

ONCOLYTIC VESICULAR STOMATITIS VIRUS INHIBITS CANCER CELL
INVASION PROCESSES

A Thesis
by
DAKOTA W. GOAD

Submitted to the Graduate School
at Appalachian State University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

August 2017
Department of Biology

ONCOLYTIC VESICULAR STOMATITIS VIRUS INHIBITS CANCER CELL
INVASION PROCESSES

A Thesis
by
DAKOTA W. GOAD
AUGUST 2017

APPROVED BY:

Maryam Ahmed, Ph.D.
Chairperson, Thesis Committee

Darren Seals, Ph.D.
Member, Thesis Committee

Chishimba Mowa, Ph.D.
Member, Thesis Committee

Zack Murrell, Ph.D.
Chairperson, Department of Biology

Max C. Poole, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Copyright by Dakota W. Goad 2017
All Rights Reserved

Abstract

ONCOLYTIC VESICULAR STOMATITIS VIRUS INHIBITS CANCER CELL INVASION PROCESSES

Dakota W. Goad
B.S. Appalachian State University

Chairperson: Dr. Maryam Ahmed

Vesicular stomatitis virus (VSV) is currently being investigated as a candidate oncolytic agent due to its capacity to kill cancer cells while exhibiting low virulence *in vivo*. My project investigates the impact of VSV on cancer cell invasion mechanisms. A main trigger for cancer metastasis is the formation of actin-rich structures known as invadopodia, which function in extracellular matrix degradation. Studies have shown that VSV can manipulate the cytoskeletal structure of cancer cells to potentiate viral replication. I hypothesized that VSV would alter invadopodia structures and inhibit their proteolytic function due to the global ability of VSV to inhibit host gene expression in infected cells. My results showed that, following infection with VSV, Src-transformed fibroblast cells formed lowered numbers of invadopodia structures, but yielded increased numbers of ring shaped invadopodia superstructures called rosettes. Overall, there was a decreased ability of these cells to degrade the extracellular matrix and express the invadopodia marker protein, Tks5, both of which correlated with the suppression of invadopodia formation. These results suggest that the alteration in invadopodia

formation by VSV is associated with the ability of the virus to decrease proteolytic degradation of the extracellular matrix. Interestingly, we found a noticeable increase in cortactin expression which may be related to the increased formation of rosette structures at later times post-infection. Our results suggest that manipulation of the cytoskeletal elements by VSV may be a mechanism by which the virus potentiates the infection process but may lead to the downstream effect of altering proteolytic activity of cancer cells to inhibit the invasion process.

Acknowledgments

I would like to thank Dr. Maryam Ahmed for her astute direction, and unyielding support throughout this entire project. I would like to acknowledge Dr. Nathan Mowa and Dr. Darren Seals for their guidance and expertise for the duration this project. I thank the Appalachian Office of Student Research for their funding which made most of this project possible. A warm thanks goes to Dylan Fehl, Brittany DeVasure, and Jack Leff for all of their countless hours in the lab helping move this project along.

Dedication

I would like to dedicate this to my father, Guthrie Wayne Goad. Words cannot convey all of the love and support you have given me throughout not only my academic career, but my entire life. Thanks Dad, this one is for you.

Table of Contents

Abstract.....	iv
Acknowledgments.....	vi
Dedication.....	vii
Foreword.....	ix
Chapter 1: Literature Review.....	1
Chapter 2: Results and Discussion.....	22
Chapter 3: Summary and Limitations.....	52
Vita.....	61

..

Foreword

Chapter 2 of this thesis will be submitted to the *Virus Research* journal, an international peer-reviewed journal owned by Elsevier B.V.; it has been formatted according to the style guide for that journal.

Chapter 1

Tumor Development

The formation of tumors, or tumorigenesis, is a process involving multiple steps and is dependent on an accumulation of mutations within cells of a specific tissue. Often tumor cells exhibit many mutations; however, only a small subset are responsible for neoplastic development (Knudson, 2001). Such mutations give rise to altered homeostasis as the transformed cells gain fitness via sustained proliferation while decreasing the rate of apoptosis and generating a pro-growth environment.

Tumors arise often, especially in older vertebrates, but most pose little threat to the host because they are localized or are of small size. Such tumors are considered benign. It is typically apparent when a tumor is benign because it contains cells that resemble and may function like normal cells. The surface interaction molecules that hold tissues together keep benign tumor cells, like normal cells, localized to their respective tissues (Lodish et al., 2000). In contrast, the cells composing a malignant tumor express some proteins characteristic of the cell type from which it arose, and a high percent of the cells grow and divide more rapidly than normal. Some malignant tumors remain localized, at least for a time. Most, however, do not remain in their original site but instead invade surrounding tissues, get into the body's vascular system, and set up areas of proliferation away from the site of their original appearance, a process called metastasis. Metastasis and the subsequent growth of tumors in other organs is the most life-threatening aspect of cancer as a disease. Cells must undergo many alterations, both genotypically and phenotypically, in order to acquire the ability to metastasize (Lodish et al., 2002).

It has long been recognized that the wound healing process and tumorigenesis are closely related. The mechanisms that regulate wound healing have been shown to promote transformation and growth of malignant cells (Dvorak, 1986). Inflammatory processes that occur during normal wound healing have been linked to the pathological state of many tumors. Normal epithelial tissue exists in a state of homeostasis where tissue regeneration is closely regulated by epithelial stem cells located within highly specialized niches. During tissue injury, replenishment of epithelial cell loss is warranted by the proliferation of these stem cells and their progeny in response to proinflammatory cytokines (Eming et al., 2007). Additionally, numerous potent growth factors are released by macrophages and lymphocytes during wound healing that promote stem cell proliferation and plasticity. Among these are morphogens that are commonly associated with embryonic development (Blanpain and Fuchs, 2014). Tissue injury induces immediate recruitment of neutrophils, which are later replaced by macrophages and lymphocytes. Infiltrating leukocytes play a major role in secretion of inflammatory cytokines, growth factors, and chemokines, which stimulate proliferation of progenitor cells and recruitment of keratinocytes and endothelial cells during the proliferative phase of wound healing (Eming et al., 2007). At this stage, granulation tissue forms, angiogenesis is induced, and new extracellular matrix (ECM) is secreted. Epithelial cells undergo epithelial–mesenchymal transition (EMT) and migrate to the edges of the wound to impart re-epithelialization of the damaged tissue. During development/wound healing, some cells can switch from an epithelial state to a mesenchymal state. This EMT program is tightly orchestrated by a number of cellular and molecular events. During tumorigenesis, the EMT may increase the motility and invasiveness of cancer

cells, and malignant transformation may actually be associated with signaling pathways that induce EMT (Boyer et al., 2000). Various processes associated with the EMT during tumor progression closely resemble those that occur during normal development/wound healing. It appears however that the molecular programs which lead to EMT during tumor progression are only a small percent of factors that induce full-fledged EMT in development/wound healing (Bellacosa et al., 2005). Invasion into surrounding tissues by cancer cells and metastasis to distant organs have been found to be critically dependent on the acquisition of EMT features in the initial cancer cells. (Kang and Massagué, 2004).

