding induces activation of phospholipase C (PLC), the primary effector of this signaling cascade. Activated PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2), releasing second messengers inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG). Released IP3 binds receptors on the endoplasmic reticulum and causes release of intracellular calcium. Diacylglycerol then activates protein kinase C, which activates MAPK signaling through Ras and Raf phosphorylation (Ringel et al., 2002). This signaling results in ERK phosphorylation and nuclear translocation, leading to a modified transcriptional profile.

In addition to the normal $G_{q/11}$ signaling, the initial characterization of KISS1R yielded additional characteristics that could help explain how KISS1 and KISS1R suppress metastasis. Ohtaki observed the formation of stress fibers and phosphorylation of FAK, both of which are signs of actin cytoskeletal reorganization (Ohtaki et al., 2001). Consistent with this theme, inhibition of migration and invasion was observed in a dose-dependent response to KP54 stimulation (Ohtaki et al., 2001). In B16 melanoma cells transfected with KISS1R or blank, administration of KP54 via osmotic pump suppressed metastasis from the murine footpad only in samples expressing KISS1R (Ohtaki et al., 2001). These observations led to the initial conclusion that KISS1R was the signaling mechanism through which KISS1 suppressed metastasis.

When examining the tissue distribution of both KISS1R and KISS1, there was overlap in the distribution of KISS1 and KISS1R expression in the neuroendocrine system. Both KISS1 and KISS1R are expressed in the brain, pituitary, spinal cord, heart, pancreas, placenta, lymphocytes,

spleen, breast, and testes (Kotani et al., 2001; Mead et al., 2007; Muir et al., 2001; Ohtaki et al., 2001). The highest expression of both KISS1 and KISS1R was found in placenta, suggesting an important interaction in both the neuroendocrine system as well as the fetal-maternal interface of the placenta. Additionally, this distribution throughout the reproductive system also hinted at a physiological function for KISS1: KISS1R signaling outside of metastasis suppression.

KISS1 and KISS1R as key modulators of puberty and fertility

While the roles of KISS1 and KISS1R lacked immediate clarity in the field of metastasis suppression, the endogenous functions of KISS1 and KISS1R were easier to decipher. The normal function of KISS1R was identified through two studies of familial idiopathic hypogonadotropic hypogonadism. These studies independently identified deletions in KISS1R which truncated the receptor and rendered it unable to signal properly (de Roux et al., 2003; Seminara et al., 2003). These studies found that KISS1 and KISS1R were critical for the onset of puberty and maintenance of fertility. Additional studies would find that even minor mutations in KISS1R were the cause for many causes of idiopathic hypogonadotropic hypogonadism, and that the syndrome could be recapitulated in mice with a KISS1R knockout (Seminara et al., 2003). Following this discovery, KISS1 and KISS1R were found to be expressed in the hypothalamus in an estrous-cycle dependent manner (Navarro et al., 2004). Additionally, stimulating the hypothalamus with KP54 stimulated luteinizing hormone (LH) surges, which indicated that KISS1 can directly induce gonadotropin releasing hormone (GnRH) release and signaling through LH and follicle stimulating hormone (FSH) (Messager et al., 2005; Navarro et al., 2004; Navarro et al., 2005). Cumulatively, these studies identified that estrogen negatively regulates KISS1 expression in the hypothalamus, and when estrogen drops during the menstrual/estrous cycle, KISS1 expression increases, stimulating KISS1R on GnRH neurons, which in turn

stimulates release of LH and FSH from the pituitary. This role of KISS1 is critical, and is evolutionarily conserved through many higher and lower evolutionary branches (Biran, Ben-Dor, & Levavi-Sivan, 2008). These observations in the hypothalamus gave rationale to the high expression of KISS1 and KISS1R in the brain and spinal cord.

While KISS1 and KISS1R have a critical role in the hypothalamic pituitary gonadal axis, KISS1 has a dual role in the realm of fertility. Circulating blood levels of KISS1 increase by roughly 900-fold during pregnancy (Horikoshi et al., 2003). Noting that placenta has the highest expression of KISS1, this suggested a role for KISS1 and KISS1R at the maternal-fetal interface of the placenta (Janneau et al., 2002). Bilban et al. showed that KP10 treatment decreased the invasiveness of primary human trophoblasts (Bilban et al., 2004). This study demonstrated that KISS1 and KISS1R were differentially expressed throughout the placenta and cooperatively function to regulate placental invasion. Immunohistochemistry analysis showed that while KISS1 is expressed by syncytiotrophoblasts near the fetal interface of the placenta, KISS1R is expressed by the syncytiotrophoblasts as well as the cytotrophoblasts on the maternal side of the placenta (Bilban et al., 2004). This expression dynamic appears to be predominantly in first term placentas, and can regulate angiogenesis on placental endothelial cells (Matjila, Millar, van der Spuy, & Katz, 2013; Ramaesh et al., 2010). Manipulations, particularly lower expression, of the KISS1/KISS1R maternal/fetal interface have been reported to be linked to pre-eclampsia and repeated loss of pregnancy, again conveying the importance of regulating placental invasion to successful pregnancy (Armstrong et al., 2009; Cartwright & Williams, 2012; Park et al., 2012; Zhang et al., 2011). As a suppressor of placental invasion, KISS1 in a normal physiological function hints at how it suppresses metastasis.

Conflicting roles for KISS1R in metastasis suppression

The initial characterization of metastin and KISS1R activation in metastasis suppression by Kotani, Muir, and Ohtaki did not definitively define the signaling which was responsible for metastasis suppression. The study by Ohtaki et al. found that KISS1R activation suppressed migration and invasion, which they attributed to the formation of stress fibers and phosphorylated FAK (Ohtaki et al., 2001). Additional studies struggled to find a common mechanism of metastasis suppression in additional models. In MDA-MB-435S cells, Becker et al. found that stimulation of KISS1R resulted in cell cycle arrest and increased rates of apoptosis (Becker et al., 2005). This observation was echoed in a study of human pituitary adenomas which overexpress KISS1 and KISS1R, where an increase in apoptotic rate was seen foll3owing treatment with KISS1 (Martinez-Fuentes et al., 2011). These observations appear to be specific to their individual models, however. A study in human fibroblasts suggested that KISS1 expression decreased NFκB nuclear translocation, resulting in decreased expression of MMP9 (Yan, Wang, & Boyd, 2001). Other studies in HUVEC (human umbilical endothelial) cells found that KP10 stimulation decreased expression of VEGF through inhibition of SP-1 binding to its promoter (Cho et al., 2009). A series of studies investigated the interactions with KISS1R and the chemokine receptor CXCR4, the receptor for the chemokine SDF-1 (Navenot, Wang, Chopin, Fujii, & Peiper, 2005). These studies claimed that KP10 treatment to cells co-expressing KISS1R and CXCR4 desensitized them to SDF-1 treatment, resulting in a suppression in invasion and migration (Navenot et al., 2005). The group then claimed that KISS1R stimulation could abrogate CXCR4/SDF-1 signaling through MAPK and Akt to inhibit pro-metastatic signaling (Navenot, Fujii, & Peiper, 2009). Cumulatively, while there is evidence that KISS1R

signaling can result in metastasis suppressing phenotypes, its role in metastasis suppression is not well understood.

The mechanism by which KISS1 and KISS1R suppress metastasis continues to increase in complexity in the case of breast cancer. While KISS1 is a broad-reaching metastasis suppressor in several cancer types, its role in breast cancer is less straightforward. This complexity in breast cancer seems to be related to the presence or absence of ERα. Lymph node positive cases of breast cancer demonstrated higher levels of KISS1 mRNA as compared to lymph node negative tumors from a 2003 study (Martin, Watkins, & Jiang, 2005). A study in ER-positive breast cancer patients found that while ER-positive tumors had less KISS1 expression than ER-negative tumors, and that in vitro re-introduction of ERα into ER-negative cells subsequently decreased KISS1 expression (Marot et al., 2007). The same study found that patients with ER-positive tumors with high KISS1 expression had significantly lower relapse-free survival than ER-positive patients with low KISS1 expression, suggesting that the feedback loop from ERα to suppress KISS1 expression could be disrupted with tumor progression (Marot et al., 2007). While this study was unable to observe statistically significant KISS1R disease data, others followed which suggested that KISS1R may have a role to play in promoting breast cancer metastasis. In a mouse mammary tumor virus–polyoma virus middle T antigen (MMTV-PyMT) model, Cho et al. developed a KISS1R heterozygous sub-strain to investigate the impact that KISS1R heterozygosity had on tumor progression and metastasis in this highly aggressive model of breast cancer (Cho et al., 2011). This study found that by depleting either KISS1 or KISS1R expression in breast epithelium delayed tumor latency, tumorigenesis, and metastasis (Cho et al., 2011). This study was pivotal in demonstrating the immediate impact that KISS1 and KISS1R signaling has on breast hyperplasia and tumor progression. A possible mechanism by which

KISS1/KISS1R promote tumorigenicity and metastasis was addressed in a report by Zajac et al., which found that KISS1R could trans-activate EGFR, a receptor tyrosine kinase with a notorious role as a tumor-promoting receptor (Zajac et al., 2011). This study directly identified the ability of KISS1R to bind and activate EGFR, but the effect appears to be dependent on a lack of ER expression (Cvetkovic et al., 2013). This observation could explain the increased invasiveness, migration, and metastasis observed in many KISS1/KISS1R positive breast cancers. Overall, there is a slowly growing body of work implying that KISS1 and KISS1R are heavily regulated by ERα and that their role in breast cancer is not a one of simple metastasis suppression.

Interestingly, the conflicted role of KISS1 and KISS1R in metastasis suppression goes deeper than model-specific signaling differences. A series of studies have demonstrated that KISS1 can suppress metastasis in cells which lack co-expression of KISS1R (Nash et al., 2007). Indeed, the initial studies characterizing KISS1 as a metastasis suppressor in human C8161.9 melanoma cells were done in lieu of KISS1R expression (Lee et al., 1996; Nash et al., 2007). The observation that of NFκB activation and MMP-9 expression was mediated without exogenous KISS1R expression or detection in HT-1080 fibroblast cells (Yan et al., 2001). Later, Liu et al. discovered that KISS1 expression resulted in a metabolic shift from a glycolytic, Warburg metabolism, towards a reliance on oxidative phosphorylation (W. Liu et al., 2014). The connection between aggressive tumors and Warburg metabolism has been inferred through the field of hypoxia (Denko et al., 2003). Hypoxia signaling can promote the transcription of glycolytic genes such as glucose transporters 1 and 3, phosphoglycerate kinase, 6-phosphofructo-2 kinase, and lactate dehydrogenases A and B (Denko et al., 2003; Ebert, Firth, & Ratcliffe, 1995; O'Rourke, Pugh, Bartlett, & Ratcliffe, 1996; Salceda, Beck, & Caro, 1996; Semenza et al.,

1994). Since the cores of even small tumors can quickly become hypoxic, it is not surprising that hypoxia plays such a driving force in metabolism and tumor progression.

Warburg metabolism was first described by Otto Warburg as the preference of tumor cells to utilize glucose as a primary metabolic substrate (Warburg, Wind, & Negelein, 1927). The preferential utilization of glucose and glycolysis by tumors has since been referred to as Warburg metabolism. By relying heavily on glycolysis, glycolytic intermediates can be used towards generation of nucleosides and amino acids (Potter, 1958; Vander Heiden, Cantley, & Thompson, 2009). In fact, the reliance of cancer cells on the Warburg effect is so widespread that it is now considered one of the hallmarks of cancer (Hanahan & Weinberg, 2011). This form of energy metabolism is not unheard of in normal tissues. Normal tissues can quickly stabilize HIF1a to primarily utilize glycolysis as an energy producing avenue in times of ischemia or acute hypoxia. Activated T-cells switch from beta-oxidation of fatty acids to a glycolytic metabolism during activation and outgrowth to enrich for biosynthetic glycolytic by-products (Gerriets & Rathmell, 2012). The transition to a glycolytic metabolism provides cancer cells with the molecular components for unchecked division.

The observation that KISS1 expression seemed to reverse the Warburg effect in cancer cells provided a possible explanation for how KISS1 suppresses metastasis independent of KISS1R signaling. In addition to suppressing glycolytic metabolism and increasing oxygen consumption, KISS1 expression was also noted to increase mitochondrial mass and function (W. Liu et al., 2014). This observation was traced backwards to the stabilization of a transcription factor, Pgc1α, in the presence of KISS1. Peroxisome proliferator-activated receptor gamma coactivator (Pgc1α) is a transcription co-activator which can bind with transcriptional co-activators to promote transcription of metabolism-related genes. While the two proteins did not physically

associate, KISS1 expression appeared to stabilize Pgc1α protein and allowed it to promote a transcriptional program associated with oxidative phosphorylation. KISS1's impact on cellular metabolism suggests that KISS1 may interfere with cellular energy sensing mechanisms, a phenotype unrelated to KISS1R signaling.

More recent observations of KISS1 suppressing metastasis in lieu of KISS1R expression give rise to several questions. Namely, if KISS1R co-expression is not required for metastasis suppression, then how does KISS1 suppress metastasis? As a secreted peptide, KISS1 has the potential to act in an autocrine, juxtacrine, or paracrine manner. In the hypothalamus and the pituitary, KISS1 is secreted from KISS1 neurons to stimulate KISS1R on GnRH neurons (Messager et al., 2005). This extracellular activity in the hypothalamus and pituitary could easily be recapitulated elsewhere in the body. Low levels of KISS1R mRNA have been detected in several metastatic sites (e.g. lung, liver, brain), but no studies have been done to determine whether KISS1R expression in the stroma is sufficient for KISS1 to suppress metastasis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). While KISS1 and KISS1R co-expression can result in inhibition of metastatic traits in vitro (such as suppression of proliferation, migration, and invasion), these phenotypes have also been demonstrated without KISS1R co-expression (Lee & Welch, 1997b; Shoji et al., 2009). Additionally, the metabolic phenotype induced by KISS1 expression was observed without co-expression of KISS1R (W. Liu et al., 2014). This gap in knowledge, presents the hypothesis that KISS1 can suppress metastasis through a non-KISS1R pathway.

Within the neuropeptide family, there is an established precedent for ligands signaling through alternate receptors (Lyubimov et al., 2010; Oishi et al., 2011). Neuropeptide receptors share an affinity for ligands with a C-terminal RF-amide motif (Walker, 1992). Within this family, there

exists variation in the specific receptors and ligands, but this preference for RF-amide as a binding site suggests a degree of promiscuity exists amongst ligands and receptors. This is true in the case of KISS1 and NPFF (neuropeptide FF), an 8-amino acid neuropeptide signaling molecule sharing a high degree of structural similarity with KP10 (Milton, 2012). KISS1, more specifically KP10, can activate NPFFR1 and 2 (neuropeptide FF receptors) with a high degree of affinity (Lyubimov et al., 2010; Oishi et al., 2011). This cross-reactivity between kisspeptins and neuropeptide receptors demonstrates the possibility that once outside of the cell, KISS1 may have a more complicated role to play in metastasis suppression than simply signaling through KISS1R.

While KISS1's ability to bind to neuropeptide receptors 1 and 2 has been described in the literature, most studies focus exclusively on the KP54 or KP10 domain of KISS1. Indeed, once it was discovered that KP54 was a receptor ligand in 2001, few groups have studied KISS1 as a metastasis suppressor without co-expression of KISS1R in their model system. In addition to this, the remaining peptide fragments of KISS1 have never been thoroughly investigated after characterization of KP54. Additionally, KISS1 processing outside of the cell releases not only KP54, but also all other kisspeptin fragments achieved by furin cleavage (Harihar et al., 2014). The extracellular nature of KISS1 processing presents the possibility that non-KP54 kisspeptins could bind and activate alternate receptors. By neglecting to study the rest of the protein, the field neglects the possibility that the metastasis suppressing capabilities of KISS1 could be resultant of more than KP54 and KISS1R signaling.

We hypothesize that non-K54 kisspeptins will possess anti-metastatic activity. In this study, we examined each theoretical cleavage product of KISS1 to systematically define the metastasis suppressing characteristics of each kisspeptin. Our results provide evidence that KP54 is not the

only domain of KISS1 necessary for metastasis suppression. These findings bring new direction to the field of KISS1 metastasis suppression and support the further research of non-KP54 kisspeptins Taken together, these data support the further study of the N-terminal domain of KISS1 as a potent suppressor of metastasis.

Chapter 2: Metastasis suppression by KP54 and nonKP54 kisspeptins

Introduction

Metastasis suppressors are a family of protein and RNA which, when expressed by a tumor, allow for primary tumor growth but prevent successful metastasis by inhibiting one or more stages of the metastatic cascade (Steeg et al., 2003). One member of this family, KISS1, is a secreted protein which inhibits metastasis at the colonization stage of metastasis (Lee et al., 1996; Lee & Welch, 1997b). KISS1 is a broadly functional metastasis suppressor which suppresses metastasis in several tumor types, including breast, melanoma, ovarian, colorectal, bladder, esophageal, thyroid, gastric, pancreatic, and endometrial cancers (Beck & Welch, 2010). When KISS1 is secreted outside of the cell it is cleaved by furin at three R/K-XX-R/K dibasic motifs to yield "kisspeptins," or smaller KISS1 peptides (Harihar et al., 2014). KISS1 can only suppress metastasis if it is secreted outside of the cell, providing evidence that it may have potential as a therapeutic peptide to maintain disseminated metastases at a dormant stage (Nash et al., 2007; Nash & Welch, 2006).

One kisspeptin derived from an internal domain of KISS1 is KP54 (formerly "metastin"), a 54-amino acid peptide which binds and activates KISS1R (AKA GPR54, AXOR12, and hO7T175). KISS1R is a $G_{q/11}$-protein coupled receptor expressed primarily in the hypothalamic-pituitary-gonadal axis (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). KISS1R activation induces the formation of focal adhesions, suppression of motility, cell cycle arrest, intracellular calcium mobilization, and suppression of MMP-9 expression (Becker et al., 2005; Cho et al., 2009; Ohtaki et al., 2001; Yan et al., 2001). Receptor binding is widely considered to be the mechanism of action for KISS1 metastasis suppression (J. F. Harms, Welch, & Miele, 2003; Ohtaki et al., 2001). However, recent studies have found that KISS1 can suppress metastasis and metastatic traits in KISS1R-deficient cells (W. Liu et al., 2014; Nash et al., 2007;

Wang, Qiao, Wang, & Zhou, 2016). Indeed, a recent study indicated that KISS1 expression can reverse the Warburg Effect by means of PGC1α stabilization in cells lacking KISS1R expression (W. Liu et al., 2014). This metabolic phenotype was not achieved when cells were treated with KP10 exogenously, suggesting that the mechanism by which KISS1 alters tumor cell metabolism and metastatic potential is independent of KISS1R interactions. Thus, an alternate hypothesis exists: the extracellular cleavage of KISS1 liberates a non-KP54 kisspeptin responsible for metastasis suppression independent of KISS1R, potentially through an alternative receptor. To test this hypothesis, however, all possible kisspeptin cleavage products must be examined to identify the region(s) of metastasis suppression.

To examine the metastasis suppressing capacities of kisspeptins resultant of furin processing, all ten theoretical kisspeptins were generated based on known dibasic cleavage sites. These constructs are referred to as the "KISS1 Manufactured Peptides," or KMPs. These KMPs were then cloned into lentiviral vectors and used to generate stable cell lines in B16-F10 murine melanoma model. Using the KMPs, we evaluated the metastasis suppression capabilities of each individual kisspeptin. Using this system as a tool, we have identified non-KP54 kisspeptins with the ability to suppress metastasis. Specifically, the N-terminal domain of KISS1 (M^{1}–K^{57} or KMP2), suppresses metastasis independent of the rest of the protein. These findings indicate that the N-terminal domain of KISS1 has anti-metastatic bioactivity.

Methods and materials

Cell lines and culture

B16F10 is a melanotic melanoma cell line derived from a C57BL/6J mouse. These cells were cultured and injected into mice ten times to derive their name and enhanced metastatic characteristics (Fidler, 1973). Cell lines were cultured in a 1:1 ratio of Dulbecco's-modified

minimum Eagle Media and Ham's F12 media with 5% fetal bovine serum, 1% l-glutamine and 0.025% non-essential amino acids. Lentiviral clones were made using Life Technologies Gateway platform and then introduced into the B16F10 cell line. The transduced cells were cultured in the presence of 4 μG/mL blasticidin (pLenti6-V5-DEST, Invitrogen) or 2.5 μG/mL puromycin (pLenti PGK Puro DEST plasmid Addgene #w529-2). A parental B16-F10 control cell line was cultured in the same conditions without selection.