The first crucial step in the process of invasion is the movement of cancer cells into tissue surrounding the tumor and the vasculature. The best understood cancer cell motility mechanism is mesenchymal cell motility. It is estimated that between 10% and 40% of carcinomas undergo an EMT and use this form of motility (Thiery et al., 2009). The EMT in cancer cells is associated with continuous changes in gene expression driven by the activation of important transcription factors like Twist1 and the Snail/Slug transcriptional repressors (Oft et al., 2002). This process results in a phenotype characterized by an elongated cell morphology with established cell-polarity and is dependent upon proteolysis to degrade the ECM. Activation of RTKs such as PDGFR α and c-Met is often the initiating event for mesenchymal motility (Zhang and Vande Woude, 2003). Subsequently, phosphatidylinositol 3, 4, 5-triphosphate (PtdIns (3, 4, 5) P3) is generated at the leading edge of the cell and activates the GTPase Rac, and recruits the Scar/WAVE family proteins and the Arp2/3 complex (Ridley et al., 2003). The Arp2/3 complex then can nucleate the formation of new filamentous actin filaments.

Oncogenic Ras proteins can also initiate these series of events by activating PtdIns-3 kinase, leading to elevated levels of PtdIns (3, 4, 5) P3. Additionally, the tumor suppressor PTEN can stimulate PtdIns (3, 4, 5) P3. Levels of Cdc42 and the recruitment of adaptor proteins can also promote actin polymerization (Pollard and Borisy, 2003). Together, these events lead to the formation of an actin-rich protrusions called invadopodia.

Invadopodia Formation and Regulation

One of the most important steps in cancer cell invasion is the development of proteolytic invadopodia structures. Invadopodia are important actin-rich protrusions of the plasma membrane from the ventral surface of the cell that are associated with degradation of the ECM during cancer invasion. After the extension of the invadopodia protrusions, small integrin-dependent focal contacts are formed which attach the new protrusions to the ECM. Some focal contacts then develop into large focal adhesions that enable actomyosin contractile force to be transmitted to the ECM (Ridley et al., 2003). The directionality of cell movement is maintained by Cdc42, which coordinates actin polymerization at the front of the cell with microtubule attachment and alignment (Ridley et al., 2003). These mesenchymal cells are then able to move through the ECM space by using proteases, such as MMPs and urokinase-type plasminogen activators that degrade ECM proteins. The formation of these invadopodia structures is driven by actin polymerization at the leading edge. Malignant tumor cells often show excessive cell protrusive activity due to irregular activation of signaling pathways that regulate actin cytoskeletal rearrangement (Wang et al., 2004).

Being an actin-based structure, invadopodia involve a large number of structural and regulatory proteins that control actin dynamics, such as Arp2/3, Ena/Vasp, and various small GTPases. Cortactin, MENA, and Tks5 also play crucial roles at invadopodia and have been implicated in tumor progression. Cortactin and MENA are both vital players in actin polymerization dynamics, so consequently their roles in tumor invasion and metastasis go beyond invadopodia to general cell migration and other actin-based cellular processes. In contrast, Tks5 is known to be more specifically involved in invadopodia formation, so its impact on tumor invasion and metastasis is thought to be largely due to its function at invadopodia (Paz et al., 2014). Given their fundamental function to recruit proteases to cell-matrix contacts for matrix remodeling, invadopodia are known to contain a large numbers of proteases. The proteases found at invadopodia include metalloproteases (both secreted and membrane-tethered MMPs), the ADAM (a disintegrin and metalloproteinase) family members, and membrane-bound serine proteases, all of which have been implicated in cancer progression and metastasis (Paz et al., 2014). The scaffolding protein Tks5 has been of particular interest during the course of this project. Tks5 has been shown to localize to and be required for the formation of invadopodia (Seals et al., 2005). Many binding partners of Tks5 have been discovered that link Tks5 to actin remodeling proteins and pericellular proteases such as ADAMs family metalloproteases and adaptor proteins Grb2 and Nck2 (Abram and Courtneige, 2003). It has been found that by siRNA knockdown of Tks5 significantly impaired the invasiveness and tumorigenicity of cancer cells (Blouw et al., 2008; Seals et al., 2005). Studies so far suggest that invadopodia-mediated cell invasion may be critical for not just progression of the metastatic cascade, but the growth of the tumor as well.

The EMT program, including the formation of invadopodia, has been shown to be orchestrated by a group of transcription factors, all of which have been implicated in tumor invasion and metastasis (Thiery et al., 2009). The transcription factor Twist1 has been shown to play critical roles in tumor metastasis in multiple tumor models (Yang et al., 2004). Twist1 was previously known to be involved in mesoderm differentiation and neural crest migration (Leptin and Grunewald, 1990) but has also been identified in a microarray-based screen for genes implicated in metastasis (Kang and Massagué, 2004). Twist1 was shown to be required for ECM invasion by inducing the formation of invadopodia. Twist1 was also found to directly induce expression PDGFR α , which then activate other kinases to promote invadopodia formation (Eckert et al., 2011). PDGFRs are cell surface tyrosine kinase receptors (RTKs) for members of the platelet-derived growth factor family. PDGFRs are important factors regulating cell proliferation, cellular differentiation, cell growth, development, and highly implemented in tumorigenesis and metastasis (Williams et al., 1988). Due to the induction of PDGFR by Twist1, Twist1 has been uncovered as a crucial upstream regulator in the formation of invadopodia. Further evidence shows that by knocking down key factors induced by Twist1 is associated with a block in invadopodia formation. Twist1 therefore represents a direct link between invadopodia and metastasis.