Lentiviral constructs and lentiviral cell line generation

KMP constructs were designed using a Kozak sequence to initiate translation and flanked by BamH1 (5') and Xho1 (3') restriction sites. These constructs were ligated into pENTR1A dual selection vector (A10462) to allow for recombination into lentiviral vectors. Lentiviral vectors used in this study were pLenti6-V5-DEST (V49610, Invitrogen) and pLenti PGK Puro DEST (Addgene #w529-2). After recombination into lentiviral vectors, plasmids were sequenced and used to make virus in 293FT cells (ThermoFisher Scientific, R70007). Briefly, 3uG of lentiviral expression vector was co-transfected with 9uG viral packaging plasmid mix via Lipofectamine 2000. 293FT cells were transfected and incubated overnight. Media was changed to fresh, complete media and incubated for an additional 24 hours. Viral supernatant was aspirated, centrifuged, and immediately used to transduce B16-F10 cells to determine viral titer. Selection media was applied 24 hours later and cells were maintained under selection until control cells with no virus had completely died. Using viral titer, B16-F10 cells were transduced, selected, and colonies were isolated. Expression was tested using RT-QPCR or western blot.

Experimental metastasis assays and animal studies

Cells were disassociated and suspended in ice-cold Hank's Buffered Saline Solution. For injection, 200 μL of cell suspension (1.0×10^4 or 5.0×10^4 cells) was injected into the lateral tail vein of 4-week-old female syngeneic C57BL/6J mice (10 per sub-clone, three sub-clones per KMP; C57BL/6J Jackson Laboratories). Mice were housed at 23ºC and given food and water ad libitum for two weeks. Upon euthanasia, gross lung metastases were quantified by light microscopy under a dissection microscope.

Statistics

Statistical tests were conducted via One Way ANOVA followed by pair-wise post-tests (Shapiro-Wilk normality test, Dunn's Method pairwise multiple pairwise comparison procedures) for experimental groups containing more than two treatment groups.

Immunofluorescence and phalloidin staining

To perform actin staining, cells were plated at $5x10^3$ cells per well and left to incubate overnight. Once cells had reached roughly 50% confluence, wells were washed twice with PBS then fixed with 3.7% paraformaldehyde for 10 minutes. The wells were washed twice more, then cells were extracted with 0.1% PBS-Triton 100 for 5 minutes. Wells were washed twice more, then stained with diluted phalloidin stain in 200 μL PBS for 20 minutes. Following this, wells were washed twice more before mounting coverslips using VectaShield with DAPI. Slides were left at 4ºC in the dark to dry before fluorescent imaging.

Anoikis resistance and clonogenicity assays

Cells were plated at $2.5x10^5$ cells per dish in 60mm low attachment dishes. Cells were cultured under normal conditions for 4 days before scraping and triturating cells for counting using a hemacytometer. Trypan blue exclusion was used to assess viability following culture in

low attachment plates. Cells were subsequently plated at $1x10^3$ live cells in triplicate in a 6-well dish. Cells were left to form colonies for 4 days. Colonies were fixed in 0.01% crystal violet and colonies were counted using ImageJ

Metabolic studies and flow cytometry

To measure mitochondrial function, B16F10 cells (2×10^4) were seeded onto a Seahorse Bioanalyzer XF plate and O2 consumption was measured sequentially following addition of electron transport chain inhibitors oligomycin (inhibitor of complex IV, ATP synthase), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; mitochondrial uncoupler), rotenone (complex I inhibitor), and antimycin A (complex III inhibitor). For flow cytometry analysis, cells were harvested, counted, and suspended to a total concentration of $1x10^6$ cells per mL in warm PBS. Cells were permeablized for 15 minutes at 37ºC and then stained with 200nM Mitotracker Red CMXRos or Mitotracker Green FM for 30 minutes in the dark at room temperature. Cells were then analyzed with flow cytometry for median fluorescence intensity.

Wound healing assay

Cells were grown to confluence in a 6-well dish. Using a 200 µl pipette tip, a scratch was introduced into the field in triplicate. The wells were washed twice with PBS to remove debris and were then incubated in serum-free media until imaging at 18 hours to assess migration. Migration was quantified using ImageJ by three measurements averaged over the distance of the scratch.

Melanosphere formation

To assess the impact of kisspeptin expression on stemness and spheroid formation, a melanosphere formation assay was performed. In a protocol adapted from (Le Coz et al., 2016),

$1x10^3$ cells were plated in each well of a 24 well ultra-low attachment plate (Corning) in serum-free DMEM F:12 containing 1X B27 (ThermoFisher Scientific), 20ng/ml murine EGF (Peprotech), and 20 ng/ml murine bFGF (Peprotech). Cells were left to incubate for 10 days before counting and imaging using the Celigo Imaging System.

RT-QPCR

RNA was harvested from cells with Zymogen's QuickRNA Prep Kit and was subsequently reverse transcribed into cDNA using iScript cDNA kit (BioRad). Expression was then tested using SYBR Green PCR reagent and primers specific to each KMP (table). Results were analyzed using comparative Ct values and delta Ct values. 18S was used as an internal control. Three KMP clones with the highest expression were chosen for *in vivo* experimentation.

FLAG ELISA

FLAG ELISA was performed as a method to validate the presence of FLAG-tagged KMP constructs. To do this, the DYKDDDDK (flag) tag ELISA Kit (Advanced Bioscience Reagents) was used per the manufacturers protocol. Briefly, 250 µg whole cell lysates were applied to a pre-warmed ELISA plate and incubated at 37C for 2 hours. Three washes were performed, followed by incubation with a detection antibody for 1 hour at room temperature. The plate was washed and TMB solution was applied, followed by stop solution. The plate was read using a plate reader and absorbance at 450nm was recorded. Values were normalized to a standard curve.

Results

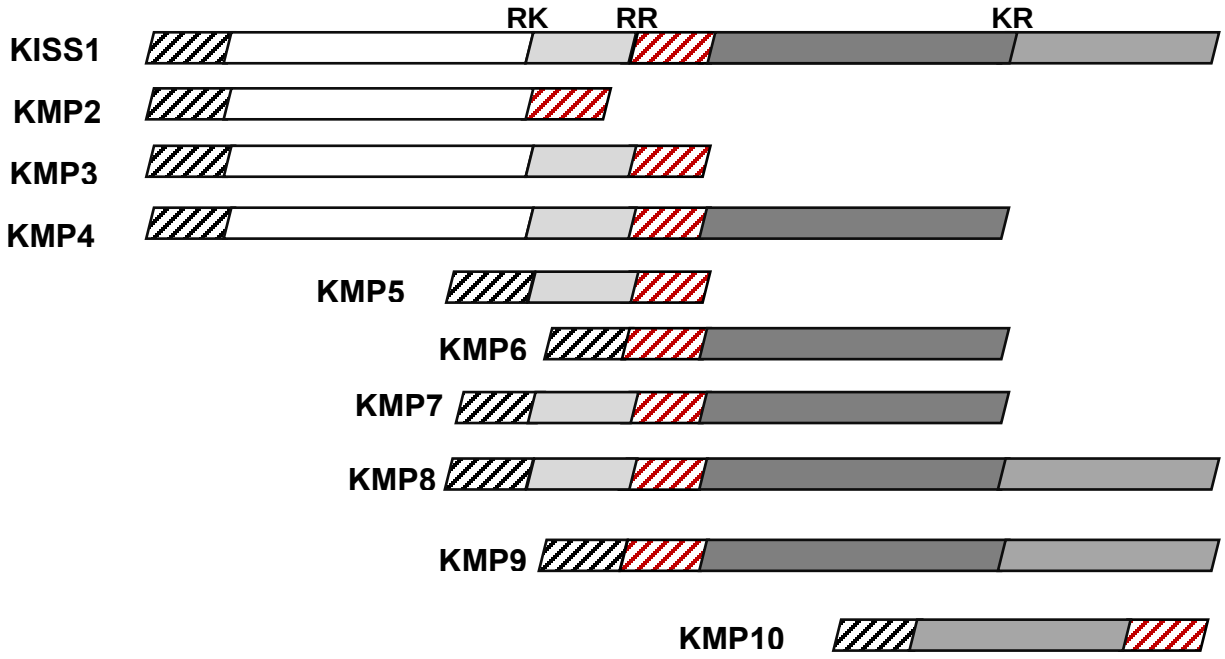
Kisspeptin design and detection

To test the metastasis suppressor capabilities of different KISS1 domains, all ten theoretical kisspeptins were designed and cloned into the Invitrogen Gateway system. As antibodies to

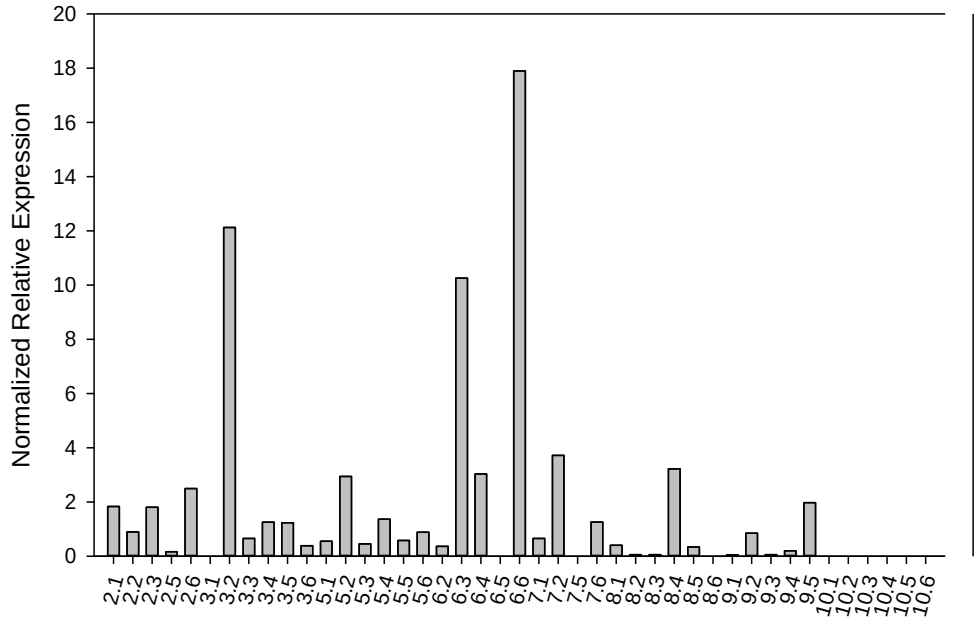
KISS1 recognize only the KP54 region of KISS1, a FLAG tag was introduced to each of these constructs (depicted with gray diagonal lines). Kisspeptins lacking the N-terminal domain of KISS1 were cloned to contain KISS1's 19-amino acid secretion sequence (black diagonal lines). These kisspeptins recapitulate the results of normal KISS1 processing by furin without confounding results from other KISS1 fragments in the extracellular environment. B16-F10 murine melanoma cells were transduced using lentivirus and tested for expression by RT-QPCR

Figure 1

A


RK
RR
KR
KISS1
KMP2
KMP3
KMP4
KMP5
KMP6
KMP7
KMP8
KMP9
KMP10

B


Normalized Relative Expression
0
2
4
6
8
10
12
14
16
18
20
2.1
2.2
2.3
2.5
2.6
3.1
3.2
3.3
3.4
3.5
3.6
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10.6

Figure 1: A, Diagram of kisspeptin construction and design. Black diagonal boxes denote KISS1's 19-amino acid secretion sequence. Red diagonal lines depict FLAG tag (DYKDDDDK). **B,** RT-QPCR analysis of KMP expression.

analysis or western blot (not pictured) (Figure 1B). Three clones with the highest expression were selected for experimental use in subsequent studies.

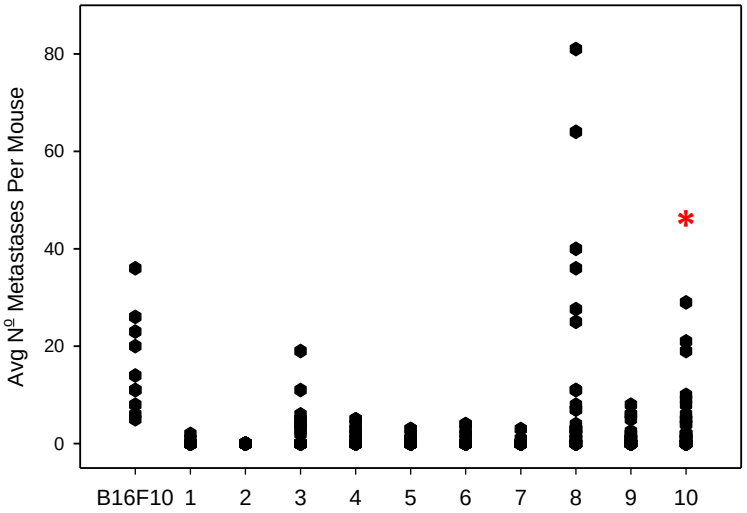
Kisspeptins differentially suppress metastasis *in vivo*

To test the ability of kisspeptins to suppress metastasis, an experimental metastasis assay was performed in syngeneic C57Bl6/J mice. The experimental metastasis assay evaluates metastatic efficacy by recapitulating the latter half of the metastatic cascade. As KISS1 functions at the final step, colonization, of metastasis, this experiment allows for evaluation of KISS1 and kisspeptin impact on metastasis suppression. Metastatic load in the lungs was quantified following injection of three cell line clones per kisspeptin (n=10 mice per clone, n=30 mice per kisspeptin). While full-length KISS1 suppresses metastasis and parental B16F10 cells did not, kisspeptins displayed variable extents of metastasis suppression (Figure 2). Interestingly, KMP6 (KP54) suppressed metastasis despite a lack of KISS1R expression in B16-F10 cells. This observation suggests that KP54 may be able to suppress metastasis by activating stromal KISS1R.

Kisspeptins which included the N-terminal domain of KISS1 and/or KP54 tended to suppress metastasis (KMP2, KMP3, KMP4, $p<0.05$ One way ANOVA). Kisspeptins which contain the C-terminal domain of KISS1 tended to not suppress metastasis (KMP8, KMP10). Overall, these results suggest that both the KP54 domain of KISS1 as well as the N-terminal domain of KISS1 (KMP2) have metastasis suppressor capabilities. The metastasis suppressor capabilities of KP54 appear to be negated by the presence of the C-terminal domain of KISS1 (KMP10). To validate the results for clones which demonstrated intra-clonal variation, an experimental metastasis assay was repeated with increased cell numbers for injection ($5x10^4$

Figure 2

A


Avg Nº Metastases Per Mouse
0
20
40
60
80
*
B16F10
1
2
3
4
5
6
7
8
9
10

B

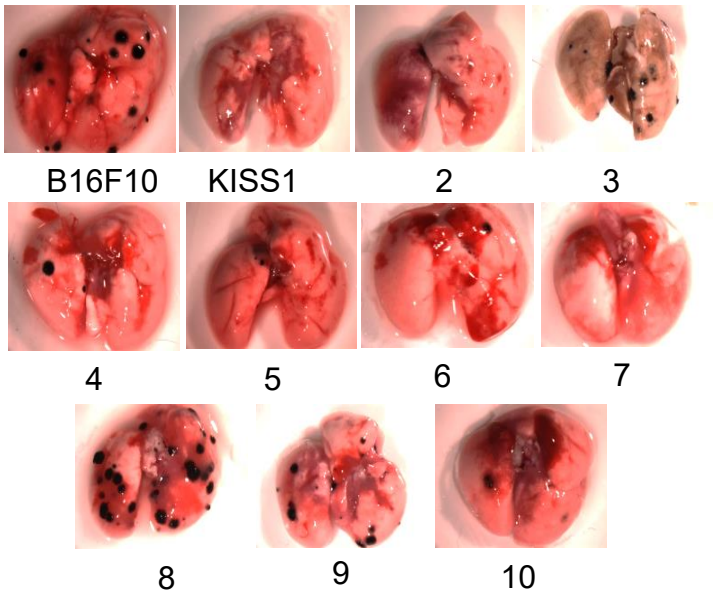

B16F10
KISS1
2
3
4
5
6
7
8
9
10

Figure 2: A, Bar graph representation of lung metastases following experimental metastasis assay. Each KMP cell line is the average number of metastases of three KMP sub-clones (n=30). Red asterisks denote KMP which are statistically similar to B16-F10 parental control by one way ANOVA analysis ($p<0.05$). **B,** Representative lung photos from the experimental metastasis assay in **2A**.

Figure 3

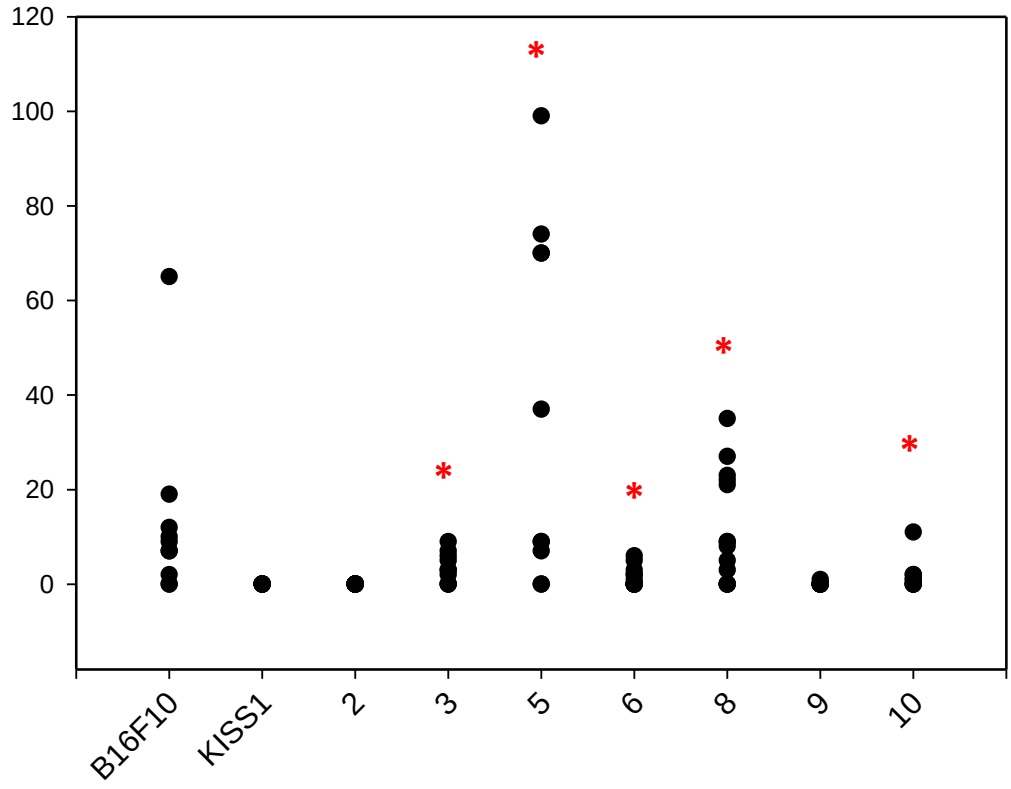


Figure 3: Bar graph representation of a repeated experimental metastasis assay of KMP expressing cell lines with increased cell density ($1x10^4 \rightarrow 5x10^4$). Red asterisks denote groups which are statistically similar to B16-F10 group ($P>0.05$, One way ANOVA followed by Dunn's Method pairwise comparison).

cells). At a 5X increase in cell number, KMP2 continued to suppress metastasis (Figure 3). Other kisspeptins demonstrated variable metastasis suppressor capabilities at an increased cell density.

At an increased cell density, KMP5 no longer suppressed metastasis. KMP8 continued to not suppress metastasis compared to parental controls. KMP10 suppressed metastasis at a higher injection density. While some kisspeptins demonstrated variable metastasis suppression abilities, KMP2 continued to completely suppress metastasis in every clone (n=15 per KMP cell line).

These data demonstrate that not all kisspeptins suppress metastasis equally. KP54 appears to be sufficient for metastasis suppression despite a lack of KISS1R co-expression. Interestingly, the N-terminal domain of KISS1, KMP2, also can suppress metastasis in an apparently KISS1R independent manner. For the remainder of these studies, the metastasis suppressor phenotype of KMP2 has been investigated.

Proliferation is not depressed by kisspeptins

One hypothesis to explain differential metastasis suppression by kisspeptins is suppression of proliferation. To test the impact of kisspeptin expression on proliferation, a proliferation assay was performed in three sub-clones of each kisspeptin (Figure 4). Clonal variation in growth was observed within each set of sub-clones. However, after five days of growth, no significant changes in proliferation were assessed between suppressor and non-suppressor kisspeptins. This leaves the conclusion that a modulation of proliferation is not likely the mechanism by which suppressor kisspeptins or KMP2 suppress metastasis.

Figure 4

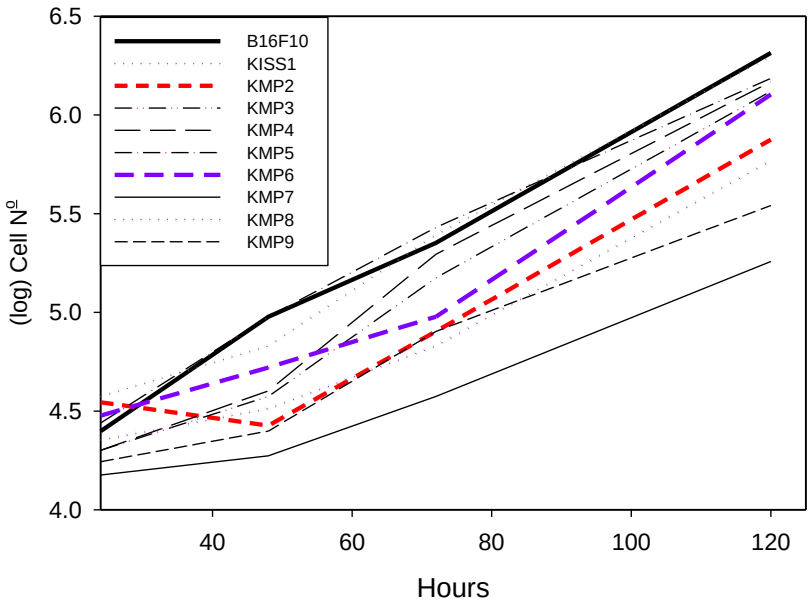

6.5
6.0
5.5
5.0
4.5
4.0
(log) Cell N°
40
60
80
100
120
Hours
B16F10
KISS1
KMP2
KMP3
KMP4
KMP5
KMP6
KMP7
KMP8
KMP9

Figure 4: Log-transformed growth curve of KMP expressing cell lines to examine growth rates of kisspeptin-expressing cells. While variation exists in growth rates between KMP expressing cell lines, suppressor kisspeptins do not uniformly suppress proliferative rates.

Cell cycle distribution and is not impacted by KMP expression

The expression of suppressor kisspeptins does not lead to a uniform suppression of proliferation. However, one mechanism of metastatic dormancy is cell cycle arrest at a secondary tissue. To evaluate cell cycle distribution with expression of kisspeptins, flow cytometry with propidium iodide was performed on parental B16-F10, KISS1-, KMP2, KMP6/KP54, and KMP8 expressing cell lines. These kisspeptins were selected to analyze effects of suppressor and non-suppressor kisspeptins on cell cycle distribution in comparison to KISS1 and parental controls. The expression of KISS1 did not induce any significant changes in cell cycle distribution as compared to parental control (Figure 5A). Cells expressing suppressor kisspeptin KMP2 contain a lower population of cells in both G0/G1 and S phases, yet a comparable population of G2/M phase cells (Figure 5B). KMP6 expression led to a slight enrichment in G2/M phase (Figure 5C). Non-suppressor KMP8 exhibited a larger population of cells in G0/G1 and G2/M phases than parental controls, suggesting an increased tendency towards proliferation with the expression of this kisspeptin (Figure 5D). These data suggest cumulatively that cells which express non-suppressor kisspeptins may have a slight tendency towards active cellular division compared to cells expressing suppressor kisspeptins.

Suppressor kisspeptins inhibit migration *in vitro*

A classic characteristic of metastatic cells is their ability to migrate and invade into surrounding tissues. In a screen of all kisspeptin-expressing lines, a wound healing assay was conducted to observe the impact of kisspeptin expression on *in vitro* migration. After introducing a scratch into a confluent field of cells, migration was evaluated over an 18-hour timeframe. KISS1 expression inhibited migration, as did KMP2, KMP3, KMP4, KMP5, and KMP6 (Figure

Figure 5

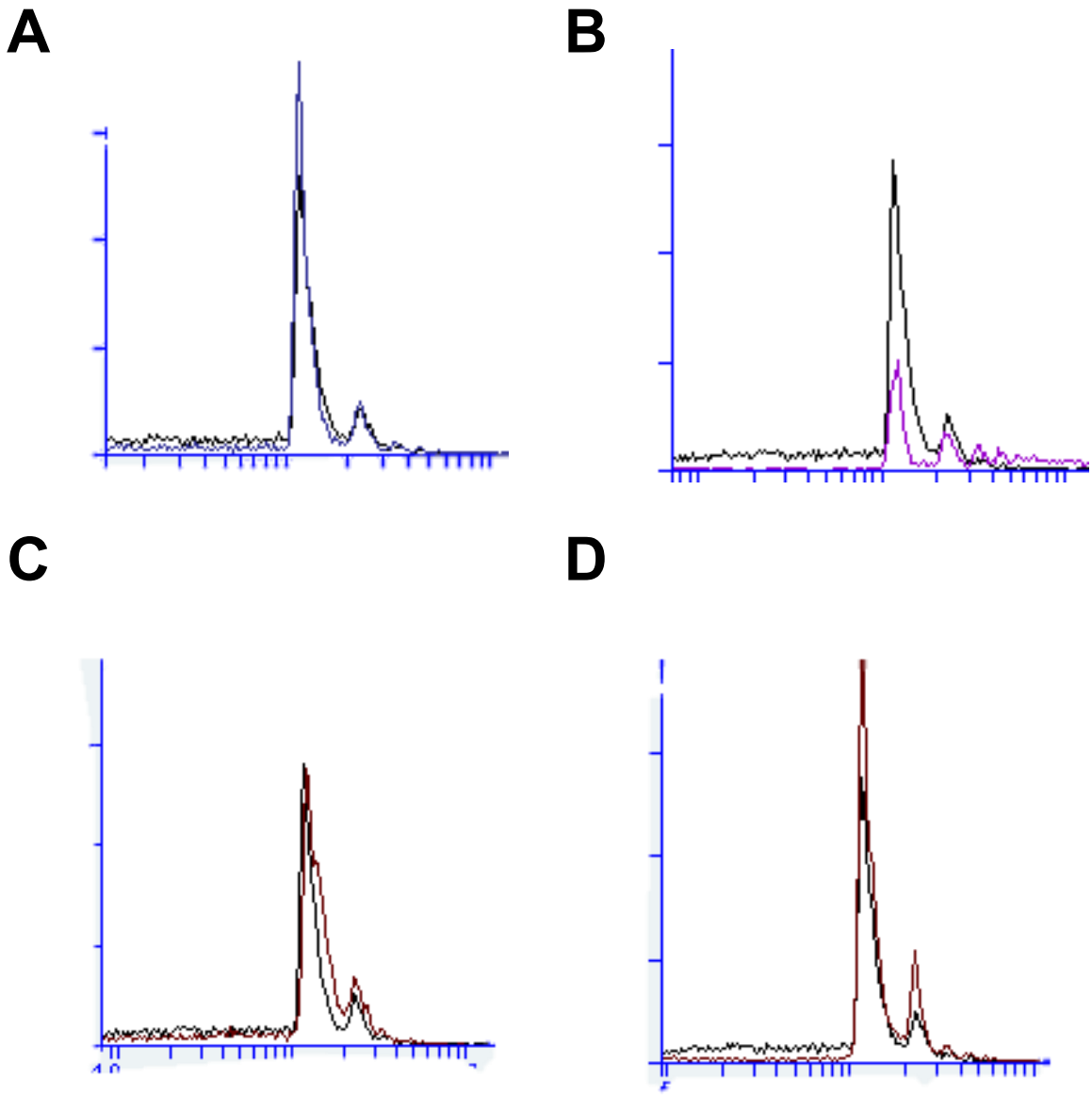


Figure 5: Cell cycle analysis of B16-F10 parental vs. KISS1 or kisspeptin expressing cells through propidium iodide staining. Black lines denote B16-F10 parental controls, colored lines correspond to KISS1 or kisspeptin expressing cell lines. A, B16F10 vs KISS1 B, B16F10 vs KMP2 C, B16F10 vs KMP6 D, B16F10 vs KMP8

Figure 6

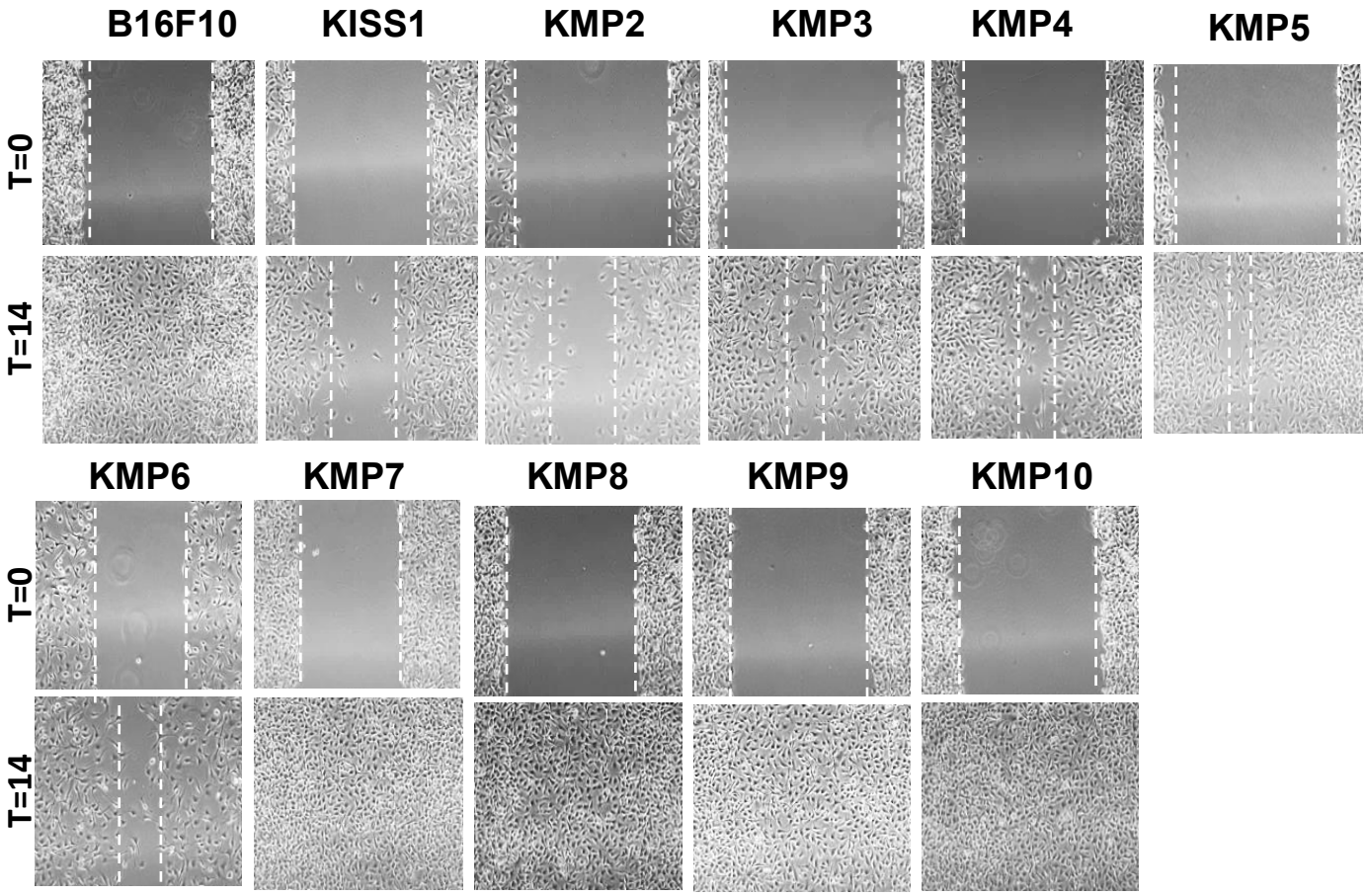

B16F10
KISS1
KMP2
KMP3
KMP4
KMP5
T=0
T=14
KMP6
KMP7
KMP8
KMP9
KMP10
T=0
T=14

Figure 6: Wound healing assay of B16-F10, KISS1, and kisspeptin expressing cell lines. White dotted lines denote migration borders at T=0 hours and T=18 hours. Kisspeptins which suppress metastasis *in vivo* tended to suppress migration *in vitro*.

Figure 7

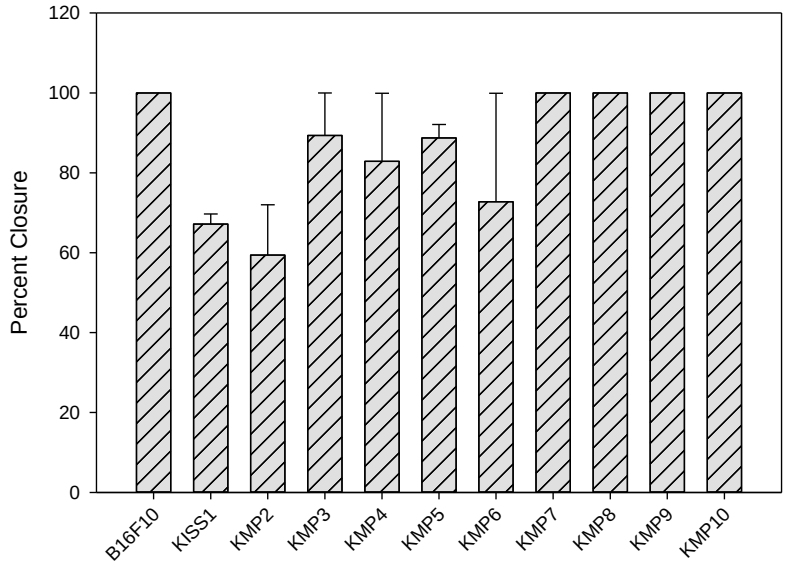

120
100
80
60
40
20
0
Percent Closure
B16F10
KISS1
KMP2
KMP3
KMP4
KMP5
KMP6
KMP7
KMP8
KMP9
KMP10

Figure 7: Quantification of wound closure at T=18 hours of migration. Two clones per kisspeptin-expressing line and three images per clone were evaluated.

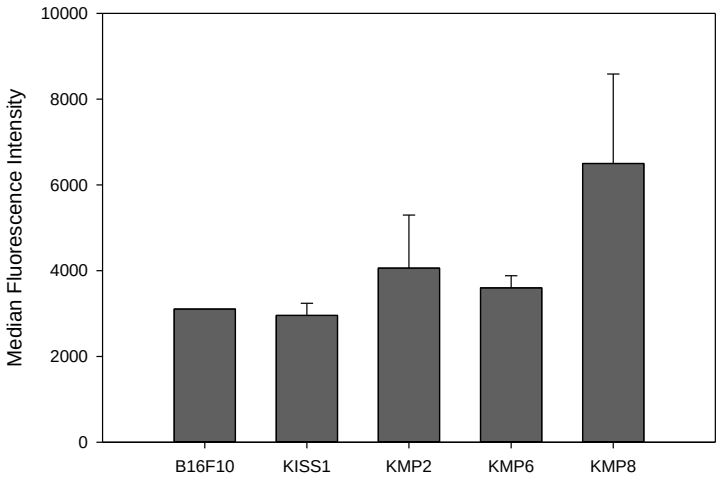
6, Figure 7). Expression of KMP7, KMP8, KMP9, and KMP10 did not suppress migration. The kisspeptin cell lines which suppressed migration tended to include the N-terminal and/or KP54 domain of KISS1. Additionally, the kisspeptins which did not suppress migration (KMP7, 8, 9, and 10) tended to contain the KP54 domain of KISS1 and/or the C-terminal domain of KISS1. The trend of migration suppression tended to correlate with *in vivo* metastasis suppression. Cumulatively, kisspeptins which suppress migration *in vitro* tend to suppress metastasis *in vivo*.

Mitochondrial mass and function is elevated by KMP2, KISS1 expression

Liu et al. reported that the expression of KISS1 elevates mitochondrial mass and oxidative phosphorylation, potentially through stabilization of PGC1α and increased mitochondrial biogenesis (W. Liu et al., 2014). To test whether suppressor kisspeptin expression results in similarly elevated mitochondrial mass, cells were stained with Mitotracker Red FM or Mitotracker Green. Mitotracker Red staining selectively stains for mitochondria with an active mitochondrial membrane potential, while Mitotracker Green stains for total mitochondrial mass. Mitotracker Green staining demonstrated that total mitochondrial load has little variation between parental B16-F10, KISS1, KMP2, and KMP6/KP54 (Figure 8A). Interestingly, KMP8-expressing cells presented an increase in mitochondrial mass (1.9 fold higher than parental control). However, Mitotracker Red staining demonstrated that KISS1 expression elevated functional mitochondrial mass 1.64-fold compared to B16-F10 parental cells (Figure 8B). Expression of KMP2 was sufficient to elevate functional mitochondrial mass 1.87-fold compared to parental controls. Expression of KMP6/KP54 did not increase mitochondrial mass, and indeed it was lower than parental controls (0.49 fold), and KMP8 expression led to slightly increased

Figure 8

A


Median Fluorescence Intensity
10000
8000
6000
4000
2000
0
B16F10
KISS1
KMP2
KMP6
KMP8

B

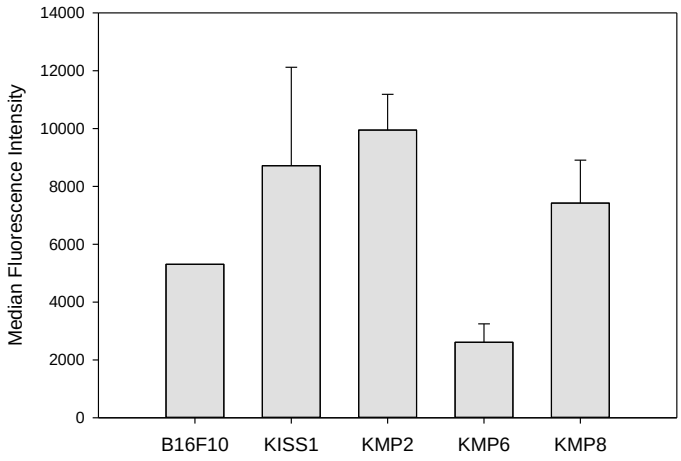

Median Fluorescence Intensity
14000
12000
10000
8000
6000
4000
2000
0
B16F10
KISS1
KMP2
KMP6
KMP8

Figure 8: A, Flow cytometry analysis of B16-F10 and KMP expressing lines with Mitotracker Green staining for total mitochondrial load. Values relative to B16-F10 parental controls. **B,** Flow cytometry analysis of Mitotracker Red staining of B16-F10 and kisspeptin expressing cell lines for mitochondria with active membrane potential. Values relative to B16-F10 parental controls.