As mentioned briefly earlier, PDGFRs may activate a family of kinases which then leads to the formation of invadopodia structures. The Src family of protein tyrosine kinases (SFKs) are a family of kinases that play key roles in regulating signal transduction downstream of diverse set of cell surface receptors. Members of the Src family show a conserved domain organization, which includes a myristoylated N-

terminal segment, followed by SH3, SH2, linker and tyrosine kinase domains, and a short C-terminal tail (Bromann et al., 2004). The ability of oncogenic forms of SFKs to induce cell transformation is due to their role in regulating cell growth. SFKs promote signaling from growth factor receptors in multiple ways, including the direct participation in pathways required for DNA synthesis as well as controlling the turnover of cell surface receptors, modifying actin cytoskeleton rearrangements and promoting cell motility and survival (Bromann et al., 2004). The basic function of Src is the transmission of external signals to the cell interior, which is accomplished by phosphorylating tyrosine residues on substrates downstream of RTKs and integrins. Notably, there is evidence that Src orchestrates tumor invasion and progression by acting on these two fundamental pathways (Abram and Courtneige, 2000). One of the most apparent phenotypes of Src-transformed cells is the formation of invadopodia. These structures are made by metastatic cancer cells and Src-transformed fibroblasts (Linder et al., 2009). It has been shown that Src activity is necessary for invadopodia formation and function, and the level of tyrosine phosphorylation at invadopodia positively correlates with the degree of ECM degradation (Bowden et al., 2006). The molecular components that make up invadopodia include proteins that facilitate actin assembly, membrane trafficking, and focal degradation. Src substrates participate in all of these functions and include the proteins cortactin (Bowden et al., 1999) N-WASp (Yamaguchi et al., 2005) Dynamin-2 (Baldassarre et al., 2003) and Tks5 (Seals et al., 2005) among others. Studies have evaluated Src activity in invadopodia formation through the ectopic expression of constitutively active Src (Stylli et al., 2009). However, these activating Src mutants are rarely found in human tumors, which instead typically contain increased levels of wild-

type (WT) Src expression and/or aberrant WT Src activity due to hyperactivation of upstream pathways (Yeatman, 2004). The exact role of WT Src in invadopodia formation and function is relatively unknown. However, we do know now that during EMT, Twist1 is an upstream activator of Src through the activity of PDGFRs.

Another potent inducer of EMT is transforming growth factor beta (TGF β). Activation of TGF β leads to the activation of different downstream substrates and regulatory proteins, inducing transcription of different target genes that function in differentiation, chemotaxis, proliferation, and activation of many immune cells (Massagué, 2012; Thiery et al., 2009). TGF β promotes EMT by activating extensive intracellular signaling that involves Smad proteins, ERK, and Jagged/Notch signaling, which are important factors for growth and development. Of particular interest in the context of invadopodia, Eckert and colleagues showed that silencing Twist1 blocked the ability of TGF β to induce PDGFR α as well as invadopodia formation (Eckert et al., 2011). In addition, Pignatelli et al. (2012) demonstrated that Hic-5 (a focal adhesion adaptor protein induced by TGF β) localized to invadopodia in TGF β -treated human epithelial breast cancer cells. They showed that Hic-5 was phosphorylated upon TGF β stimulation and that this phosphorylation was required for TGF β -induced invadopodia formation and invasion, thereby further emphasizing the role of EMT-inducing genes in invadopodia regulation.

Role of Immune Cells in Invasion

In addition to transcription factors, immune cells infiltrating the tumor microenvironment play key roles in the invasion process. In the final phase of wound

healing, wound contraction and differentiation of fibroblasts to myofibroblasts result in the formation of fibrous (scar) tissue (Singer and Clark, 1999). Likewise, chronic inflammation has been linked to tumorigenesis, tumor progression, and metastasis in many different cancers. The tumor microenvironment and chronic wounds share many features. Infiltrating leukocytes present within the tumor and associated stroma stimulate tumor growth, invasion, and angiogenesis. Tumor-associated macrophages (TAM), tumor-associated dendritic cells (TADC), and tumor infiltrating lymphocytes are a source of proinflammatory mediators in the tumor microenvironment. Environmental stimuli such as hypoxia and DNA damaging agents stimulate secretion of chemokines from tumor cells that recruit pro-tumor inflammatory cells and help to shape the pro-tumor immune response (Qian and Pollard, 2010). The relationship between the wound healing process and tumorigenesis is complex with many factors and stages. Actually, it is now known that TAMs promote cancer progression and metastasis (Pollard, 2004) and a paracrine interaction between tumor cells and macrophages has been identified *in vivo* that is required for invasion and intravasation (Wyckoff et al., 2004). Clinical studies have shown that the presence of TAMs in primary tumors correlates with poor prognosis in several human cancers (Goede et al., 1999; Leek et al., 1996). TAMs secrete a variety of growth factors, chemokines, and matrix-modifying enzymes that facilitate carcinoma cell migration and invasion, angiogenesis, and matrix remodeling. Macrophages are known to form podosomes that are similar to invadopodia in molecular composition (Linder, 2009). Because podosomes are usually observed at the leading lamella in migrating macrophages, they are thought to function as adhesion structures in cell motility and chemotaxis (Evans et al., 2003). When cultured on fluorescently labeled

gelatin coverslips, primary macrophages derived from mouse bone marrow form podosomes that organize into large ring shaped clusters called rosettes. These structures also degrade the underlying gelatin matrix. As macrophages release MMPs, they have been thought to act as producers of proteinases that infiltrate into and degrade the ECM. In addition to producing MMPs, macrophages have been shown to directly degrade ECM through podosomes. This matrix degradation seems to be a conserved function of podosomes as it has been observed in various other cell types including smooth muscle cells, endothelial cells, and transformed fibroblasts (Burgstaller and Gimona, 2005). The podosome mediated migration and breakdown of ECM by TAMs, subsequently releases sequestered growth factors that help feed a positive feedback loop during cancer progression. Indeed, the ability of cells to form podosomes/invadopodia structures is critical for the metastatic process, thus revealing a sexy target for therapeutic strategies against metastatic cancers.

Do all invasive cancer cells need invadopodia structures to invade surrounding tissues? Numerous studies have shown that areas of matrix degradation spatially correlate with invadopodia structures. This could be due to the proteolytic nature of invadopodia structures. Invadopodia have been shown to exhibit an association of integrins along with membrane type-1 matrix metalloproteinase (MT1-MMP) and not just active enzymes like MMP-2 and MMP-9 alone. It has been shown that reducing the proteolytic activity in invadopodia with the MMP-9 and MMP-2 inhibitor batimastat (BB-94) decreases invasion significantly. This suggests that cancer cell invasion is dependent upon proteolytically active invadopodia (Kelly et al., 1998). The necessity of invadopodia during the course of invasion creates a need for more targeted therapeutic

strategies that can either inhibit their formation, or render them incapable of proteolytic degradation.

Cancer Therapies

Many therapeutic options for cancer exist. The primary therapies include surgery, radiation therapy, hormonal therapy, chemotherapy, targeted therapy and palliative care. What treatments are used can depend on the location, type, and grade of the cancer, including the overall health of the patient. The treatment goal may or may not be curative. Complete removal of the cancerous tissue without damaging other parts of the body is the ideal outcome of treatment that can sometimes be accomplished by surgery, but the tendency of cancers to invade adjacent tissues or to spread to distant sites by microscopic metastases often limits its effectiveness, while chemotherapy and radiotherapy can have adverse effects on normal, non-cancerous cells. Therefore, treatment with non-negligible adverse effects may be accepted in some cases; and besides curative intent, practical aims of therapy can also include suppressing the cancer to a subclinical state and maintaining that state for a decent quality of life and palliative care without curative intent for advanced-stage metastatic cancers (Uppuluri et al., 2015).