Figure 9

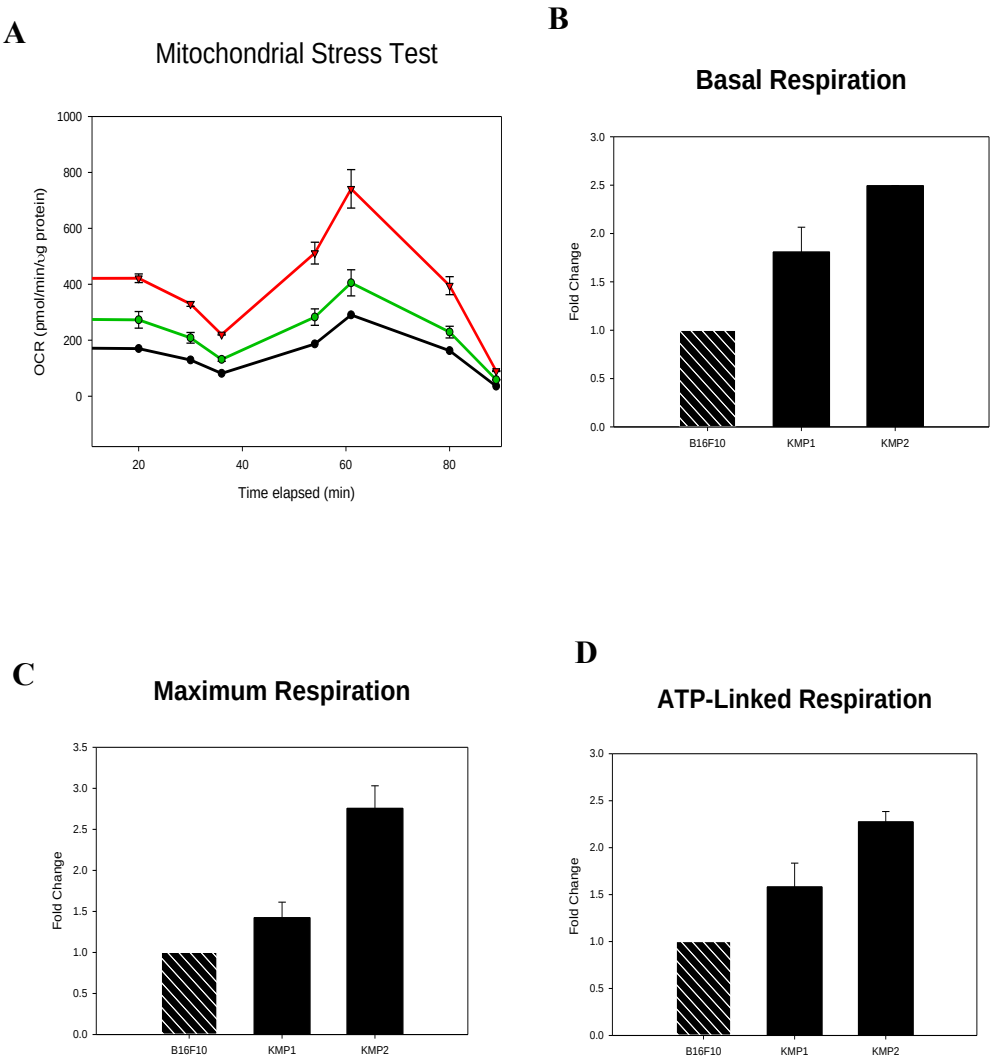

A
Mitochondrial Stress Test
OCR (pmol/min/ug protein)
Time elapsed (min)
B
Basal Respiration
Fold Change
B16F10
KMP1
KMP2
C
Maximum Respiration
Fold Change
B16F10
KMP1
KMP2
D
ATP-Linked Respiration
Fold Change
B16F10
KMP1
KMP2

Figure 9: A, Seahorse Bioanalyzer X7 oxygen consumption rate (OCR) for B16F10 (black), B16-F10 KISS1 (green), B16-F10 KMP2 (red) normalized to protein content. **B**, Basal oxygen consumption calculated from oxygen consumption rate. Expression of KISS1 and KMP2 both increase B16-F10 basal oxygen consumption compared to parental control (diagonal lines). **C**, Maximum respiration for both KISS1- and KMP2-expressing cell lines is elevated compared to parental control. **D**, ATP-linked respiration calculated from OCR is increased in both KISS1- and KMP2- expressing cell lines compared to parental control.

functional mitochondrial biomass (1.39 fold). These data show that the expression of kisspeptins variably alters functional mitochondrial load without largely impacting total mitochondrial mass. Additionally, the increase in functional mitochondrial mass was induced by KMP2, which lacks the KISS1R binding motif. Therefore, this effect does not appear to be resultant of KISS1R stimulation.

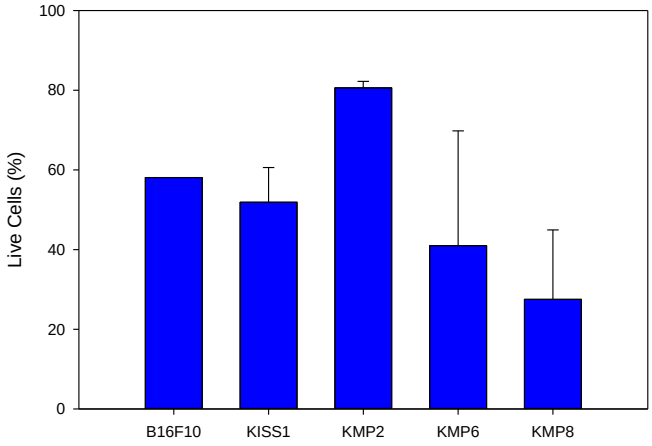
To further investigate the similarities between KISS1 and KMP2's impact on mitochondrial function, oxygen consumption rate was examined using a Seahorse BioAnalyzer MitoStress Test. Overall normalized oxygen consumption levels were elevated in both KISS1 and KMP2 cell lines (Figure 9A). When testing for basal oxygen consumption, both KISS1 and KMP2 elevated basal oxygen consumption levels (Figure 9B). Additionally, KISS1 and KMP2 both elevated ATP-linked respiration compared to parental control (Figure 9C). Maximum respiration was elevated with both KISS1 and KMP2 expression as well (Figure 9D). Cumulatively, these data confirm that the expression of KISS1 elevates functional mitochondrial mass. This phenotype is consistent with KMP2 expression, suggesting that KMP2 could be the kisspeptin which is responsible for the metabolic phenotype observed with KISS1 expression.

Resistance to anoikis and clonogenicity is altered by KMP2 expression

A key metastatic trait is the ability to resist anoikis, or programmed cell death due to lack of adhesion to other cells or a basement membrane (Yawata et al., 1998). To test if suppressor kisspeptin expression induces a sensitivity to anoikis and results in inhibited colonization, cells were cultured under low attachment conditions for 96 hours before assessing viability by trypan blue exclusion. Parental control cells were 59% viable after low attachment culture, and KISS1

Figure 10

A


Live Cells (%)
100
80
60
40
20
0
B16F10
KISS1
KMP2
KMP6
KMP8

B

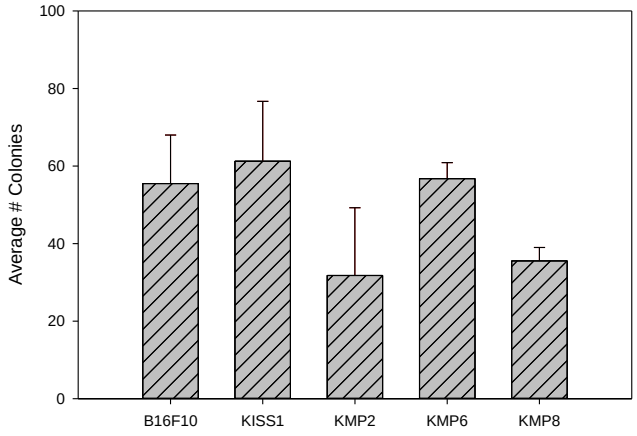

Average # Colonies
100
80
60
40
20
0
B16F10
KISS1
KMP2
KMP6
KMP8

Figure 10: A, Cell viability by trypan blue exclusion was measured after 96 hours under low attachment conditions. Cells expressing KMP2 have a higher viability than those expressing KISS1, KMP6, or KMP8. **B**, $1x10^4$ viable cells were re-plated and allowed to form colonies to evaluate clonogenicity following culture under low attachment conditions. Despite having the highest viability following low attachment, KMP2-expressing cells have the lowest clonogenicity.

expressing cells were at 53%, suggesting no large change following KISS1 expression (Figure 10A). KMP6/KP54 cell viability was depressed to 40%, and non-suppressor KMP8 viability was at 25%. Surprisingly, KMP2 expression elevated cell viability to 82%. These data suggest that suppressor KMP do not result in decreased viability following low attachment conditions.

Following 96 hours in low attachment conditions, $1x10^4$ viable cells were plated in tissue culture plates and a clonogenic assay was conducted for 96 additional hours. B16-F10 parental controls formed roughly 55 colonies on average, and KISS1 expressing cells formed 62 (Figure 10B). KMP6/KP54 expressing cells formed 60 colonies on average, and KMP8 cells formed 38. KMP2 expressing cells formed 30 colonies. There was little change with KISS1 or KMP6/KP54 expression on anoikis resistance or clonogenicity. KMP8, a non-suppressor kisspeptin, demonstrated low viability in low attachment and a consequently low clonogenicity. Expression of KMP2 resulted in higher viability following low attachment conditions, indicating a resistance to anoikis, yet lower clonogenicity.

Actin cytoskeletal organization is not modulated by KMP expression

One of the notable phenotypes induced by KISS1R stimulation is the formation of stress fibers and focal adhesions (Ohtaki et al., 2001). To examine the impact of kisspeptin expression on actin cytoskeletal dynamics, phalloidin staining was performed. No changes in dendritic projections, cellular morphology, or stress fiber formation was observed between kisspeptin expressing cell lines and KISS1 (Figure 11). The expression of KISS1R in B16-F10 parental cells is undetectably low. Together, these data suggest that KMP2 expression has no discernable impact on cytoskeletal organization.

KMP2-3XFLAG protein is detectable by ELISA and suppresses metastasis

The metastasis suppressor characteristics demonstrated by KMP2 have been characterized using a construct tagged with a single FLAG tag (DYKDDDDK). However, the small molecular weight of KMP2 (estimated 7.02 kDa) results in a challenge to identify by western blot. To verify protein abundance of KMP2, a 3X-FLAG tag was introduced to the C-terminal domain of KMP2 to replace the single FLAG tag. This construct was used to generate new lentiviral cell lines in B16-F10. To test for expression and abundance of KMP2-3XFLAG, a FLAG ELISA was performed (Figure 12A). The three highest expressing KMP2-3XFLAG cell lines (clones 2, 3, and 4) were selected for an experimental metastasis assay to verify that the introduction of the 3X-FLAG tag did not interfere with metastasis suppression (Figure 12B). This experiment confirmed that the introduction of 3X-FLAG tag increases the detection of KMP2 and does not interfere with KMP2 metastasis suppression.

Figure 11

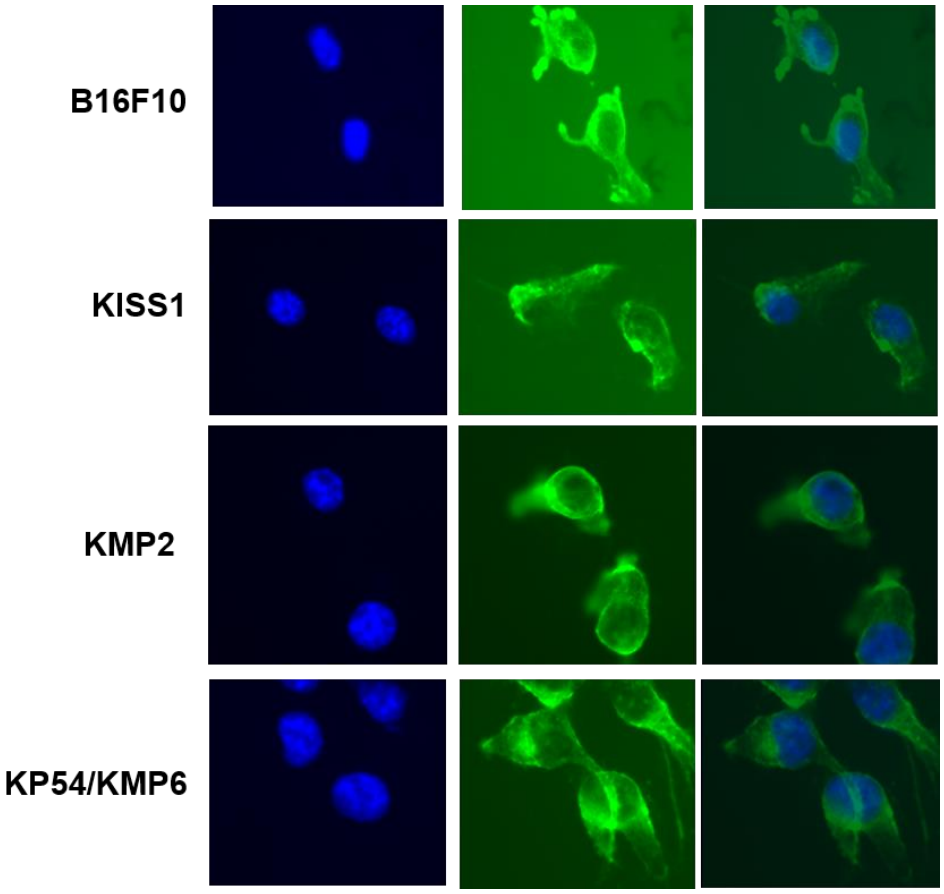
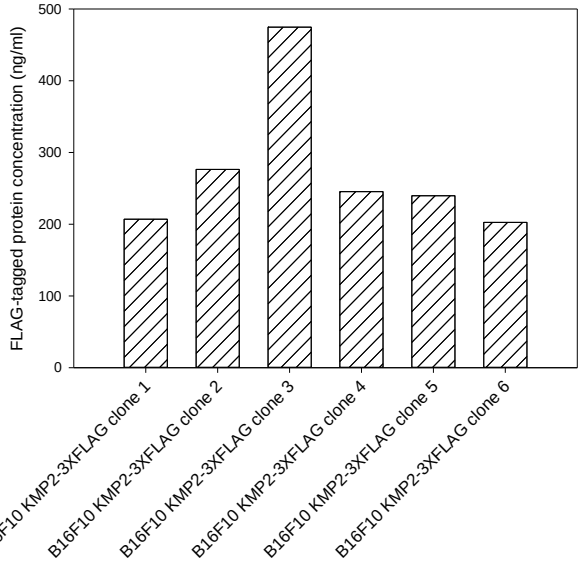


Figure 11: Phalloidin staining for actin networks demonstrates no large cytoskeletal reorganizations following kisspeptin or KISS1 expression in B16-F10 cells.

Figure 12

A


FLAG-tagged protein concentration (ng/ml)
500
400
300
200
100
0
B16F10 KMP2-3XFLAG clone 1
B16F10 KMP2-3XFLAG clone 2
B16F10 KMP2-3XFLAG clone 3
B16F10 KMP2-3XFLAG clone 4
B16F10 KMP2-3XFLAG clone 5
B16F10 KMP2-3XFLAG clone 6

B

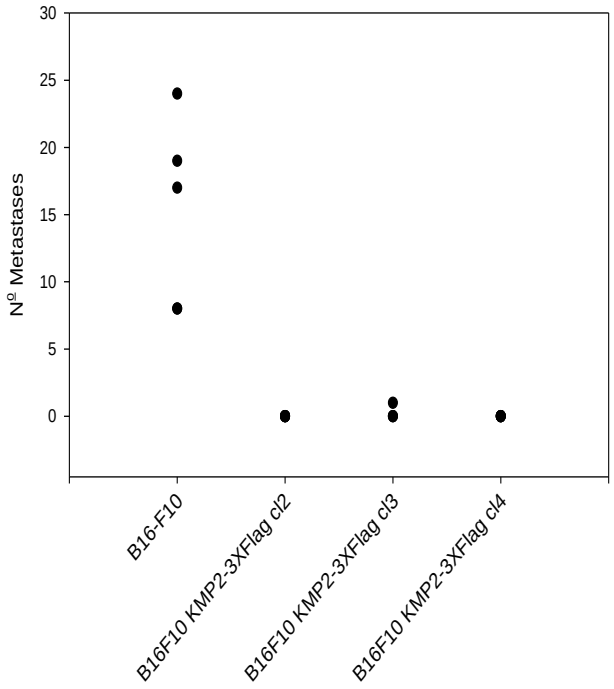

Nº Metastases
30
25
20
15
10
5
0
B16-F10
B16F10 KMP2-3XFlag cl2
B16F10 KMP2-3XFlag cl3
B16F10 KMP2-3XFlag cl4

Figure 12: A, KMP2-3XFLAG constructs are readily detectable by FLAG ELISA. The top three expressing clones (clones 2, 3, and 4) were chosen for use in experimental metastasis assay. **B,** KMP2-3XFLAG-expressing cells suppress metastasis *in vivo* compared to parental control.

KMP2 expression inhibits melanosphere formation

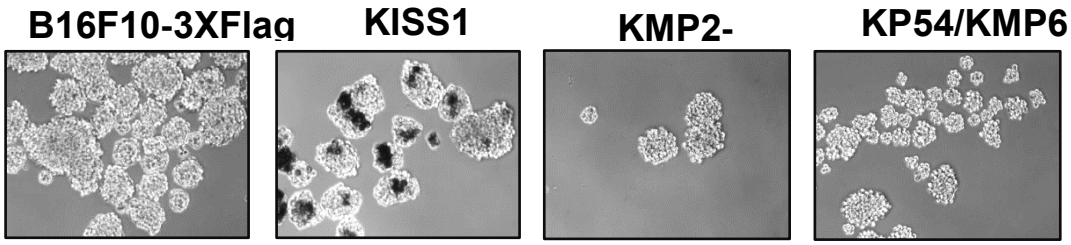
A strong indicator of stemness and propensity to metastasis is the ability for cancer cells to form spheroids. The ability of melanoma cells to form spheroids, called melanospheres, has been noted as an indicator of invasive and metastatic potential. To test the ability of KISS1 and kisspeptins to inhibit melanosphere formation, a melanosphere assay was performed using ultra low attachment plates. Cells were cultured for 10 days before imaging and analysis. Both number of melanospheres and abundance of cells remaining were evaluated. Compared to control, KISS1 had no large impact on melanosphere formation (Figure 12). Expression of KMP6 or KP54 did not inhibit melanosphere formation. However, KMP2-expressing cells formed 10-fold fewer spheroids. These data suggest that the expression of KMP2, in lieu of the remaining KISS1 protein, inhibits melanosphere formation and melanoma stem-like characteristics. This observation supports the other data that KMP2 suppresses metastasis through a mechanism independent of KP54 and KISS1R expression.

Conclusions and discussion

KISS1 is a metastasis suppressor protein which must be secreted to suppress metastasis (Nash & Welch, 2006). We have previously reported that KISS1 is processed to kisspeptins outside of the cell by furin, a prohormone convertase (Harihar et al., 2014). However, processing at dibasic cleavage sites does not appear to be critical for metastasis (Harihar et al., 2014). One interpretation of these data is that KP54 liberation and KISS1R binding are not necessary for metastasis suppression. These observations lead to the possibility that non-KP54 domains of KISS1 could play a role in metastasis suppression. This study demonstrates

Figure 13

A


B16F10-3XFlag
KISS1
KMP2-
KP54/KMP6

B

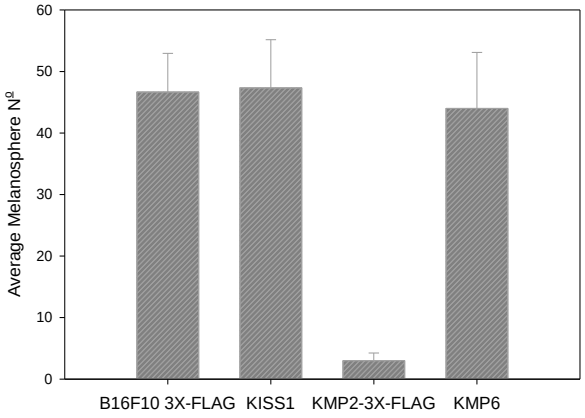

Average Melanosphere N°
60
50
40
30
20
10
0
B16F10 3X-FLAG
KISS1
KMP2-3X-FLAG
KMP6

Figure 13: A, Melanosphere formation is inhibited by KMP2-3XFLAG expression, but not KISS1 expression. Melanospheres formed by KISS1-expressing cells are larger than those formed by vector controls. **B,** Quantification of total melanospheres formed demonstrates extreme inhibition of melanosphere formation by KMP2 expression.

that other kisspeptin fragments of KISS1, namely the N-terminal domain (KMP2), possess the ability to suppress metastasis.

However, besides KMP2, multiple kisspeptins suppressed metastasis. Statistically, KMP8 and KMP10 were the only kisspeptins which did not initially suppress metastasis. The domain that these two kisspeptins share is the C-terminal domain of KISS1 (K122-Q145). The presence of this domain on KMP8 blocked the seemingly anti-metastatic impact of KP54, which is also included on KMP8. KMP10 by itself is a short (~20aa) peptide, but could potentially block protein-protein interactions by blocking protein folding dynamics. Subsequently, the KMP10 domain could prevent successful furin cleavage to liberate the KISS1R binding motif in lieu of the remaining protein. KMP10 expression did not suppress metastasis, which could be due to a lack of bioactivity of this domain or low expression in KMP10 cells (Figure 1B). The presence of KMP10, the C-terminal domain of KISS1 in the two kisspeptins which did not suppress metastasis suggests an inhibitory effect of this domain on the metastasis suppressor capabilities of KP54.