Cancer is not actually a single disease, but a class of diseases; therefore, it is unlikely that there will ever be a single cure for cancer any more than there will be a single treatment for all infectious diseases. The treatment options depend on the type of cancer, stage of the disease, whether or not the cancer has spread, and treatments administered in the past and the patient's general health. Stages II-IV cancers are differentiated on the

degree to which the tumor has spread. Stages II and III indicate larger cancers or tumors that have grown deeper into nearby tissue. They may have also spread to lymph nodes but not to other parts of the body. Stage IV cancer is used to classify cancer that has metastasized to other organs of the body (Donohoe et al., 2017). Once cancer spreads, it can be extremely difficult to control. Although some types of metastatic cancer can sometimes be cured with current treatments, most cannot. The goal of these treatments however is to stop or slow the growth of the cancer and to relieve symptoms caused by it, to both prolong and increase the quality of the patients' life. Many late stage cancer patients have endured unforgiving chemotherapy treatment regimens with only minimal benefits, or worse, to discover the cancer was completely unresponsive. When accepting common treatments, most patients are not aware that chemotherapy is effective in only 2% of late stage cancers after a five-year period. Despite the National Cancer Institute's forty years of scientific research, stage III and IV chemotherapy-driven cancer treatments have not progressed. In fact, the treatments typically do not work. Therefore, it is critical that more effective, targeted treatment options are developed (Faguet, 2005).

Oncolytic Viruses

Recently, oncolytic viruses (OVs) have been studied as potential cancer therapeutics, a field known as oncolytic virotherapy. Oncolytic virotherapy is centered on the preferential replication of viruses in cancer cells and their subsequent spread within a tumor without causing damage to normal, non-cancerous tissue (Russell et al., 2012). This results in multiple self-perpetuating rounds of infection, replication, lysis and spread throughout the tumor, sparing host non-tumor cells. In addition, virus replication within a tumor may help stimulate the immune system by prompting the release of

cytokines and by liberating tumor antigens (Ries et al., 2000). Genetic manipulation has enabled for the rapid expansion of OV's in the recent past, giving rise to a broader range of potentially pathogenic viruses to be engineered for targeting and safety (Cattaneo et al., 2008). Some of hallmarks of cancer (Hanahan and Weinberg, 2011) provide a permissive environment for OV replication; including sustained proliferation, avoidance of apoptosis, evasion of growth suppressors, genomic instability and evasion of the immune system. This may be possible because in many cases, proliferative and antiviral signaling pathways are mutually antagonistic. Hence, development of enhanced proliferative signaling in tumor cells may lead to suppression of antiviral pathways. For example, it is now known that most tumor cells are resistant to the antiproliferative effects of interferons (IFNs) (Einhorn and Strander, 1993) and actually, several different defects in the IFN signal transduction pathway have been identified in different types of tumors (Wong et al., 1997). The resistance of tumor cells to the antiproliferative effects of IFNs may make cancer cells compatibly more permissive to infection with a variety of viruses, including vesicular stomatitis virus (VSV). Many studies have demonstrated the ability of OV's to preferentially destroy cancer cells *in vitro* and *in vivo*, however; the effects of OV's on the invasive mechanisms of cancer cells, including invadopodia formation and protease activity, remain unclear.

VSV is a rhabdovirus that primarily infects cattle, horses and swine in the wild, but can also infect humans amongst other species (Mead et al., 2000). The two main serotypes found in the Americas are New Jersey (VSV-NJ) and Indiana (VSV-Ind), whereby the New Jersey serotype causes most clinical diseases. Cases of human infection from VSV are rare, however, if infected; it is usually asymptomatic with

occasional reports of mild-like flu symptoms (Roberts et al., 1999). VSV is a negative sense RNA virus which replicates entirely in the host cell's cytoplasm. The small 11kb genome of VSV consists of a single strand of RNA, which is completely coated by the viral nucleoprotein. In the course of infection, VSV synthesizes five sub-genomic mRNAs that encode its five different proteins (Fields, 1996).

The nucleoprotein, phosphoprotein, large polymerase protein along with other specific host cell proteins are responsible for both viral transcription and replication. In addition, viral binding to target cells is mediated by the viral glycoprotein, and the matrix protein (229 amino acids) has a crucial role in the viral assembly and budding, and plays a critical role in early phase infection, aiding the virus to avoid host antiviral responses (Black and Lyles, 1992). This is accomplished by two mechanisms: interruption of host cell transcription and the blocking of mRNA export from the nucleus. In both cases, the role of the matrix protein is to inhibit the expression of the host cell's antiviral gene products, enabling the virus to replicate unhindered (Black and Lyles, 1992). In healthy humans, VSV may at first enter and infect host cells, but initiation of the innate and adaptive immune responses will suppress viral replication and clear out infected cells. So while the sensitivity of malignant cells to VSV may be due to a higher propensity to apoptose, it is most likely their inherent defects in innate immune signaling pathways (due to the general genomic instability of malignant cells and the high levels of proliferative signaling pathways) that allow for unchecked viral replication and infection (Parato et al., 2005). Cancer cells are known to generally have defective immune signaling (Rabinovich et al., 2007), which is important for proliferation and escape from host immunosurveillance. In addition, it has been shown that the defective

immune regulatory system, including dysregulation of interferon activation, is an important factor in regulating replication of VSV in cancer cells (Stojdl et al., 2000). Also, cancer cells can proliferate continuously due to deregulated translation which subsequently allows for high levels of viral protein products and replication within the cells, leading to cell death (Balachandran and Barber, 2004). The ability of VSV to preferentially replicate in and subsequently kill malignant cells has been well established in cultured cells as well as mouse xenografts of human cancer cells (Balachandran et al., 2001; Diaz et al., 2007; Ebert et al., 2003; Ebert et al., 2005). Additional studies into the biology of VSV, its oncolytic activity, and its regulation in malignant cells as well as additional testing in animal models for efficacy and safety, may bring VSV oncolytic therapy closer for treatment of cancer patients. For the experiments presented within this project, recombinant virus obtained from an infectious cDNA clone containing a wild-type (wt) M protein (rwt virus) was compared to an isogenic recombinant having a single point mutation that substitutes an arginine for methionine at position 51 of the 229 amino acid M protein (rM51R-M virus). That point mutation renders the virus defective in its ability to inhibit host gene expression, but does not compromise the expression of viral genes or the production of infectious progeny (Ahmed et al., 2003; Kopecky et al., 2001). One of many potential concerns when administering oncolytic viruses as treatments is the associated toxicity, which could be lethal in immune compromised individuals. The rM51R mutant virus is an exciting oncolytic option because not only is the virus avirulent and safer than its intact M protein counterpart (VSV rwt), but also has the ability to induce a tumor specific immune response due to its inability to shut down host gene expression (Ahmed et al., 2003).