One notable observation from this study was validation that KP54 can suppress metastasis in murine cells lacking expression of KISS1R. These data support previous observations that KISS1 suppresses metastasis in C8161.9 and MelJuSo human melanoma lines lacking endogenous KISS1R expression (Harihar et al., 2014; W. Liu et al., 2014; Nash et al., 2007). Rather than suggesting KP54 does not signal through KISS1R, we instead suggest that these data support the hypothesis that KISS1 and KP54 can signal in a juxtacrine manner to suppress metastasis. As a secreted peptide, KISS1 is often studied in its ability to signal in an intracrine manner. However, the impact of KP54 stimulation on the metastatic microenvironment has not been thoroughly investigated. Low levels of KISS1R expression have been reported in

lung tissue, which presents a hypothesis for the mechanism of KP54-mediated metastasis suppression (Kotani et al., 2001). These data suggest that KP54 can signal through stromal KISS1R as a mechanism of metastasis suppression. Future studies to further define the role of KP54 and KISS1R signaling in the metastatic microenvironment would provide a benefit to the field.

Much of this study focused on the characterization of KMP2 as a metastasis suppressor peptide. In this work, we have demonstrated that KMP2 expression is sufficient to suppress metastasis *in vivo*, suppress migration *in vitro*, and elevate mitochondrial mass and function. These phenotypes are also observed with the expression of full-length KISS1. We have also demonstrated that KMP2 expression without the remaining KISS1 protein is a potent inhibitor of melanosphere formation, which indicates that the KMP2 domain may be more potent in its suppressor capabilities in lieu of the remaining protein. Because KMP2 does not contain the LRF-amide KISS1R binding motif, KMP2 metastasis suppression is independent of KISS1R binding. As these phenotypes are present in both *in vivo* and *in vitro* assays, this presents the hypothesis that KMP2 utilizes an intracrine signaling mechanism of metastasis suppression.

The data indicate that KMP2 is a potent metastasis suppressor domain of KISS1. However, the mechanism by which KMP2 suppresses metastasis remains undefined. As a secreted peptide, KMP2 is likely to bind a receptor or other membrane-bound protein to induce metastasis suppression. KISS1 binding to non-receptor proteins has been described in the literature. When characterizing MMP9's ability to cleave KISS1 at Gly-118 and Leu-119, a highly stable disulfide binding interaction between KISS1's Cys-53 and pro-MMP9 and pro-MMP2. This cysteine lies within the KMP2 domain, and this binding interaction suggests that

other proteins could potentially interact with this region. Further studies identifying the exact mechanism of KMP2 metastasis suppression will be necessary.

KISS1 and kisspeptins are a valuable tool to study in the quest to develop better anti-metastatic therapeutics. While most chemotherapeutics target both tumors and their metastases, these drugs can select for a resistant population of cancer cells. Additionally, conventional chemotherapeutics and radiation can have potent side effects, resulting in decreased quality of life for patients. KISS1 has potential for use as an anti-metastatic therapeutic, but the short half-life of proteins makes them poor drugs unless they are a candidate for a stapled peptide. Another downside to KISS1 as an anti-metastatic therapeutic is off-target side effects in the hypothalamic-pituitary-gonadal axis. KISS1 has been administered therapeutically as a stimulator of GnRH, LH, and FSH release (George et al., 2017). Recently, administration of KP54 has been found to increase libido and limbic brain activity (Comninos et al., 2017). These off-target effects, while not necessarily deleterious, are undesirable when choosing the basis for a therapeutic. While the exact mechanism of KMP2 metastasis suppression remains undescribed, KMP2 does not signal through KISS1R and would not induce the reproductive side-effects consequent of KISS1:KISS1R signaling. Further studies could implicate KMP2 as a basis for a stapled peptide therapeutic.

In conclusion, this study is the first to investigate the metastasis suppressor capabilities of KP54 and non-KP54 kisspeptins. We have supplemented the growing literature that identifies KP54 as suppressing metastasis through juxtacrine signaling in addition to intracrine signaling through KISS1R. Additionally, we found that the N-terminal domain of KISS1, herein referred to as KMP2, possesses metastasis suppressor capabilities comparable to that of the full-length protein. KMP2 expression alone is sufficient to suppress metastasis *in vivo* and metastatic

characteristics *in vitro*. These data suggest that, while the mechanism is currently undetermined, KMP2 by itself may hold potential as an anti-metastatic therapeutic.

Chapter 3: Impact of metarrestin treatment on metastatic melanoma

Introduction

Cancer is a disease characterized by genomic instability and abnormal cellular behavior (Hanahan & Weinberg, 2011). This genomic instability can lead to broad heterogeneity between patients with the same tumor types. This heterogeneity extends to a tumor's tendency to metastasize (Fidler, 1978). From increasing discoveries of new biomarkers and improved technology to detect circulating tumor cells, the field is improving in its ability to discern which cancers have a propensity towards metastasis (Alix-Panabieres & Pantel, 2014; Faratian & Bartlett, 2008; Ransohoff, 2007). However, many of these biomarkers are specific to a certain tumor type and reliability varies from biomarker to biomarker (Ransohoff, 2007). There remains a need to more precisely target and treat disseminated metastases, as metastasis is at the root of most the morbidity and mortality associated with cancer. Drug distribution to metastases can be accomplished by most conventional chemotherapeutics, however many of these have harmful side-effects such as neuropathy and cardiotoxicity (Bhave, Shah, Akhter, & Rosen, 2014; Fehrenbacher, 2015). Additionally, tumors and metastases can grow resistant to initially effective treatments (Nakazawa, Paller, & Kyprianou, 2017; Sharma, Hu-Lieskovan, Wargo, & Ribas, 2017). This gap in therapy leaves a persistent need to develop a better method to discern metastatic potential and treat disseminated metastases.

One strategy for targeting metastases is to target structures absent in normal tissue. The perinucleolar compartment (PNC) is a structure composed of RNA-binding proteins and RNAs which is physically associated, yet distinct from, the nucleolus (Huang, Deerinck, Ellisman, & Spector, 1997). Components of the PNC include CDK13, PTB, CUG-BP1, KSRP, Raver 1 and 2, Rod1, as well as newly synthesized RNA Pol III transcripts for a series of genes (Gromak et al., 2003; Philips, Timchenko, & Cooper, 1998; Savkur, Philips, & Cooper, 2001; Valcarcel &

Gebauer, 1997). The complete structure and function of the PNC has yet to be thoroughly described. The PNC is a dynamic structure which disassembles during mitosis and is reassembled during G1 phase, but PNC-negative cells can also give rise to PNC-positive daughter cells (Huang et al., 1997). The PNC is found exclusively in cancer cells, and appears to be enriched in metastatic cells (Kamath et al., 2005; Norton et al., 2008). A panel of human solid tumor cancer cell lines found that PNC can be found in a broad spectrum of cancers (Norton et al., 2008). As a cellular structure present in only malignant cells of many cancer types, the PNC is a promising candidate for therapeutic targeting.

A high throughput screen was performed to identify compounds which target and disassemble the PNC (Frankowski et al., 2010; Norton et al., 2009). From this screen, one compound, ML246 (hereafter metarrestin), was discovered to efficiently disassemble the PNC with low cellular toxicity (Frankowski et al., 2010). Metarrestin was later found to be effective in targeting and inhibiting metastases in breast cancer patient-derived xenograph, human prostate, and pancreatic cancers (manuscript in preparation). Metarrestin has no discernable impact on normal cell populations which harbor no PNC. This potent impact on PNC disassembly and targeting of metastatic cells makes metarrestin a lucrative compound with therapeutic potential.

Melanoma is an aggressively metastatic cancer with the highest mortality rate of all skin cancers (Siegel et al., 2016). In the panel of human solid tumor cancer cell lines, two human melanoma cell lines were evaluated and found to be PNC-positive (Norton et al., 2008). This observation aligns with the highly metastatic nature of melanoma. From this observation, it is hypothesized that metarrestin treatment could inhibit melanoma from successful metastatic outgrowth.

To date, no experiments characterizing the impact of this compound on a melanoma model have been conducted. This study evaluated the efficacy with which metarrestin can arrest disseminated melanoma metastases. To examine the impact of metarrestin treatment on disseminated metastases, a series of experimental metastasis assays were performed in conjunction with metarrestin treatment. The impact of metarrestin treatment on orthotopic tumor growth was also tested. Cumulatively, this study found that metarrestin treatment attenuated primary tumor growth and delayed tumor onset, but had no significant effect on targeting disseminated metastases. This study provides evidence that metarrestin could have an impact on treating tumors with low PNC prevalence, but is less effective in treating metastases in PNC-low tumor types.

Materials and methods

Cell line and culture

C8161.9 is a highly metastatic sub-clone of the human melanoma cell line C8161 (Welch et al., 1991). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 growth medium (ThermoFisher Scientific). Growth medium was supplemented with 5% fetal bovine serum, 1% L-glutamine, and 0.25% non-essential amino acids. For experiments, cells were detached using 2 mM EDTA in Ca2+- and Mg2+-free Dulbecco's phosphate-buffered saline (DPBS). Cells were tested and found to be free of *Mycoplasma spp.* contamination by a PCR-based assay (ATCC, Universal Mycoplasma Detection Kit).

Drug preparation

For treatment, ML246 was suspended in 5% N-Methylpyrrolidine (NMP, Sigma M79204), 20% polyethylene glycol-400 (PEG- 400, Sigma 202398) and solubilized via sonication and

vortexing. Large drug crystals were disrupted by drawing the mixture through a syringe fitted with a 28G needle. This mixture was stored at room temperature. Immediately prior to injection, 75% total volume 10% HP-β-cyclodextrin was added to the solution.

Immunofluorescence Staining

C8161.9 cells were plated on 8-well chamber slides to reach 70% confluence within 24 hours. The following day, cells received vehicle or metarrestin treatment at 10uM for 24 hours. Cells were fixed using 4% paraformaldehyde and stained for SH-54, which stains for polypyrimidine tract binding protein (PTB), a nuclear protein included in the PNC. Briefly, cells were washed following fixation, permeablized with 0.1% Triton-100 in PBS, and blocked in 3% BSA. Slides were incubated in 1:300 SH-54 antibody overnight, then washed, blocked and incubated with secondary antibody conjugated to Texas Red fluorophore. Coverslips were mounted using medium containing DAPI for nuclear staining. Slides were imaged and assessed for PNC prevalence using fluorescence microscopy.

Statistics

Statistical tests were conducted via One Way ANOVA followed by pair-wise post-tests (Shapiro-Wilk normality test) for experimental groups containing more than two treatment groups. The experiment testing metarrestin against macroscopic metastases was analyzed via t-test. The spontaneous metastasis assay was insufficiently powered for statistical analysis.

Animal studies

Animals used in this study were Hsd: Athymic Nude-Foxn1Nu (Envigo). Animals were maintained at 23ºC and given food and water ad libitum.

Metarrestin treatment on microscopic metastases

C8161.9 cells ($2x10^5$) were plated 48 hours prior to intravenous injection into the lateral tail vein. One week after injection, mice received daily (Monday – Friday) injections of either vehicle (5% NMP, 20% PEG-400, 75% v/v 10% HP-β-cyclodextrin), 6.25 mg/kg, 12.5 mg/kg, or 25 mg/kg metarrestin treatment in a 200 uL injection volume. Mice received injections for three weeks until week 5 of the experiment, upon which the mice were euthanized and lungs were harvested for evaluation of gross metastases.

Metarrestin treatment on macroscopic metastases

C8161.9 cells ($2x10^5$) were plated 48 hours prior to intravenous injection into the lateral tail vein. Two weeks after injection, mice received daily (Monday – Friday) injections of either vehicle (5% NMP, 20% PEG-400, 75% v/v 10% HP-β-cyclodextrin) or 25 mg/kg metarrestin treatment in a 200 uL injection volume. Mice received these injections for three weeks until week 5 of the experiment, upon which the mice were euthanized and lungs were harvested for evaluation of gross metastases.

Preventative treatment with metarrestin

C8161.9 cells were plated to reach 75% confluence in 24 hours. Metarrestin treatment (10uM) was administered for 24 additional hours prior to injection, upon which the cells ($2x10^5$) were injected through the lateral tail vein of nude mice. One treatment group received daily intraperitoneal metarrestin treatment (25mg/kg) and the other two groups were monitored until the completion of the study.

Metarrestin impact on orthotopic tumor growth

C8161.9 cells were plated 48 hours prior to injection, upon which cells ($1x10^6$) were injected intradermally into the flank of nude mice. Treatment of either metarrestin (25mg/kg) or vehicle was implemented intraperitoneally the day following injection and continued daily for the duration of the 6-week experiment. Animals were euthanized and the tumors were measured and resected.

Results

PNC in C8161.9 are disassembled by metarrestin treatment

To evaluate the abundance of PNC in C8161.9 melanoma, immunofluorescence was performed to stain for PTB (Figure 1). PTB is a nuclear protein which is normally widely distributed throughout the nucleus. In PNC-positive metastatic cells, PTB will accumulate in the PNC in bright, distinct puncta. C8161.9 cells were stained for PTB and PNC abundance was quantified. Despite the highly metastatic nature of C8161.9 cells, the PNC prevalence in C8161.9 is only 8.2% (Table 1). This level of PNC abundance is relatively low compared to other cell lines which have been evaluated (Norton et al., 2008). Following a 24-hour treatment with 10 μM metarrestin, PNC were completely disassembled in C8161.9 (Figure 1). These experiments illustrated the relatively low abundance of PNC in C8161.9 cells and the efficiency with which metarrestin treatment disassembles them.

Cellular pre-treatment with metarrestin suppresses metastatic outgrowth

To test whether PNC depletion prior to injection would diminish successful metastatic seeding and outgrowth, C8161.9 cells were pre-treated with 10 μM metarrestin for 24 hours. The following day, pre-treated and untreated cells were injected into the lateral tail vein of nude mice for an experimental metastasis assay (Figure 2). The treatment group receiving exclusively

metarrestin pre- treated cells (n=10) displayed no suppression in metastasis (Figure 3). However, the treatment group which received metarrestin pre-treated cells as well as 25 mg/kg metarrestin s.i.d. displayed a statistically significant suppression in metastases. The combined effect of pre-treatment with daily treatments appears to be sufficient to prevent successful metastatic colonization and outgrowth. This effect is likely due to an initial sensitization and prevention of later outgrowth.

Table 1

Treatment Group (C8161.9)	PNC (+)	Total Cells	PNC Prevalence (%)
Non-treated	16	303	5.6
Vehicle	17	162	10.7
Overall	**33**	**465**	**8.2**

Figure 1. C8161.9 cells have detectable PNC by immunofluorescence staining for PTB, a PNC protein component. Arrows indicate PNC localization. Perinucleolar compartments in C8161.9 are disassembled with metarrestin treatment.

Figure 1

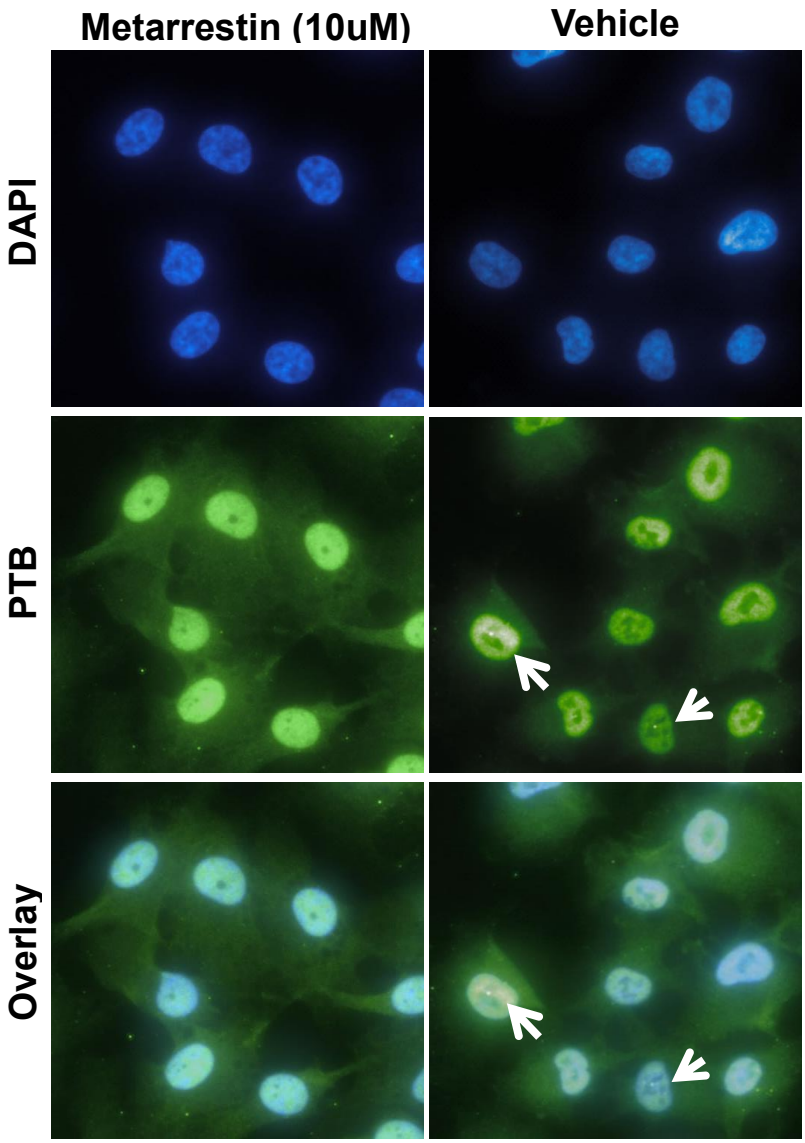


Table 1. SH-54 staining for PNC in non-treated and vehicle-treated cells demonstrates a low PNC abundance in C8161.9.

Figure 2

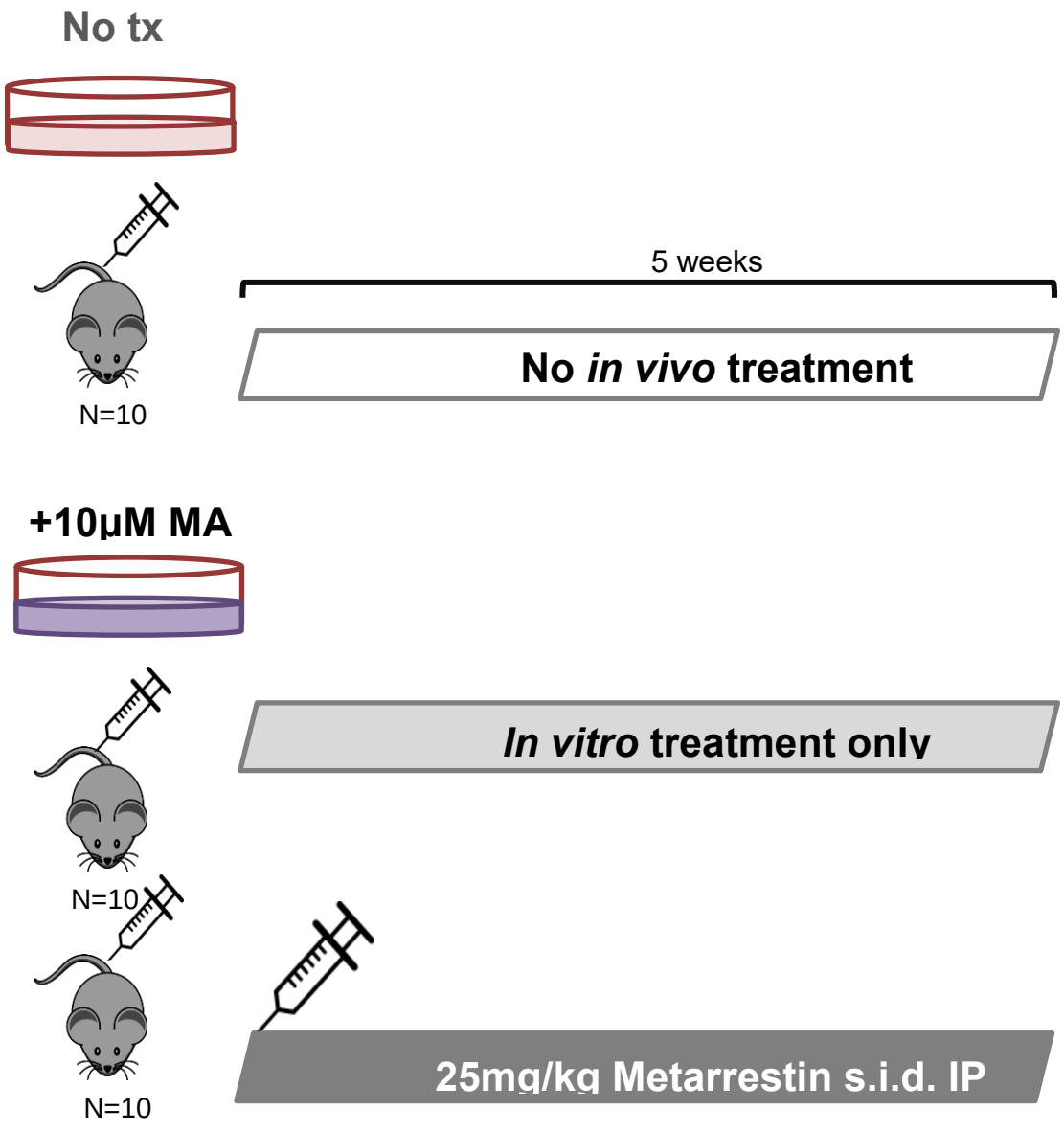

No tx
5 weeks
No *in vivo* treatment
N=10
+10μM MA
In vitro treatment only
N=10
25mg/kg Metarrestin s.i.d. IP
N=10

Figure 2. Experimental design to test the impact of metarrestin pre-treatment on cells before metastatic seeding with or without adjuvant metarrestin *in vivo* treatment.

Figure 3

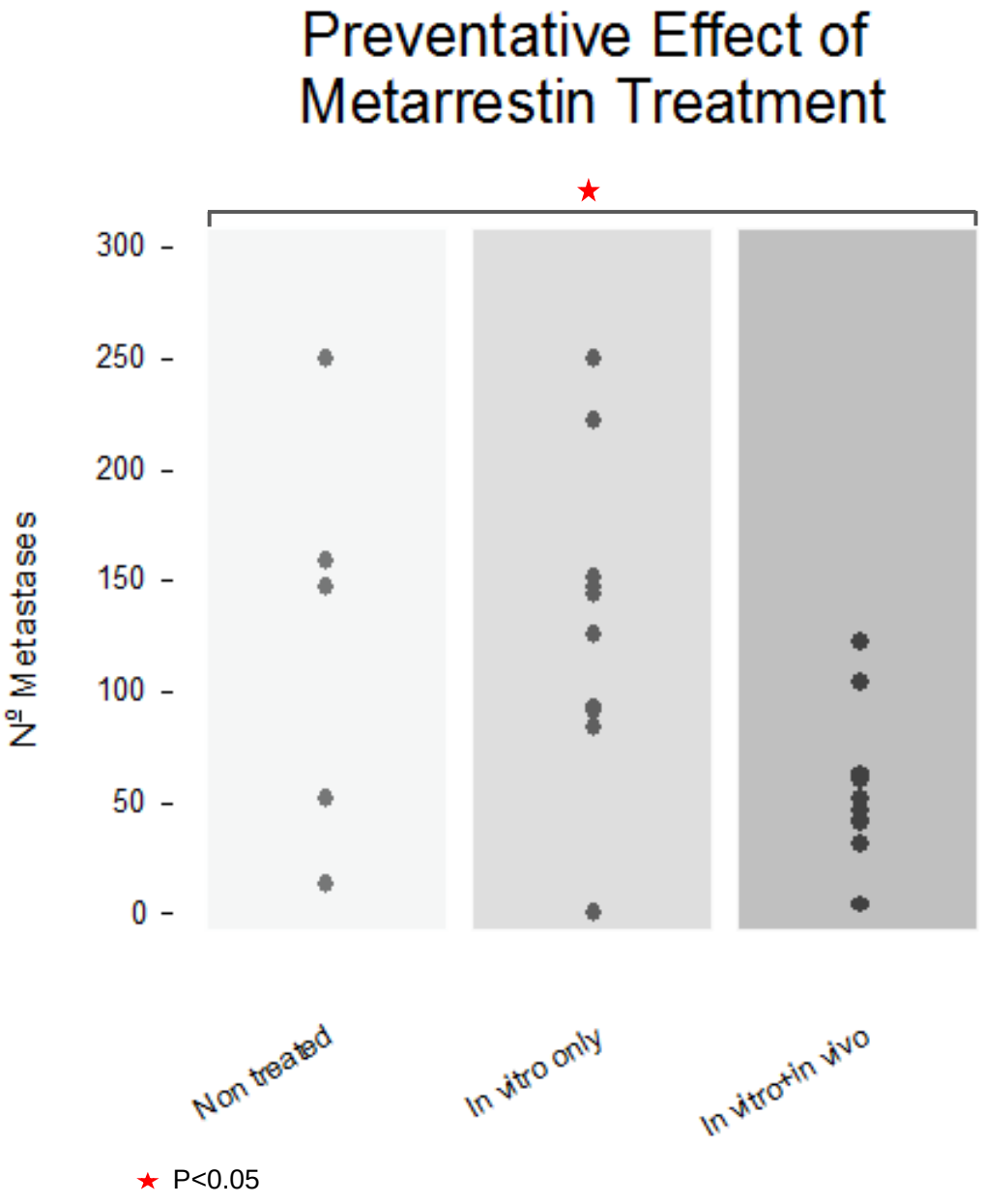

Preventative Effect of Metarrestin Treatment
Nº Metastases
300
250
200
150
100
50
0
Non treated
In vitro only
In vitro+in vivo
★ P<0.05

Figure 3. Impact of metarrestin pre-treatment in cells prior to injection with or without subsequent *in vivo* dosing. While treatment with metarrestin suppresses gross metastasis numbers, pre-treatment in conjunction with in vivo treatment has a statistically significant depression in metastasis numbers. Groups were compared by One Way ANOVA (P=0.02) followed by pairwise comparisons using Holm-Sidak method (Non-treated vs. in vitro and in vivo treatment, p=0.05).

Microscopic metastases are not suppressed by metarrestin treatment

Many patients presenting with highly invasive and undifferentiated tumors are likely to harbor micro- or macroscopic metastases at the time of diagnosis. To test the ability of metarrestin treatment to arrest disseminated microscopic metastases, an experimental metastasis assay was performed. Metarrestin treatment commenced seven days following intravenous injection, upon which metastases contain roughly 10 cells (John F. Harms & Welch, 2003). Seven days following injection, mice received five weeks of s.i.d. intraperitoneal metarrestin treatment at a range of doses: 25 mg/kg, 12.5, 6.25, or vehicle (Figure 4). No visible toxicity or signs of distress upon treatment were observed in the animals. While metarrestin treatment did trend towards lower metastatic load (188 average untreated vs. 138, 126, 142 treated), these results were not statistically significant (Figure 5; One way ANOVA, P=0.357). These results suggest that metarrestin treatment combined with low abundance of PNC is an inefficient way of targeting disseminated microscopic metastases in this model of metastatic melanoma.

Macroscopic metastases are not inhibited by metarrestin treatment

For the purposes of this experiment, macroscopic metastases are defined as metastatic lesions roughly 100 cells in size. Disseminated metastases are estimated to reach this size two weeks after metastatic seeding (John F. Harms & Welch, 2003). These lesions can impede proper tissue function and cause a decrease in quality of life. To test the effectiveness of metarrestin treatment on macroscopic metastases, an experimental metastasis assay was

Figure 4

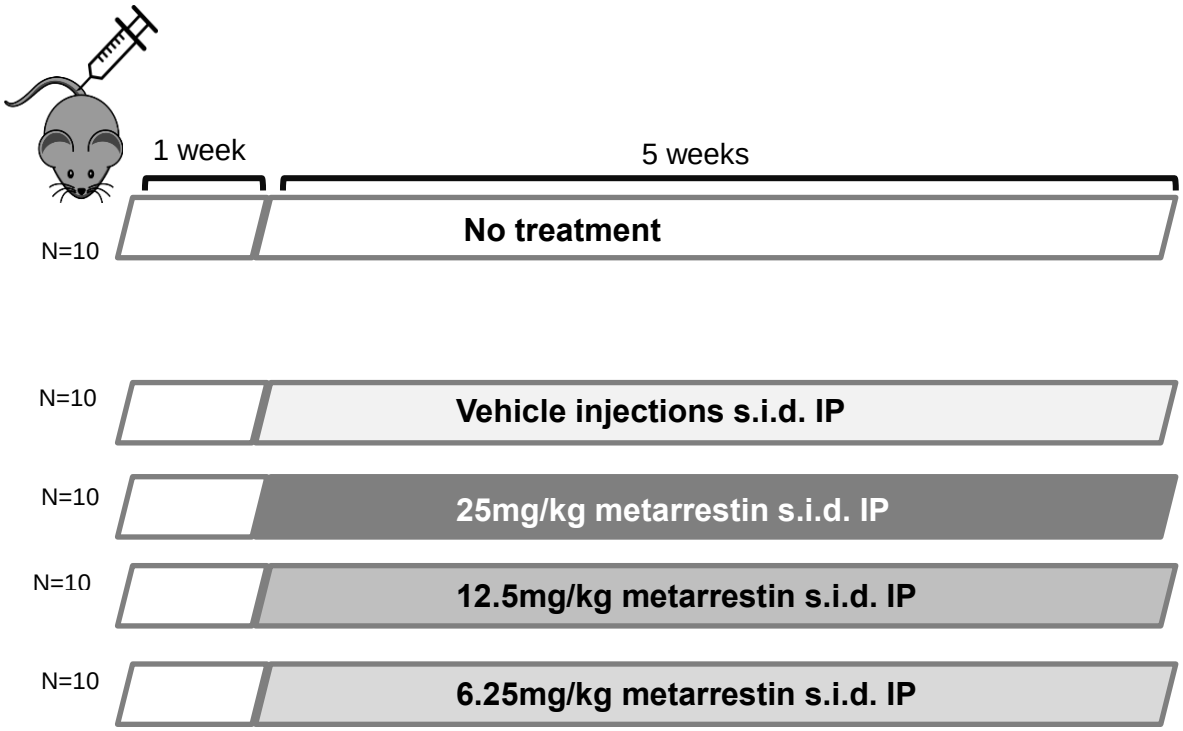

1 week
5 weeks
No treatment
N=10
N=10
Vehicle injections s.i.d. IP
N=10
25mg/kg metarrestin s.i.d. IP
N=10
12.5mg/kg metarrestin s.i.d. IP
N=10
6.25mg/kg metarrestin s.i.d. IP

Figure 4. Experimental design to test the impact of metarrestin treatment on microscopic metastases.

Figure 5

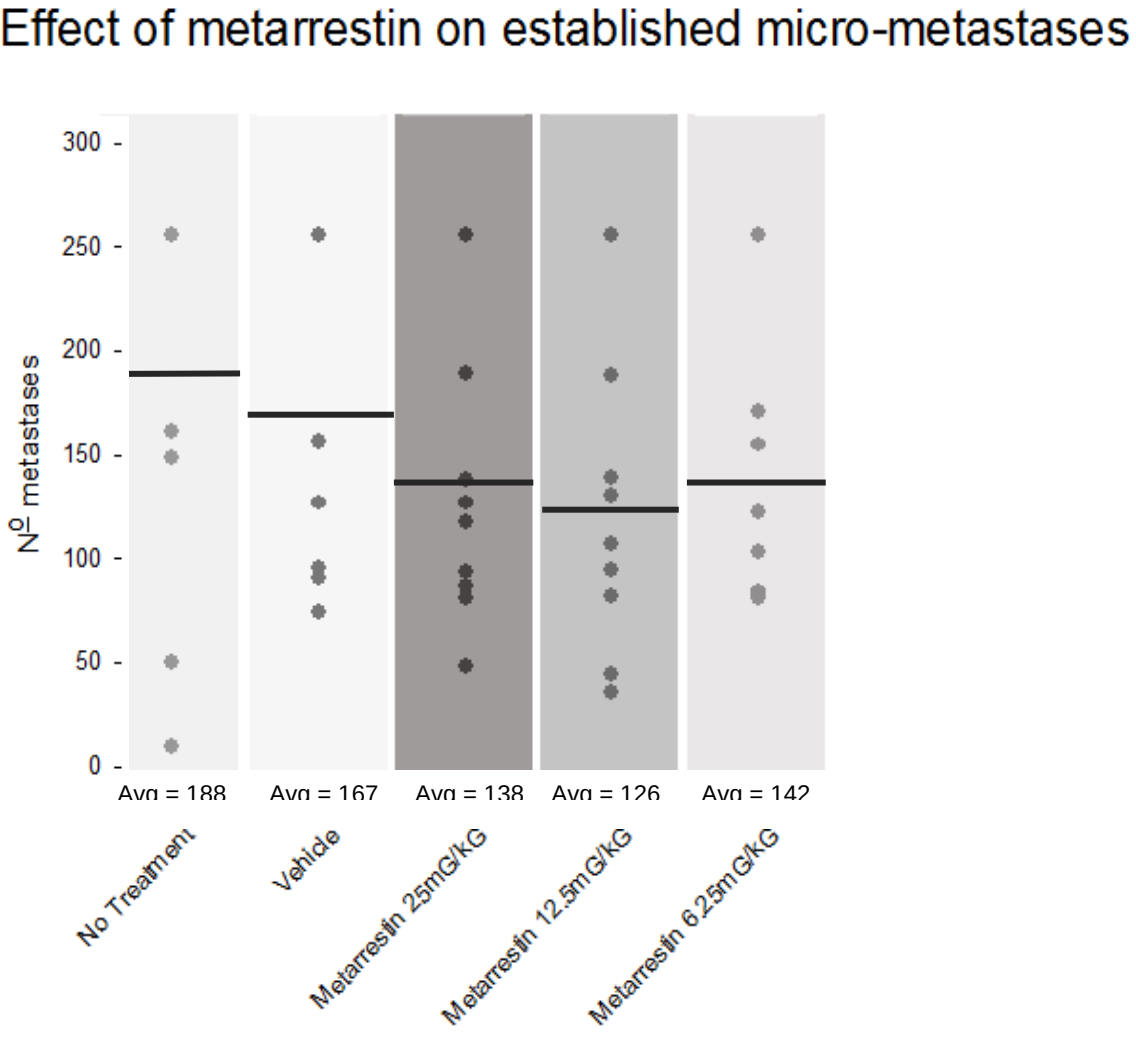

Effect of metarrestin on established micro-metastases
Nº metastases
300
250
200
150
100
50
0
Avg = 188
Avg = 167
Avg = 138
Avg = 126
Avg = 142
No Treatment
Vehicle
Metarrestin 25mG/kG
Metarrestin 12.5mG/kG
Metarrestin 6.25mG/kG

Figure 5. One week following intravenous injection of cells, metastases are estimated to be roughly 10 cells. Treatment commenced one week following metastatic seeding with metarrestin doses of at 25mg/kg, 12.5mg/kg, and 6.25mg/kg resulted in a trend of metastasis suppression, yet was statistically insignificant by one-way ANOVA ($p>0.05$).

Figure 6

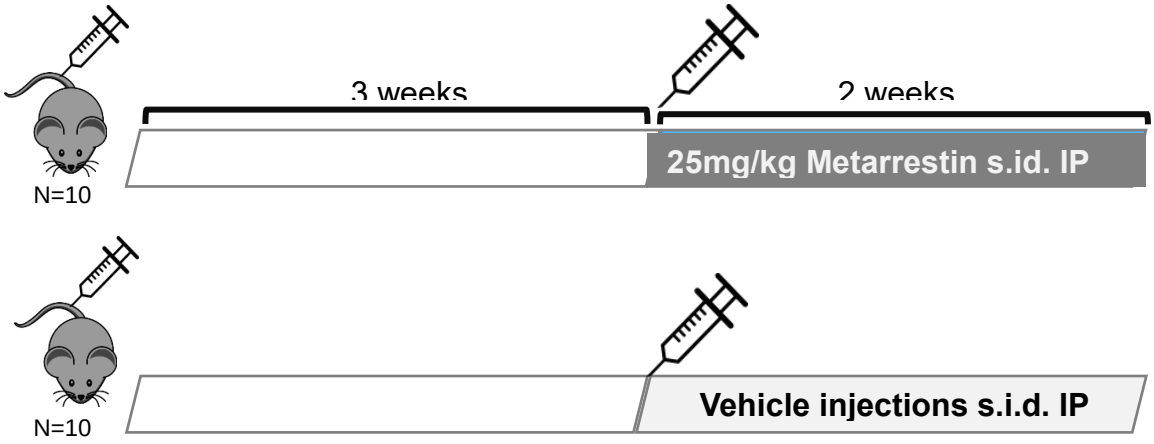

3 weeks
2 weeks
25mg/kg Metarrestin s.id. IP
N=10
Vehicle injections s.i.d. IP
N=10

Figure 6. Experimental design to test the impact of metarrestin treatment on macro-metastases. Cells were seeded, left to grow into metastases for three weeks, before receiving vehicle or metarrestin (25mg/kg) treatment s.i.d.

Figure 7

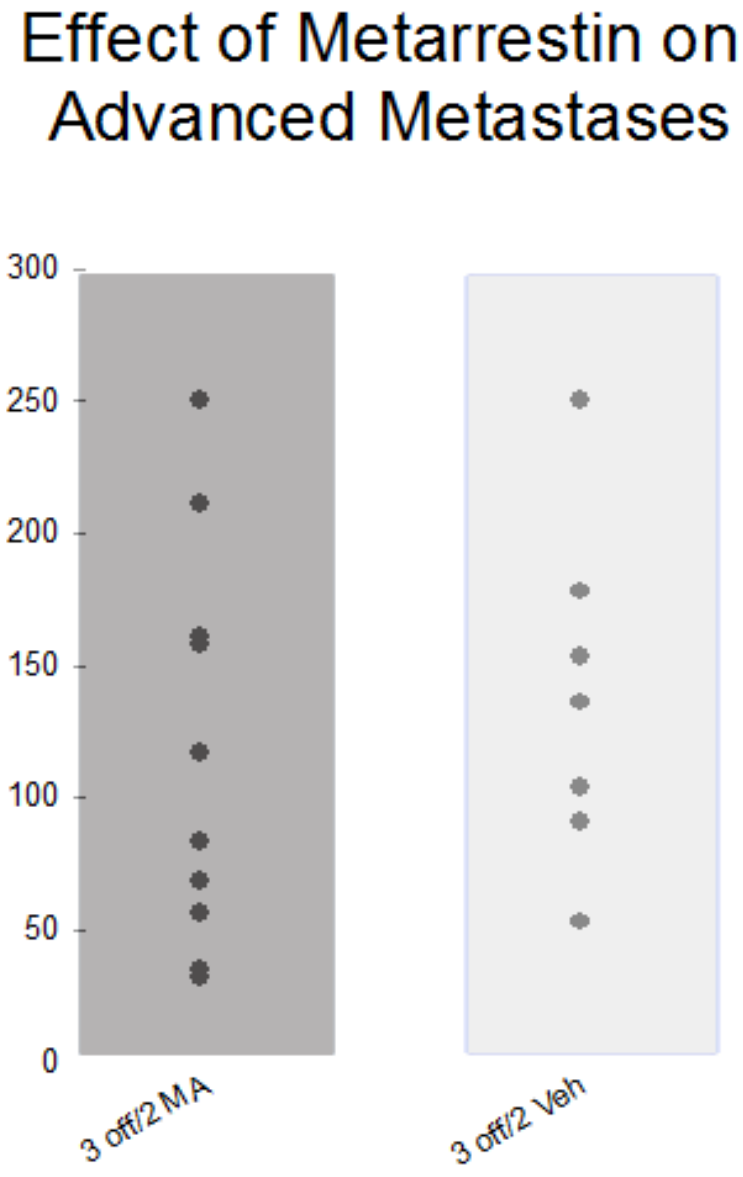

Effect of Metarrestin on Advanced Metastases
300
250
200
150
100
50
0
3 off/2 MA
3 off/2 Veh

Figure 7. Three weeks after cell seeding, metastases are expected to be roughly 100 cells. Treatment with metarrestin at this established metastasis stage does not have a statistically significant impact on metastasis. However, the metarrestin treatment group (left) has a lower mean than vehicle-treated mice (right, 118 vs 155) and there was a higher number of animals with lower metastasis count, suggesting that treatment had a small effect on suppressing metastasis.

performed. Mice were maintained for three weeks following injection, upon which metastases are estimated to be roughly 100 cells in size. At this point, mice received s.i.d. IP injections of either vehicle or 25 mg/kg metarrestin treatment (Figure 6). No visible toxicity or signs of distress upon treatment were observed in the animals. Compared to vehicle treated mice (average number metastases= 155), mice receiving two weeks of metarrestin treatment had a slight decrease in metastases (Figure 7; average number of metastases = 118). However, this effect was not statistically significant (two-tailed p=0.270, t-test). This treatment regimen combined with the advanced nature of these metastases appears to be insufficient to suppress or arrest metastatic outgrowth.