In the context of this project, we sought to determine the effects of both rwt and rM51R-M VSV infection on the invasive mechanisms of Src transformed NIH 3T3 murine fibroblasts. This included assessing overall cell viability post infection with VSV, testing the ability of VSV to alter the morphological characteristics of invadopodia structures including subsequent ECM degradation, and a screen for expression of many critical proteins necessary for invadopodia assembly and maturation.

References

- Abram, C.L., Seals, D.F., Pass, I., Salinsky, D., Maurer, L., Roth, T.M., Courtneidge, S.A., 2003.** The adaptor protein fish associates with members of the ADAMs family and localizes to podosomes of Src-transformed cells. *Journal of Biological Chemistry*, 278(19), 16844-16851.
- Abram, C.L., Courtneidge, S.A., 2000.** Src family tyrosine kinases and growth factor signaling. *Experimental Cell Research*, 254(1), pp.1-13.
- Ahmed, M., McKenzie, M.O., Puckett, S., Hojnacki, M., Poliquin, L., Lyles, D.S., 2003.** Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. *Journal of Virology*, 77(8), 4646-4657.
- Balachandran, S., Porosnicu, M., Barber, G.N., 2001.** Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis. *Journal of Virology*, 75(7), 3474-3479.
- Balachandran, S., Barber, G.N., 2004.** Defective translational control facilitates vesicular stomatitis virus oncolysis. *Cancer Cell*, 5(1), 51-65.
- Baldassarre, M., Pompeo, A., Beznoussenko, G., Castaldi, C., Cortellino, S., McNiven, M.A., Luini, A. and Buccione, R., 2003.** Dynamin participates in focal extracellular matrix degradation by invasive cells. *Molecular Biology of the Cell*, 14(3), 1074-1084.
- Black, B.L. and Lyles, D.S., 1992.** Vesicular stomatitis virus matrix protein inhibits host cell-directed transcription of target genes in vivo. *Journal of Virology*, 66(7), 4058-4064.
- Blanpain, C., Fuchs, E., 2014.** Plasticity of epithelial stem cells in tissue regeneration. *Science*, 344(6189), 1242281.
- Blouw, B., Seals, D.F., Pass, I., Diaz, B., Courtneidge, S.A., 2008.** A role for the podosome/invadopodia scaffold protein Tks5 in tumor growth in vivo. *European Journal of Cell Biology*, 87(8), 555-567.
- Boyer, B., Vallés, A.M., Edme, N., 2000.** Induction and regulation of epithelial–mesenchymal transitions. *Biochemical Pharmacology*, 60(8), pp.1091-1099.
- Bowden, E.T., Onikoyi, E., Slack, R., Myoui, A., Yoneda, T., Yamada, K.M., Mueller, S.C., 2006.** Co-localization of cortactin and phosphotyrosine identifies active invadopodia in human breast cancer cells. *Experimental Cell Research*, 312(8), 1240-1253.
- Bowden, E.T., Barth, M., Thomas, D., Glazer, R.I., Mueller, S.C., 1999.** An invasion-related complex of cortactin, paxillin and PKC μ associates with invadopodia at sites of extracellular matrix degradation. *Oncogene*, 18-31.
- Bromann, P.A., Korkaya, H., Courtneidge, S.A., 2004.** The interplay between Src family kinases and receptor tyrosine kinases. *Oncogene*, 23(48), 7957-7968.

- Burgstaller, G., Gimona, M., 2005.** Podosome-mediated matrix resorption and cell motility in vascular smooth muscle cells. *American Journal of Physiology-Heart and Circulatory Physiology*, 288(6), H3001-H3005.
- Cattaneo, R., Miest, T., Shashkova, E.V., Barry, M.A., 2008.** Reprogrammed viruses as cancer therapeutics: targeted, armed and shielded. *Nature Reviews Microbiology*, 6(7), 529-540.
- Diaz, R.M., Galivo, F., Kottke, T., Wongthida, P., Qiao, J., Thompson, J., Valdes, M., Barber, G., Vile, R.G., 2007.** Oncolytic immunovirotherapy for melanoma using vesicular stomatitis virus. *Cancer research*, 67(6), pp.2840-2848.
- Donohoe, K.J., Cohen, E.J., Giammarile, F., Grady, E., Greenspan, B.S., Henkin, R.E., Millstine, J., Smith, G.T., Srinivas, S., Kauffman, J. and Ahuja, S., 2017.** Appropriate use criteria for bone scintigraphy in prostate and breast cancer: Summary and Excerpts. *Journal of Nuclear Medicine*, 58(4), 14N-17N.
- Dvorak, H.F., 1986.** Tumors: wounds that will not heal. Similarities between tumor stroma generation and wound healing. *New England Journal of Medicine*, 315(26), 1650-9.
- Ebert, O., Harbaran, S., Shinozaki, K., Woo, S.L., 2005.** Systemic therapy of experimental breast cancer metastases by mutant vesicular stomatitis virus in immune-competent mice. *Cancer Gene Therapy*, 12(4), 350-358.
- Ebert, O., Shinozaki, K., Huang, T.G., Savontaus, M.J., García-Sastre, A., Woo, S.L., 2003.** Oncolytic vesicular stomatitis virus for treatment of orthotopic hepatocellular carcinoma in immune-competent rats. *Cancer Research*, 63(13), 3605-3611.
- Eckert, M.A., Lwin, T.M., Chang, A.T., Kim, J., Danis, E., Ohno-Machado, L., Yang, J., 2011.** Twist1-induced invadopodia formation promotes tumor metastasis. *Cancer Cell*, 19(3), 372-386.
- Einhorn, S., Strander, H., 1993.** Interferon treatment of human malignancies-a short review. *Medical Oncology*, 10(1), 25-29.
- Eming, S.A., Krieg, T., Davidson, J.M., 2007.** Inflammation in wound repair: molecular and cellular mechanisms. *Journal of Investigative Dermatology*, 127(3), 514-525.
- Evans, J.G., Correia, I., Krasavina, O., Watson, N. and Matsudaira, P., 2003.** Macrophage podosomes assemble at the leading lamella by growth and fragmentation. *The Journal of Cell Biology*, 161(4), 697-705.
- Faguet, G.B., 2008.** *The war on cancer: an anatomy of failure, a blueprint for the future.* Dordrecht: Springer.
- Fields, B.N., 1996.** Epstein-Barr virus and its replication. Chapter 75 Epstein-Barr virus. *Fields virology*.
- Goede, V., Brogelli, L., Ziche, M., Augustin, H.G., 1999.** Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. *International Journal of Cancer*, 82(5), 765-770.