Metarrestin treatment attenuates orthotopic tumor onset and growth

Although PNC are enriched in metastatic cells, PNC-positive cells are also present in primary tumors. Previous studies have indicated high prevalence of PNC in breast cancer tumors, suggesting that the primary tumor could also be targeted by metarrestin treatment (Kamath et al., 2005). To test the ability of metarrestin treatment to suppress growth of orthotopic tumors, cells were injected intradermally into the flank of nude mice. The day following inoculation, mice received either vehicle or 25 mg/kg s.i.d. IP metarrestin treatment (Figure 8). Mice were evaluated for tumor onset and size. Interestingly, metarrestin treatment delayed tumor onset by 10 days (Table 2; 14 days to 24 days, vehicle vs. metarrestin treated). Additionally, final tumor size and weight were suppressed in metarrestin treated animals (Figure 9). These data show that even in a PNC-low tumor type, metarrestin treatment can still have a potent effect on delaying tumor onset and growth.

Figure 8

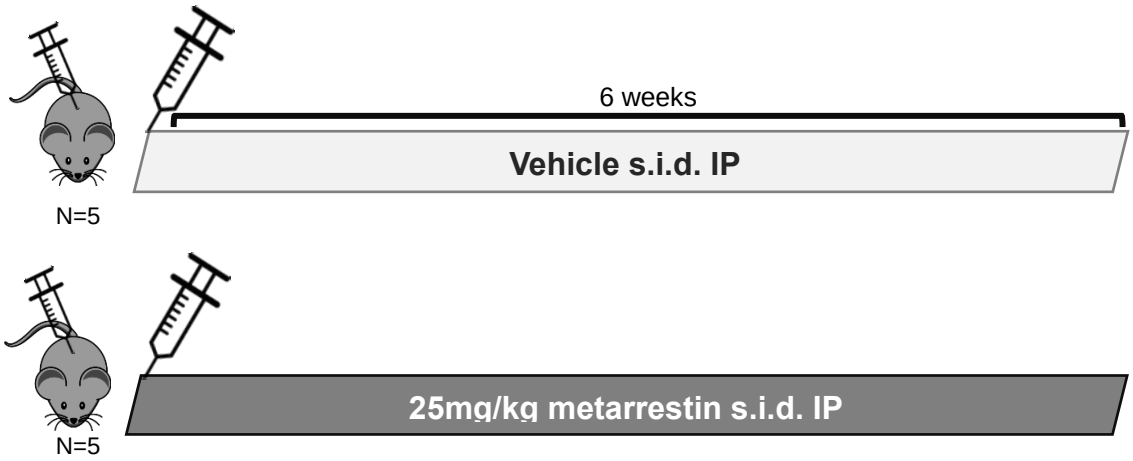
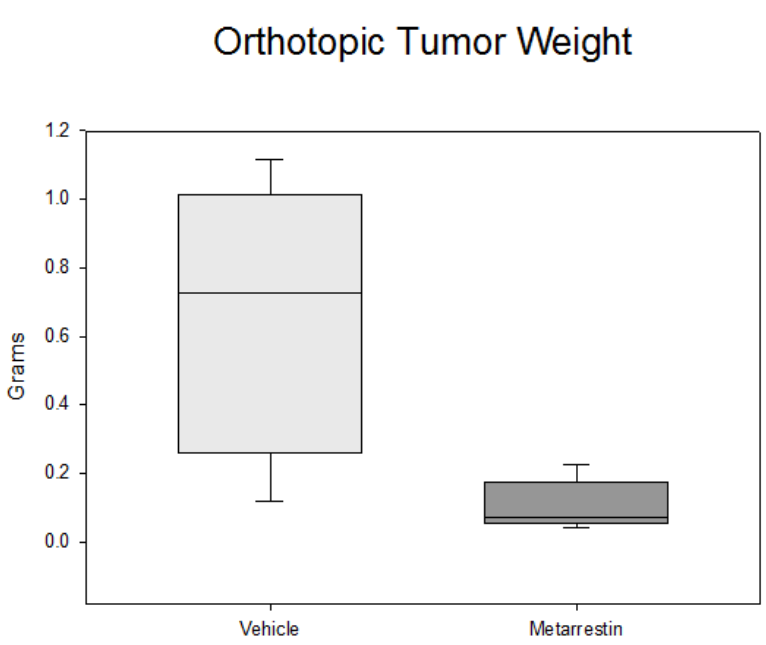


Figure 8. Experimental design to test the impact of metarrestin treatment on orthotopic tumor formation and progression. Mice were intradermally injected with $1x10^6$ cells and given either daily vehicle or metarrestin (25mg/kg) IP injections and monitored for tumor onset and progression.

Figure 9

A


Orthotopic Tumor Weight
Grams
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Vehicle
Metarrestin

B

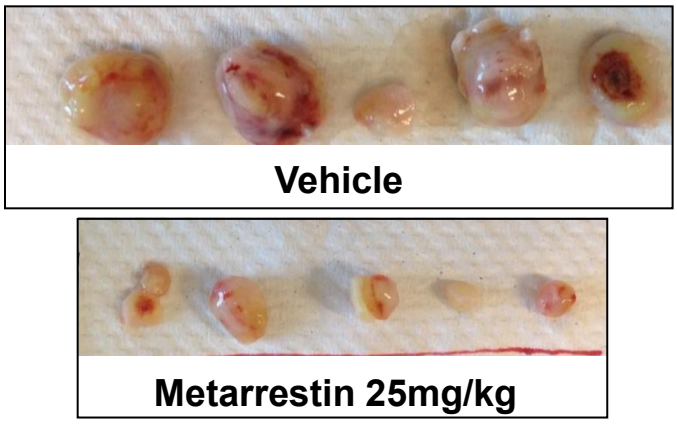

Vehicle
Metarrestin 25mg/kg

Figure 9. **A**, Orthotopic tumor weight of intradermal tumors with daily vehicle or metarrestin (25mg/kg) treatment. Metarrestin treatment has a tumor suppressive impact on tumor weight. **B**, Treatment with metarrestin delays tumor onset by 10 days, suggesting that while metarrestin treatment delays tumor onset, it does not prevent eventual tumor growth. ***Note**: No distant metastases were observed in either treatment group.

Table 2

Treatment group	Time to Tumor Onset (days)
Vehicle	14
Metarrestin (25mg/kg)	24

Table 2. Time to tumor onset in metarrestin or vehicle-treated mice. Times were determined by average days to tumor onset for all mice in each group (n=5 per group).

Discussion

This study describes the impact of metarrestin, a molecule which disassembles the perinucleolar compartment, and its ability to arrest disseminated melanoma metastases. Metarrestin, which has suppresses metastasis in breast cancer PDX, pancreatic cancer, ovarian cancer, and prostate cancer, seems to suppress orthotopic tumor growth and metastatic colonization in PNC-positive cell lines. These data suggest that in a clinical setting, metarrestin treatment may not be the best course of action for patients presenting with a low PNC abundance and a likelihood of disseminated metastases.

However, not all data from this study were insignificant. Pre-treating cells with metarrestin prior to injection coupled with daily metarrestin treatment caused a significant suppression of metastasis. Although this scenario is clinically unrealistic, it does shed light on possible applications of metarrestin. Prior to metastatic dissemination, pre-treatment with metarrestin seemed to decrease cell viability. This initial decrease in viability coupled with daily injections of metarrestin seemed to prevent metastatic outgrowth, implying that PNC-positive cells can arise from PNC-negative cells. Additionally, this experiment demonstrated that preventative metarrestin treatment could be beneficial for patients presenting with very early-stage tumors.

One of the more striking observations from this study was the impact of metarrestin treatment on orthotopic tumor onset and growth. Perinucleolar compartment prevalence has been demonstrated to increase with tumor progression and in metastatic lesions (Norton et al., 2008). Given the low abundance of PNC in C8161.9 cells (8.2%), the effect of metarrestin treatment on orthotopic tumor growth was striking. The ability of metarrestin treatment to delay tumor onset by 10 days suggests that this treatment regimen was effectively killing PNC-positive cells from

the onset of treatment. This treatment appears to select for PNC-negative cells, which appeared to be in lower abundance earlier in tumor progression. However, since metarrestin-treated tumors did eventually grow, this suggests that metarrestin treatment is ineffective in fully attenuating PNC-negative tumor growth.

The duration of this experiment did not allow for the observation of disseminated metastases in vehicle- or metarrestin-treated animals, but future studies would benefit from this experiment. Despite their eventual ability to form tumors, metarrestin treatment appears to keep PNC-positive cells at bay. A hypothesis from the results of this study would be that metarrestin-treated primary tumors will produce fewer metastases than vehicle-treated tumors. As surgical resection is one of the more prevalent cancer treatments, an experiment studying the ability of metarrestin treatment to suppress outgrowth of seeded metastases would provide additional clinical relevance to the study.

While there were valuable insights to be learned from this series of experiments, there are limitations to its interpretation. To begin, C8161.9 cells have a low abundance of PNC. These experiments provide evidence that metarrestin is likely to be ineffective in treating disseminated metastases from PNC-low tumors. However, performing these experiments in a melanoma model with a higher abundance of PNC would provide more clear data as to whether metarrestin could serve as an anti-metastatic therapeutic. An additional limitation to this study is the manner of drug treatment. Metarrestin and vehicle treatments were administered by intraperitoneal injections Monday through Friday for five weeks following metastatic inoculation. Mice respond with stress upon handling by humans, and this has been reported to modify metastatic responses to the experimental metastasis assay (Moynihan, Brenner, Ader, & Cohen, 1992). To avoid this daily stressor, these experiments could be performed using a surgically implanted osmotic pump.

Finally, there exists a challenge in extrapolating studies performed in mice to a human model. The experiments examining metastasis bypassed the first half of the metastatic cascade and pre-selected the metastatic site. Drug delivery to other metastatic sites, such as bone or brain, is likely dissimilar from the lung. No toxicity was observed from the dosing paradigm used in this study, but human side effects may be different and discourage use. Additionally, the nature of the preventative treatment experiment is impossible to recapitulate in a human disease model.

In conclusion, this study examined the impact of metarrestin, a PNC-disassembling compound, on C8161.9 melanoma tumors and metastases. C8161.9 has a low abundance of PNC, but still appears sensitive to metarrestin treatment on orthotopic tumor growth. In addition, metarrestin pre-treatment appears to rapidly eliminate PNC-positive C8161.9 cells prior to injection, and subsequent metarrestin treatment was sufficient to attenuate pre-treated metastatic lesions. Despite the low PNC abundance in C8161.9, this study still observed trends towards metastasis suppression following metarrestin treatment. This trend, combined with the potent suppression of orthotopic tumor onset and growth, suggests that metarrestin could serve as an adjuvant therapy in specific tumor types. In tumors with a high PNC prevalence, metarrestin is likely to be an effective adjuvant in conjunction with the standard of care. Another role for metarrestin could be in highly aggressive tumor types with low progression-free survival. Assuming the low toxicity observed in mice is consistent with a human model, patients with highly aggressive tumors could likely benefit from metarrestin treatment. Based on these results, further studies are recommended in melanomas with a higher PNC prevalence. These results suggest that, in tumor types with higher PNC abundance, metarrestin could be a powerful clinical inhibitor of tumor growth and metastasis.

Chapter 4: Discussion

Conclusions

Metastasis is an insidious process which is responsible for much of morbidity and mortality associated with cancer. Methods to therapeutically target metastases often overlap with therapies effective at targeting the primary tumor. This paradigm neglects the heterogeneity of metastases, and can result in microscopic metastatic lesions which lie dormant before forming macroscopic metastatic lesions. The heterogeneity of metastases and the inability to fully eradicate them upon treatment with chemotherapy is a lingering problem in the realm of cancer treatment. To target metastases in an adjuvant manner, one approach is to target certain features which are gained throughout metastatic progression, such as the PNC. Another interpretation to solving the problem of metastasis is to re-introduce factors lost in metastatic progression, such as metastasis suppressor genes. Therapeutic re-introduction of metastasis suppressors and/or targeting of metastases-enriched structures such as the PNC could serve as a strategy for adjuvant therapy to prevent successful metastasis.

Chapter 2: Metastasis suppression by KP54 and non-KP54 kisspeptins

KISS1 is a secreted neuropeptide which suppresses metastasis in a broad spectrum of cancers (Beck & Welch, 2010; Nash et al., 2007). Upon secretion, KISS1 is cleaved by furin into kisspeptins at three dibasic sites (Harihar et al., 2014). This cleavage event releases several kisspeptins into the extracellular environment. One of these kisspeptins is KP54, the neuropeptide ligand to KISS1R, a $G_{q/11}$-protein coupled receptor (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001). However, expression of KISS1 has been documented to induce phenotypes inconsistent with KISS1R activation, and KISS1 can suppress metastasis in cells lacking KISS1R expression (W. Liu et al., 2014; Mitchell et al., 2006; Nash & Welch, 2006). These observations beget two hypotheses: that KP54 suppresses metastasis through paracrine

signaling, and that other kisspeptins resultant of furin processing are responsible for metastasis suppression. This study examined these hypotheses by investigating the metastasis suppressor capabilities of all ten kisspeptins resultant of furin cleavage.

One observation of note from this study was the observation that expression of KP54 is sufficient to suppress metastasis in lieu of KISS1R co-expression. This observation supplements the growing body of literature evidencing a stromal or paracrine mechanism of action for KP54-mediated metastasis suppression. While KP54 was not the focus of this study, future studies are advised to further examine the role of KP54 in metastasis suppression. Detectable levels of KISS1R have been reported in the stroma of both human and mouse lung tissue (Kotani et al., 2001). Future studies examining the necessity of stromal KISS1R would further elaborate on the relevance of stromal KP54:KISS1R interactions. This could be accomplished by utilizing an experimental metastasis assay in KISS1R-deficient or haplo-insufficient mice. Alternately, studies investigating the impact of KISS1R agonist (RF9) or antagonist (p234) treatment to animals bearing seeded lung metastases would shed further light on the relevance of KISS1R signaling in KP54-mediated metastasis suppression.

Kisspeptin-54 contains the characteristic RF-amide motif shared by other ligands of the neuropeptide family. Recent reports have identified promiscuous *in vitro* binding activities between ligands and receptors of the neuropeptide family, namely neuropeptide FF receptors 1 and 2 (Lyubimov et al., 2010; Milton, 2012; Milton, Chilumuri, Rocha-Ferreira, Nercessian, & Ashioti, 2012; Oishi & Fujii, 2016; Oishi et al., 2011). These neuropeptide receptors are G_s G-protein coupled receptors highly expressed throughout the central nervous system which modulate nocioception (Bonini et al., 2000; Elshourbagy et al., 2000; Hinuma et al., 2000). As melanocytes arise from embryonic neural crest cells, a potential explanation for KP54

suppressing metastasis in lieu of KISS1R co-expression is the aberrant expression of NPFFR1 or 2. However, QPCR analysis showed that the levels of NPFFR1 and 2 were low in C8161.9 human melanoma cells compared to SK-N-MC neuroblastoma control (NEW FIG?). Therefore, unless the effect of KP54 signaling through NPFFR1/2 is potent even at low levels, the mechanism of KP54-mediated metastasis suppression is more likely resultant of stromal KISS1R interactions.

The main data of interest from this study were the data demonstrating that non-KP54 kisspeptins suppress metastasis. Specifically, the N-terminal domain of KISS1, KMP2 in this study, was a potent suppressor of metastasis (Figure 2A, Figure 3, Figure 12B). This study is the first to not only examine kisspeptins lacking the KP54 domain, but to also characterize their impact on metastasis suppression. The N-terminal domain of KISS1 has been herein demonstrated to suppress metastasis *in vivo*, as well as several metastatic characteristics such as motility, melanosphere formation, and clonogenicity *in vitro*. This observation significantly redefines the field of study examining KISS1 as a metastasis suppressor by broadening the spectrum of metastasis-suppressing domains.

This study characterized both the impacts of KMP2 on *in vitro* characteristics of metastasis and on *in vivo* metastasis suppression. However, within the boundaries of this study, the exact mechanism of KMP2-mediated metastasis suppression remains unexplained. As KMP2 is a kisspeptin liberated outside of the cell by furin cleavage, a hypothesis for the mechanism of KMP2 metastasis suppression is that KMP2 is the ligand to a heretofore unidentified cell surface receptor. To answer this lingering question, an unbiased genome-wide CRISPR/Cas9 knockout screen was performed in KMP2-expressing cells (Appendix 1). Preliminary studies show that transduction with this genome-wide knockout library led to a loss of KMP2-induced metastasis

suppression (Appendix 1, Figure 2). Deep sequencing of lung metastases from this study will reveal genes silenced to recover metastatic proficiency. This study will identify relevant signaling molecules involved in the KMP2-mediated metastasis suppression pathway and shed light on this novel mechanism of metastasis suppression.

While the mechanism of metastasis suppression remains presently incomplete, KMP2 shows promise as a metastasis suppressor peptide. A translational application of this finding would be to use KMP2 as a basis for a therapeutic peptide. Peptides have inherent challenges as biological therapeutics, as they can be readily degraded by extracellular proteases. However, the field of stapled peptides, or peptide stabilized by a molecular brace, has gathered momentum in clinical trials. Staples "lock" peptides into a specific conformation, increase resistance to protease cleavage, and enhance cell permeability (Blackwell et al., 2001; Chang et al., 2013; Higueruelo, Jubb, & Blundell, 2013). Using KMP2 as a peptide backbone, addition of a staple to increase stability could support the concept of KMP2 as a possible anti-metastatic therapeutic peptide.

Chapter 3: Impact of metarrestin treatment on melanoma metastases

The studies discussed in chapter three outlined the efficacy of metarrestin, a small molecule capable of disassembling the PNC, treatment on disseminated melanoma metastases. Metarrestin has been found to be an effective inhibitor of metastasis in breast cancer and pancreatic cancer models (manuscript in preparation). In this experimental model of human melanoma, metarrestin treatment elicited a modest and statistically insignificant impact on metastasis outgrowth. While there was a slight trend towards metastasis suppression, these data were insignificant and clinically irrelevant, as metarrestin-treated animals still bore a large metastatic load (>100 metastases per animal). These data are not altogether unexpected,

however, given that we observed a low (8.2%) prevalence of PNC in C8161.9 (Chapter 3, Table 1).

Given the low abundance of PNC in C8161.9, however, metarrestin treatment was not wholly ineffective. Metarrestin treatment suppressed orthotopic tumor onset and growth. No visible metastases were observed in the lungs collected from tumor-bearing animals of either treatment group (data not shown). Similarly, pre-treatment of cells in vitro prior to cell seeding, followed by metarrestin in vivo treatment led to a robust suppression of metastasis. Metarrestin treatment in low-PNC models of metastasis may be most effective in larger cell populations rather than micro- or macroscopic metastases. A hypothesis from these data is that metarrestin treatment targets PNC-positive tumor cells with a higher propensity to metastasis, and ultimately suppresses spontaneous metastasis in this tumor model. To test this hypothesis, future studies would extend the timeframe of the experiment to observe for spontaneous metastases from metarrestin- or vehicle-treated animals with surgical resection of primary tumors.

The low degree of overall metastasis suppression observed by metarrestin treatment in the C8161.9 model of human metastatic melanoma does not negate the potential therapeutic benefit of metarrestin. Indeed, several challenges exist in the translation of drug development from mice to humans. Cell-line derived xenograph models targeting oncogenic proteins have low success in predicting efficacy in human treatments (Johnson et al., 2001). These human melanoma studies were performed in an immune-compromised mouse model, neglecting any possible interplay from the host immune system. Future studies would benefit from examining a spontaneous mouse model of cancer and metastasis, such as a MMTV-PYMT or HER2-Neu transgenic mouse model of breast cancer. These models would allow for observation of metarrestin treatment on spontaneous breast cancer tumors and metastasis in an immune-

competent animal. Additionally, maximum tolerated dose studies examined doses up to 125 mg/kg with no visible toxicity, suggesting that increased dosing could improve treatment efficacy without additional toxicity to the animals. Further characterization of metarrestin as a therapeutic molecule is necessary before moving into human studies.