- Hanahan, D., Weinberg, R.A., 2011.** Hallmarks of cancer: the next generation. *Cell*, 144(5), 646-674.
- Kang, Y., Massagué, J., 2004.** Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell*, 118(3), 277-279.
- Kelly, T., Yan, Y., Osborne, R., Athota, A., Rozypal, T., Colclasure, J.C., Chu, W., 1998.** Proteolysis of extracellular matrix by invadopodia facilitates human breast cancer cell invasion and is mediated by matrix metalloproteinases. *Clinical and Experimental Metastasis*, 16(6), 501-512.
- Knudson, A.G., 2001.** Two genetic hits (more or less) to cancer. *Nature Reviews Cancer*, 1(2), 157-162.
- Kopecky, S.A., Willingham, M.C., Lyles, D.S., 2001.** Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. *Journal of Virology*, 75(24), 12169-12181.
- Larue, L., Bellacosa, A., 2005.** Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene*, 24(50), 7443-7454.
- Leek, R.D., Lewis, C.E., Whitehouse, R., Greenall, M., Clarke, J., Harris, A.L., 1996.** Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Research*, 56(20), 4625-4629.
- Leptin, M., Grunewald, B., 1990.** Cell shape changes during gastrulation in *Drosophila*. *Development*, 110(1), 73-84.
- Linder, S., 2009.** Invadosomes at a glance. *J Cell Sci*, 122(17), 3009-3013.
- Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000.** *Molecular cell biology* 4th edition. National Center for Biotechnology Informations Bookshelf.
- Massagué, J., 2012.** TGF β signalling in context. *Nature Reviews Molecular Cell Biology*, 13(10), 616-630.
- Mead, D.G., Ramberg, F.B., Besselsen, D.G., Maré, C.J., 2000.** Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice. *Science*, 287(5452), 485-487.
- Oft, M., Akhurst, R.J., Balmain, A., 2002.** Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nature cell biology*, 4(7), 487-494.
- Parato, K.A., Senger, D., Forsyth, P.A., Bell, J.C., 2005.** Recent progress in the battle between oncolytic viruses and tumours. *Nature Reviews Cancer*, 5(12), 965-976.
- Paz, H., Pathak, N., Yang, J., 2014.** Invading one step at a time: the role of invadopodia in tumor metastasis. *Oncogene*, 33(33), 4193-4202.

- Pignatelli, J., Tumbarello, D.A., Schmidt, R.P., Turner, C.E., 2012.** Hic-5 promotes invadopodia formation and invasion during TGF- β -induced epithelial-mesenchymal transition. *J Cell Biol*, jcb-201108143.
- Pollard, J.W., 2004.** Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews Cancer*, 4(1), 71-78.
- Pollard, T.D., Borisy, G.G., 2003.** Cellular motility driven by assembly and disassembly of actin filaments. *Cell*, 112(4), 453-465.
- Qian, B.Z., Pollard, J.W., 2010.** Macrophage diversity enhances tumor progression and metastasis. *Cell*, 141(1), 39-51.
- Rabinovich, G.A., Gabrilovich, D., Sotomayor, E.M., 2007.** Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.*, 25, 267-296.
- Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., Horwitz, A.R., 2003.** Cell migration: integrating signals from front to back. *Science*, 302(5651), 1704-1709.
- Ries, S.J., Brandts, C.H., Chung, A.S., Biederer, C.H., Hann, B.C., Lipner, E.M., McCormick, F., Korn, W.M., 2000.** Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). *Nature Medicine*, 6(10), 1128-1133.
- Roberts, A., Buonocore, L., Price, R., Forman, J., Rose, J.K., 1999.** Attenuated vesicular stomatitis viruses as vaccine vectors. *Journal of Virology*, 73(5), 3723-3732.
- Russell, S.J., Peng, K.W., Bell, J.C., 2012.** Oncolytic virotherapy. *Nature Biotechnology*, 30(7), 658-670.
- Seals, D.F., Azucena, E.F., Pass, I., Tesfay, L., Gordon, R., Woodrow, M., Resau, J.H., Courtneidge, S.A., 2005.** The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. *Cancer Cell*, 7(2), 155-165.
- Singer, A.J., Clark, R.A., 1999.** Cutaneous wound healing. *New England Journal of Medicine*, 341(10), 738-746.
- Stojdl, D.F., Lichty, B., Knowles, S., Marius, R., Atkins, H., Sonenberg, N., Bell, J.C., 2000.** Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. *Nature Medicine*, 6(7), 821-825.
- Stylli, S.S., Stacey, T.T., Verhagen, A.M., Pass, I., Courtneidge, S.A., Lock, P., 2009.** Nck adaptor proteins link Tks5 to invadopodia actin regulation and ECM degradation. *Journal of Cell Science*, 122(15), 2727-2740.
- Thiery, J.P., Acloque, H., Huang, R.Y., Nieto, M.A., 2009.** Epithelial-mesenchymal transitions in development and disease. *Cell*, 139(5), 871-890.
- Uppuluri, L.L., Garge, V.N., Kadam, V.J., 2015.** Evaluation of anti-angiogenesis activity of neem root using zebra fish model. *International Journal of Pharmaceutical Sciences and Research*, 6(6), 2437.

- Wang, W., Goswami, S., Lapidus, K., Wells, A.L., Wyckoff, J.B., Sahai, E., Singer, R.H., Segall, J.E., Condeelis, J.S., 2004.** Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. *Cancer Research*, 64(23), 8585-8594.
- Williams, L.T., Escobedo, J.A., Keating, M.T., Coughlin, S.R., 1988.** Signal transduction by the platelet-derived growth factor receptor. In *Cold Spring Harbor Symposia on Quantitative Biology* Cold Spring Harbor Laboratory Press. Vol. 53, 455-465.
- Wong, L.H., Krauer, K.G., Hatzinisiriou, I., Estcourt, M.J., Hersey, P., Tam, N.D., Edmondson, S., Devenish, R.J., Ralph, S.J., 1997.** Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3 γ . *Journal of Biological Chemistry*, 272(45), 28779-28785.
- Wyckoff, J., Wang, W., Lin, E.Y., Wang, Y., Pixley, F., Stanley, E.R., Graf, T., Pollard, J.W., Segall, J., Condeelis, J., 2004.** A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Research*, 64(19), 7022-7029.
- Yamaguchi, H., Lorenz, M., Kempiak, S., Sarmiento, C., Coniglio, S., Symons, M., Segall, J., Eddy, R., Miki, H., Takenawa, T., Condeelis, J., 2005.** Molecular mechanisms of invadopodium formation. *The Journal of Cell Biology*, 168(3), 441-452.
- Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., Weinberg, R.A., 2004.** Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell*, 117(7), 927-939.
- Yeatman, T.J., 2004.** A renaissance for SRC. *Nature Reviews Cancer*, 4(6), 470-480.
- Zhang, Y.W., Vande Woude, G.F., 2003.** HGF/SF-met signaling in the control of branching morphogenesis and invasion. *Journal of Cellular Biochemistry*, 88(2), 408-417.

Chapter 2

1. Introduction

Oncolytic viruses (OVs) are being widely exploited as alternative treatment options for many types of cancers due to their natural and/or engineered ability to preferentially target and lyse cancer cells while sparing normal healthy cells. (Atherton and Lichty, 2013; Barlett et al., 2013) Some hallmarks of cancer as described by Hanahan and Weinberg (2011) provide a permissive environment for OV replication; including sustained proliferation, avoidance of apoptosis, evasion of growth suppressors, genomic instability, and evasion of the immune system. This may be possible because in many cases, proliferative and antiviral signaling pathways are mutually antagonistic. Hence, development of enhanced proliferative signaling in tumor cells may lead to suppression of antiviral pathways. For example, it is now known that most tumor cells are resistant to the antiproliferative effects of type I interferons (IFNs) (Einhorn and Strander, 1993; Wong et al., 1997). The resistance of tumor cells to the antiproliferative effects of IFNs may make cancer cells more permissive to infection with a variety of viruses, including vesicular stomatitis virus (VSV).

VSV is a natural oncolytic agent that selectively replicates in cells with type I IFN antiviral defects, while normal tissues with intact antiviral responses are resistant to infection and killing by VSV (Hastie and Grdzlishvili, 2012). It has been shown that VSV can directly destroy various types of cancer cells *in vitro* and *in vivo* (Hastie and Grdzlishvili, 2012). For the experiments presented within this project, recombinant virus obtained from an infectious cDNA clone containing a wild-type (wt) matrix (M)

protein (rwt virus) was compared to an isogenic recombinant having a single point mutation that substitutes an arginine for methionine at position 51 of the 229 amino acid M protein (rM51R-M virus). This point mutation renders the virus defective in its ability to inhibit host gene expression, but does not compromise the expression of viral genes or the production of infectious progeny (Ahmed et al., 2003; Kopecky et al., 2001). Viruses show high potential as oncolytic agents due to their effectiveness at killing various cancer types in the absence of disease, thus making them effective, yet safer treatment options.

Cancer causes approximately 730,000 deaths annually and nearly 90% of them are associated with metastasis (Mehlen and Puisieux, 2006). Metastasis results from various molecular cascades through which cancer cells disseminate from the primary tumor and travel to distant sites of the body where they can recolonize and form a secondary tumor. A cascade of adhesive interactions, invasive processes, and response to chemotactic stimuli characterizes the metastatic process. For many of these processes to occur, cells must acquire the ability to breach the basement membrane (BM) and move through tissues. The BM breach occurs through localized remodeling of the extracellular matrix (ECM), which is mediated through the formation of structures called invadopodia, or “invasive feet”. Invadopodia are actin-rich membrane protrusions formed by invasive cancer cells, which facilitate focal degradation of the BM and ECM by the localized proteolytic activity of matrix metalloproteinases (MMPs). The ability of cancer cells to form invadopodia is often related to their invasive and metastatic potentials and there is strong evidence indicating that invadopodia are key cellular structures that regulate

metastasis of many cancers (Blouw et al., 2008). Because invadopodia structures are necessary for cancer cell metastasis, they represent attractive targets for cancer therapies.

In this study, we sought to investigate whether oncolytic VSV could alter invadopodia structures in cancer cells in order to attenuate the process of metastasis. Previous studies have shown that VSV utilizes cellular factors, which are known components of the invadopodia assembly and maturation pathways during the course of viral entry, transport of viral proteins within the cytoplasm, and viral budding. Therefore, we hypothesized that the formation of invadopodia would be modulated by both rwt and rM51R-M viruses. However, the mechanisms by which these viruses alter invadopodia structures may differ due to differences in the ability of these viruses to shut down host gene expression in infected cells. To carry out our studies, we utilize highly aggressive Src transformed NIH 3T3 murine fibroblasts, which are known to form robust invadopodia structures and are susceptible to killing by rwt and rM51R-M viruses. We found that both viruses were effective at inhibiting the ability of these cells to form invadopodia structures. The decrease in invadopodia correlated with a decrease in the ability of cells to degrade the ECM. Furthermore, we found that the invadopodia protein marker Tks5 expression was significantly decreased after rwt virus infection, even as the expression of cortactin was drastically increased. These data indicate that oncolytic VSV can not only selectively lyse tumor cells, but may act to also inhibit the ability of tumor cells to metastasize.

2. Materials and methods

2.1 Cell lines and virus

Src transformed NIH 3T3 murine fibroblasts, also called 527s, were propagated in DMEM containing 10% Fetal Bovine Serum (FBS). LNPT prostate cancer cells were acquired by Dr. Darren Seals (Appalachian State University) and were grown in RPMI 1640 containing 10% FBS. SNB19 glioblastoma cells were grown in DMEM containing 10% FBS. Hela cells were grown in DMEM containing 10% FBS. rwt and rM1R-M viruses were a generous gift from Dr. Douglas Lyles at Wake Forest University School of Medicine (Winston Salem, NC) and have previously been described (Whitlow et al., 2006). Virus stocks were prepared in Baby Hamster Kidney (BHK) cells as described (Kopecky et al., 2001).

2.2 Measurement of cell viability

527 and LNPT cells were plated onto 96-well dishes and allowed to adhere for 3-5 hours. Cells were then challenged with rwt and rM51R-M viruses at multiplicity of infections (MOIs) of 0.1 and 10 pfu/cell (plaque forming units/cell) for 6, 14, and 24 hours. The high MOI (10 pfu/cell) was used to measure the cytopathic effects of VSV in a synchronous infection while the lower MOI (0.1 pfu/cell) allowed for the measurement of the effects of virus spread in culture. At 6, 14, and 24 hours post infection, cell viability was measured by an MTT assay (Cell Proliferation Kit 1; Roche Diagnostics, Indianapolis, IN). Cells that were not challenged with VSV served as controls in this assay. Experiments were run in triplicate and performed 3-5 times.

2.3 Wound Healing Assay

Src NIH 3T3 fibroblast cells were seeded into a 60mm tissue culture dish and were allowed to grow to 90% confluency in DMEM medium supplemented with 10% FBS. A scratch was made in the monolayer of cells with a 1-100ul pipette tip and the plate was washed twice with cell medium to remove cell debris. 5.0 mL of medium was gently re-added into the scratched plates and cells were infected with either rwt or rM51R-M virus at MOIs of 0.1, 1.0 and 10 pfu/cell. Cells were incubated for 24 hours and the migration progress was monitored every four hours via microscopy using AxioVision 4.8 camera software. Images were analyzed with Adobe Photoshop using the Ruler Tool to measure the width of the scratch at three locations per time point. Data was normalized as a percentage of the original width of the scratch at 0 hours.

2.4 ECM degradation assay

ECM degradation assays were performed as previously described (Martin et al., 2012).