Significance

These studies have shed further light on the complexity and viability of targeting and treating disseminated metastases. The study of KISS1 and its kisspeptin fragments has identified a novel metastasis suppressor domain, herein KMP2, and has characterized its ability to suppress metastasis. KMP2 is a 56-amino acid peptide capable of suppressing metastasis, enhancing mitochondrial function, and decreasing stemness and spheroid formation abilities of metastatic cells. This metastasis suppression seems to be completely independent of KISS1R activation. Further study of KMP2 and its specific mechanism of metastasis suppression will elaborate on its viability as the basis for an anti-metastatic therapeutic.

There exists a large and pressing need for better drugs to target metastases. Patients diagnosed with more advanced tumors or disseminated metastases generally have decreased long term survival. At the point of metastasis, patients are often considered beyond any measurable help or benefit of treatment. Treatments which could extend the progression-free survival of patients with metastatic cancers with minimal side effects are worthy of further study. Drugs which can extend the lifespan or quality of life for patients with advanced disease can be fast-tracked by the FDA. For example, Palbociclib (Ibrance) is a drug specifically catered to patients with metastatic ER+/HER2- breast cancer. This treatment doubled progression-free survival when administered in conjunction with letrazole, the standard of care and was subsequently fast-tracked to FDA approval by the granting of “breakthrough therapy” status. This example

demonstrates the potential benefit that anti-metastatic therapies can provide to a patient population in desperate need. Metarrestin and KMP2 both serve as examples of molecules with a large therapeutic potential which can help patients with metastatic disease. Further study of each of these molecules could lead to the development of effective and safe anti-metastatic therapeutic treatments.

Chapter 5: References

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Appendix I: A genome-wide CRISPR screen to identify KMP2 metastasis suppression signaling pathways

Introduction

Chapter 2 describes novel anti-metastatic activity by the N-terminal domain of KISS1, referred to as KMP2.KMP2 expression was demonstrated to suppress metastasis in vivo, suppress migration in vitro, and elevate mitochondrial function in vitro (Chapter 2). These characterizations demonstrated that KMP2 suppresses metastasis and metastatic traits in a manner comparable to full-length KISS1. This mechanism of metastasis suppression appears to be independent of KISS1R expression or stimulation, as KMP2 does not contain the LRF-amide binding motif required for KISS1R activation. However, ruling out KISS1R signaling leaves the question as to the functional mechanism of metastasis suppression utilized by KMP2.

As KMP2 and other kisspeptins are peptide fragments which require secretion to suppress metastasis, it is hypothesized that KMP2 signals through a receptor to induce metastasis suppression. This mechanism is suspected to be intracrine, as many of the metastasis suppressor phenotypes have been described *in vitro* without stromal interactions. However, receptor signaling is not the exclusive mechanism of signaling for extracellular peptides. To avoid the inherent bias of specifically investigating receptors, a genome-wide CRISPR/Cas9 knockout screen was performed in cells expressing KMP2. The results of this screen suggest that KMP2 utilizes complex signaling pathways to suppress metastasis.

Methods and Materials

Cell lines and culture

B16F10 is a melanotic melanoma cell line derived from a C57BL/6J mouse. These cells were cultured and injected into mice ten times to derive their name and enhanced metastatic characteristics (Fidler, 1973). Cell lines were cultured in a 1:1 ratio of Dulbecco's-modified

minimum Eagle Media and Ham's F12 media with 5% fetal bovine serum, 1% l-glutamine and 0.025% non-essential amino acids. Lentiviral constructs of KMP2-3XFLAG were in developed in pENTR1A vector through the Life Technologies Gateway platform and then recombined into pLenti PGK Puro DEST plasmid for stable expression (Addgene #w529-2). Cells were transduced with this plasmid and viral packing particles (Life Technologies ViraPower Viral Packaging Mix). To select for expressing clones, cells were transduced and selected with 2.5µg puromycin for 14 days. Resistant clones were isolated and tested for expression.

GeCKO CRISPR/Cas9 plasmid library preparation

Mouse GeCKOv2 CRISPR knockout pooled library was a gift from Feng Zhang and obtained through Addgene (catalog #1000000052) (Sanjana, Shalem, & Zhang, 2014; Shalem et al., 2014). Both library A and library B were used in this study, ensuring 6X redundancy in gene targeting. Libraries were amplified as per depositor's instructions. Briefly, Lucigen Endura electrocompetent cells (#60240) were electroporated, and antibiotic-free bacterial broth was used to plate electroporated bacteria on ampicillin agar plates. Plates were inverted and incubated at 37C overnight. Colonies were scraped and suspended in antibiotic-free broth before pelleting and weighing the pellet. DNA was isolated using one column of a Qiagen Maxiprep Spin Kit per 0.4g bacterial pellet. Plasmids from library A and library B were pooled upon plasmid purification.

Viral Library Preparation

293FT cells (Invitrogen) were used to generate lentiviral GeCKO library pools. 293FT cells were plated to reach 90% confluence. Cells were transfected with 3 µg pooled library plasmids and 9 µg of equimolar packaging mix (VSV-G and PAX2) using Lipofectamine 2000. Transfection

media was applied to 293FT cells and incubated overnight. The following day, transfection media was exchanged for fresh complete media. Twenty-four hours later, media was harvested and centrifuged at 5000G for 15 minutes at 4C. Viral supernatant was stored in cryovials at -80C until transduction.

Experimental metastasis assay

B16-F10 KMP2-3XFLAG clone 3 cells were tested for expression and plated in four 25cm plates. Three plates of cells were transduced at an MOI of 1 with the GeCKO library virus generated from 293FT cells. Cells were transduced, and maintained under selection for 7 days until injection. Cells were disassociated and suspended in ice-cold Hank's Buffered Saline Solution. For injection, 200 µl of cell suspension (5.0×10^4 cells) was injected into the lateral tail vein of 4-week-old female syngeneic C57BL/6J mice. Four mice received B16-F10 KMP2-3XFLAG clone 3 control cells to ensure metastasis suppression. Twenty mice received B16-F10 KMP2-3XFLAG + Library transduced cells. Mice were housed at 23ºC and given food and water ad libitum for two weeks. Upon euthanasia, gross lung metastases were quantified by light microscopy under a dissection microscope. Lungs were imaged and flash frozen using liquid nitrogen. Tissues were stored at -80C until processing.

Tissue preparation and genomic DNA extraction

200 mg samples from three lungs bearing a high metastatic load (>50 metastases per sample) were selected for genomic DNA extraction. DNA extraction was performed as per the depositor's protocol. Briefly, flash frozen tissues were ground using a mortar and pestle and digested using NK lysis buffer (50 mM Tris, 50 mM EDTA, 1% SDS, pH 8) and proteinase K overnight at 55°C. The next day, 30 µl RNase A (Qiagen) was added to each sample and

incubated 25 times before incubation at 37°C for 30 minutes. Samples were cooled on ice before adding 2ml chilled 7.5 M ammonium acetate to precipitate proteins. Samples were vortexed and centrifuged at 5000G for 20 minutes. Supernatant was decanted into a new tube, to which isopropanol was added and samples subsequently inverted. Samples were centrifuged at 5000G for 10 minutes. Supernatant was carefully aspirated from genomic DNA pellets and 70% ethanol was added to each tube. Samples were inverted and centrifuged at 5000G for 2 minutes. Supernatant was removed and carefully aspirated from the genomic DNA pellet. The pellet was left to air dry for 1 hour before adding 350 µl of elution buffer to each sample. Samples were incubated at 65°C for 20 minutes, then left at room temperature overnight. Samples were vortexed briefly and concentration was determined using a Nanodrop (Thermo Scientific). Samples were pooled and stored at -20°C for further analysis.

Library preparation and deep sequencing

To analyze the distribution of gRNA sequences present in genomic DNA, two steps of PCR were performed to amplify samples from pooled DNA samples. First, PCR v2 primers were utilized to add PCR priming sites to inserts (Table 1). PCR was performed using 7.5 µG genomic DNA as template DNA and Phusion Flash High Fidelity Master Mix PCR cycling conditions (ThermoFisher Scientific). Specific conditions for this reaction were annealing temperature of 55°C and 20 cycles. From the PCR mix, 5µl was utilized for the template basis of PCR#2. PCR#2 consisted of an equivalent mix of F1-F12 concatenated ultramer primers containing Illumina barcodes and the R1 concatenated primer (Table 2). Reactions were mixed using a 1:1 mixture of forward:reverse primers in 50 µL reaction volume, using Phusion Flash High Fidelity Master Mix. The reaction was cycled for 27 cycles to achieve appropriate band density (Figure 3) PCR products were then loaded into a 2% agarose gel and the 370 bp band was gel purified

Table 1

V2Adaptor_F	AATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCG
V2Adaptor_R	TCTACTATTCTTTCCCCTGCACTGTtgtgggcgatgtgcgctctg

Table 1. V2 primers used to add priming sites to CRISPR/Cas9 sequences integrated into genomic DNA sequences.

Table 2

R1	CAAGCAGAAGACGGCATACGAGATAAGTAGAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTtTCTACTATTCTTTCCCCTGCACTGT
F1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTtAAGTAGAGtcttgtggaaaggacgaaacaccg
F2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTatACACGATCtcttgtggaaaggacgaaacaccg
F3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgatCGCGCGGTtcttgtggaaaggacgaaacaccg
F4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTcgatCATGATCGtcttgtggaaaggacgaaacaccg
F5	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTtcgatCGTTACCAtcttgtggaaaggacgaaacaccg
F6	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTatcgatTCCTTGGTtcttgtggaaaggacgaaacaccg
F7	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgatcgatAACGCATTtcttgtggaaaggacgaaacaccg
F8	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTcgatcgatACAGGTATtcttgtggaaaggacgaaacaccg
F9	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTacgatcgatAGGTAAGGtcttgtggaaaggacgaaacaccg
F10	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTtAACAATGGtcttgtggaaaggacgaaacaccg
F11	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTatACCTGTATCtcttgtggaaaggacgaaacaccg
F12	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTgatAGGTCGCAtcttgtggaaaggacgaaacaccg

Table 2. Concatenated primers used to amplify CRISPR/Cas9 gRNA inserts from PCR #1 with primers from Table 1. Primers contain Illumina barcodes and a staggered sequence variation to avoid monotemplate issues during sequencing.

using the QiaQuick Gel Extraction Kit (Qiagen) and optimized conditions (Taiwo et al., 2012). Isolated DNA was eluted in water and checked for concentration using the Nanodrop.

Deep sequencing experimental planning was performed in collaboration with the KUMC Genomics Core. The Agilent Bioanalyzer was used to verify the quality of the gel extraction. Following this, QPCR was performed to verify the presence of Illumina adaptors on the isolated band. Validated samples will be sequenced using the Illumina Rapid Read 150 cycle sequencing procedure and a high concentration of PhiX (15-25%).

Results

Transduction with GeCKO library disrupts KMP2 metastasis suppression

Following intravenous injection and two weeks of incubation, lungs were evaluated for metastatic load (Figure 1). Mice receiving injections of B16-F10 KMP2-3XFLAG cells presented with no detectable metastases (n=4). These results are consistent with previous experiments (Figure 1A). However, mice bearing KMP2-3XFLAG + library transduced cells displayed a broad spectrum of metastatic load (Figure 1B). Four animals had zero metastatic burden. The remaining 16 animals bore a metastatic load. These data support the inherent redundancy in the GeCKO system. These data also support the hypothesis that there are several signaling effectors crucial to KMP2's mechanism of metastasis suppression, possibly through receptor activation or other means.

GeCKO sequences are detectable in lung metastases

Using isolated genomic DNA from three lungs bearing a high metastatic load, PCR was performed in two rounds to amplify the gRNA inserts from metastases. The first round of PCR, using v2 adaptor primers (Table 1) added priming sites to sequences. The second round of PCR

Figure 1

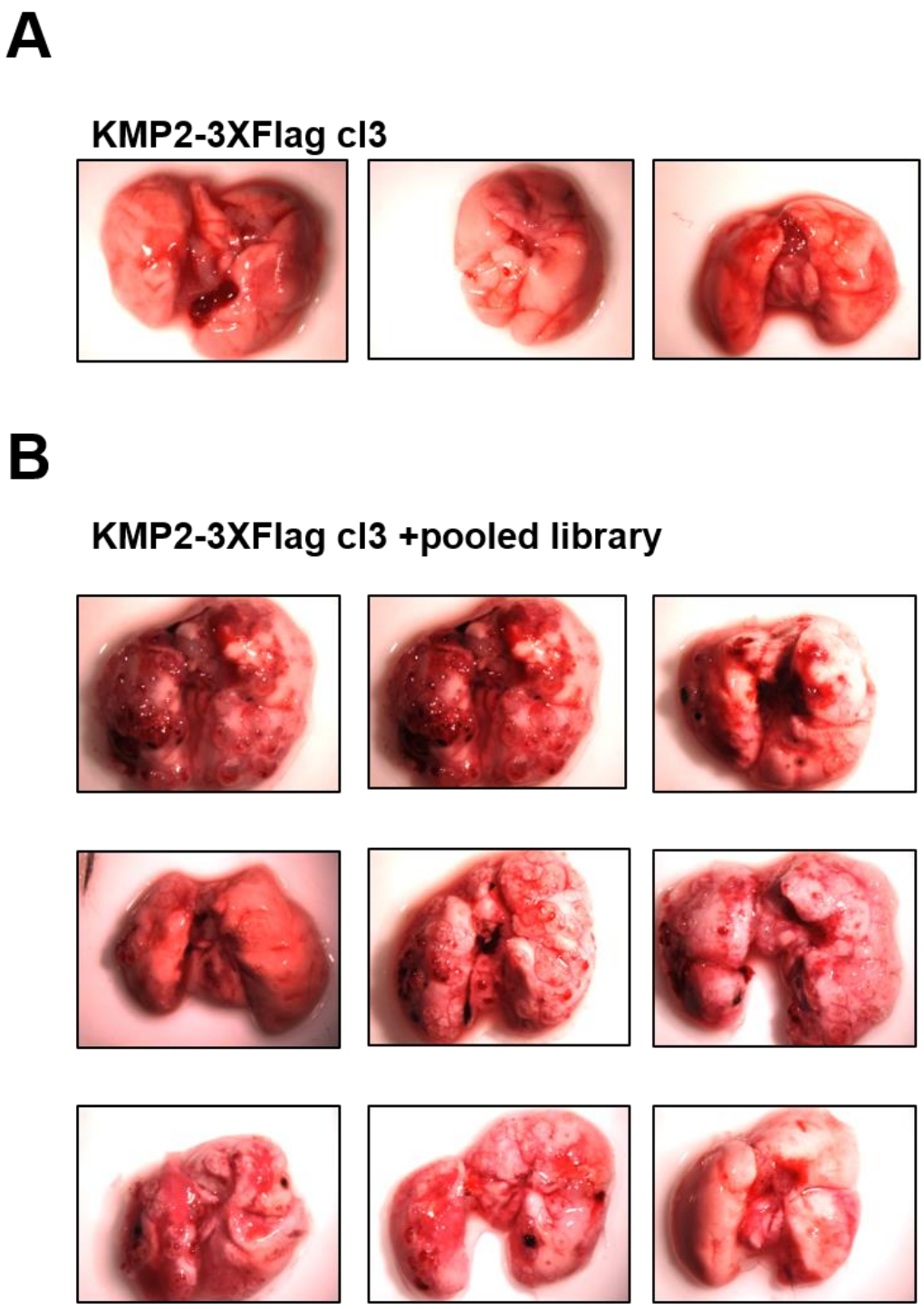

Figure 1: A, Representative photos of lungs from animals injected with B16-F10 KMP2-3XFLAG cells. These lungs bear no metastatic load. **B,** Representative lung photos from animals injected with B16-F10 KMP2-3XFLAG + library cells. Metastatic lesions vary in size. Most metastases are amelanotic, an anomaly for this cell line.

Figure 2

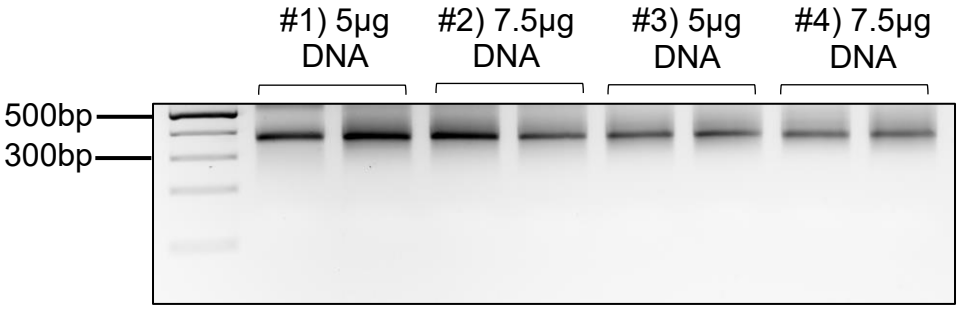

Figure 2: Agarose gel (2%) containing PCR products of PCR from two separate conditions: #1-2) 5 and 7.5 μg of genomic DNA input from PCR#1 reaction using 2X PCR Master Mix (ThermoFisher Scientific) and #3-4) 5 and 7.5 μg of genomic DNA input from PCR#1 reaction using 2X Phusion Flash High Fidelity Master Mix. Sample #4 was selected for use in sequencing reactions.

further amplified gRNA inserts and added Illumina adaptors to flank gRNAs (Table 2). The expected product from these consecutive rounds of PCR is 370 bp. This band was obtained and purified for sequencing (Figure 2).

Conclusions and future directions

The findings of this study indicate that KMP2 suppresses metastasis through an undoubtedly complex signaling pathway. Sequencing information produced from this study will be used to identify silenced genes present in metastases expressing KMP2-3XFLAG. Studies utilizing genome-wide knockout screens have been performed in both *in vitro* and *in vivo* platforms (Katigbak et al., 2016; Shalem et al., 2014). Results from this study will shed light on the mechanism of KMP2 metastasis suppression, but also genes which can regulate metastasis and override metastasis suppressor signaling.

Genes identified by this experiment are likely to be relevant KMP2 signaling effectors. However, the sheer volume of metastases detected suggests the presence of off-target hits. Despite the potent anti-metastatic impact of KMP2 expression, silencing of genes such as pro-apoptotic factors (Bcl-2 family proteins, caspases, etc.) or tumor suppressor genes (p53), or additional factors may be sufficient to overcome suppression. In order to discern off-target gene hits from KMP2 signaling gene hits, a number of strategies will be employed. Analysis of sequencing results will commence with determining the number of hits per gene identified. Genes targeted more frequently in more metastases will receive more attention during analysis due to abundance. Special attention will be given to cell surface molecules and receptors identified by this screen, due to the secreted nature of KMP2 and the hypothesis that KMP2 binds to a receptor to induce metastasis suppression. Next, pathway analysis will be performed

on the sequencing data set to identify signaling pathways shared by the identified genes. These strategies will stratify the data to define off target hits from KMP2-related hits.

Following the identification and characterization of impacted signaling pathways, these hits must be functionally validated. To confirm these hits, individual genes at the initiation of impacted signaling cascades will be targeted for knockout and rescue studies. Additionally, receptors and cell surface molecules identified by this screen will be selected for further analysis. Individual CRISPR/Cas9 targeting vectors will be developed to target these selected genes, and these knockouts will be developed in KMP2 expressing cells. Following the development of these knockout lines, cells will be injected into mice via experimental metastasis assay to evaluate metastatic efficiency. If these genes are critical for metastasis suppression, the silencing of the individual gene in the presence of KMP2 should be sufficient to prevent metastasis suppression. Subsequently, a rescue plasmid will be introduced into knockout lines which does not contain the CRISPR/Cas9 gRNA target sequence to recover expression of the gene of interest. These clones will be used to perform additional experimental metastasis assays to validate the recovery of metastasis suppression.

In conclusion, this study has conducted an unbiased genome-wide CRISPR screen to identify signaling pathways involved in KMP2-induced metastasis suppression. The data generated by this study indicate that there are a broad number of signaling effectors critical for metastasis suppression. These studies will further elaborate on the mechanism by which KMP2 suppresses metastasis.