Details are provided below:

a) *Production of Oregon Green 488-gelatin coated coverslips.*

Glass coverslips (13mm), in a 12-well dish, were cleaned in 20% nitric acid and washed with PBS. Coverslips were coated with poly-L-lysine, placed in 0.5% glutaraldehyde and washed again with PBS. Oregon green 488-conjugated gelatin was reconstituted according to the manufacturer's protocol (Molecular Probes G-13187) and diluted into eight parts unlabeled gelatin 100µL of gelatin mixture (at 40°C) was pipetted onto each coverslip and sodium borohydride (NaBH₄) was added to each well to reduce

and inactivate residual glutaraldehyde. After washing with sterile PBS, coverslips were equilibrated at least 24 hours in complete media appropriate to the cell type to be assayed.

b) Plating and processing of cells on Oregon green 488 –gelatin coated coverslips to assay ECM degradation

Cells were seeded onto a coverslip within each well at 70% confluence. The cells were given 3-5 hours for full adherence. The cells were then challenged with rwt and rM51R-M VSV at MOIs of 0.1 and 10 pfu/cell for 6 and 14 hours. This assay was run in duplicate and performed 3-4 times. Post infection, each well was aspirated and washed with PBS. To fix the cells, 0.4% paraformaldehyde was added to each well for 15 minutes at room temperature. The wells were washed with PBS, followed by permeabilization by adding 0.4% Triton-X for 4 minutes at room temperature. The wells were washed in PBS to remove all Triton-X. A piece of parafilm was labeled and put into a moist chamber. A 5.0% donkey serum and 0.5% Alexa flour conjugated phalloidin in PBS was made and was dropped onto the parafilm for each coverslip. Forceps were used to lift coverslips and inverted onto the drops for 30-45 minutes in the dark at room temperature. Forceps were used to return coverslips to the 12-well dish, followed by washing with PBS. Coverslips were then mounted onto glass microscope slides using small aliquots of Prolong Gold DAPI. The coverslips were then stored in the dark overnight.

c) Quantification of fluorescent gelatin degradation by measuring normalized matrix degradation

Each coverslip was observed using fluorescent microscopy. 10 random images were taken for each coverslip. Images were opened using ImageJ (<http://www.macbiophotonics.ca/imagej/>). Scale and measurements were selected by choosing “Analyze, Set measurements.” Using the fluorescent gelatin image, total area of degradation was calculated. The threshold of the image was set (Image, Adjust, and Threshold) to the upper and lower intensity values to select the areas of degradation. In some cases the gelatin was not completely flat when images were acquired. Corrections for uneven background illumination across the gelatin were accomplished by subtracting the background (Process, Subtract background) or by filtering with a bandpass filter (Process, FFT, Bandpass filter). The total area degraded was measured by particle analysis (Analyze, Analyze particles). In the Analyze particles window, particle size >0 was chosen to remove noise from the selection. “Display results” and “Summarize” were checked to show measurements. The “Total area” measurement was copied onto a spreadsheet.

2.5 Invadopodia/Rosette Morphology Assay

Glass coverslips were processed as previously described in section 2.3.1; however, the coverslips were not coated with gelatin. Cells were seeded onto coverslips and infected with VSV and processed as described in section 2.3.2. Ten random images were taken of each coverslip using fluorescent microscopy. Image sequences were imported (File, Import, Image sequence) for each set of coverslip treatments into ImageJ (<http://www.macbiophotonics.ca/imagej/>). The nuclei were counted via cell counter (Plugins, Analyze, Cell counter). Single, actin based punctate invadopodia and complete circular rosette structures were manually counted for each individual image and data show the number of invadopodia or rosette structures per cell, normalized to mock. This assay was run in duplicate and performed 3-6 times.

2.6 Western blot analysis

527 and LNPT cells were grown to approximately 75% confluence in 6-well dishes and infected with rwt or rM51R-M VSV at MOIs of 0.1 and 10 pfu/cell for 6 and 14 hours. As controls, cells were mock-infected. At 14 hours post-infection, cells were directly lysed in RIPA buffer containing aprotinin. Protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Scientific). 20 μ g total protein for each sample was subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels. The proteins were transferred onto a nitrocellulose membrane and blocked in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T, pH 7.6) and 5.0% dry condensed milk. Immunoblots were probed with antibodies against Tks5, c-Src, Cortactin, Dynamin 2, and β -actin (all from Cell Signaling Technologies) overnight

at 4°C in 5.0% BSA + 0.1% TBS-T. β -actin was used as an internal standard. Blots were incubated with goat anti-rabbit horseradish peroxidase (HRP) linked secondary antibodies. Dura or Pico (Thermo Scientific) luminol and stable peroxide substrate were used to visualize the signal on GeneMate Bluelite Autorad film. ImageQuant TL v2005 was used to quantify relative band intensities. Experiments were carried out 3-5 times. Data was normalized to levels of β -actin in each lane and expressed relative to mock samples. Statistical analyses were performed using Student's t-test.

3. Results

3.1 *Src NIH 3T3 cells are susceptible to VSV infection*

In order to select a cancer cell line with high metastatic potential, various cancer cell lines were screened for their ability to form invadopodia structures and susceptibility to VSV infection. Src transformed NIH 3T3 murine fibroblasts have constitutively active Src expression and form prominent invadopodia structures. LNPT prostate cancer cells which express Tks5 and SNB19 glioblastoma multiforme (GBM) cells are also innately invasive with demonstrated invadopodia competence. Cell viability was measured at 6, 14, and 24 hours post infection with VSV at MOIs of 0.1 and 10 pfu/cell. For these studies, two different strains of VSV were tested. rwt virus is a recombinant wild-type strain of VSV, while rM51R-M virus is isogenic to rwt virus, but contains a methionine to arginine substitution at position 51 of the M protein sequence (Black et al., 1993). This mutation renders the virus defective at inhibiting host gene expression, including expression of genes in the host antiviral response (Ahmed et al., 2003). We and others have shown that M protein mutants, such as rM51R-M virus, are selective oncolytic agents due to their ability to target tumors *in vivo* with absence of disease (Ahmed et al., 2004; Lun et al., 2006; Stojdl et al., 2003). Our results (Fig. 1) indicate that 527 cells were susceptible to infection with both rwt and rM51R-M viruses, especially at later times post-infection. 6 hours post-infection represents an early time point in the life cycle of VSV where cells are starting to produce progeny virions but virus-induced cytopathology is limited. At this time point, a significant decrease in cell viability was observed in cells infected with rM51R-M virus at an MOI of 10 pfu/cell (approximately

20% as compared to mock-infected cells), but not with rwt virus. By 24 h post-infection, a later time in the life cycle of the virus, the viability of cells infected with rwt and rM51R-M viruses decreased to approximately 50%. No changes in cell viability were observed when cells were infected at an MOI of 0.1pfu/cell except at 24h post infection with r-M51R-M. These results indicate that both viral strains have the capacity to kill 527 cells *in vitro* when cells are infected with a high concentration of virus.

However, these cells are relatively resistant to VSV-induced cell death at low MOIs, thus demonstrating the aggressive nature of these cancer cells.

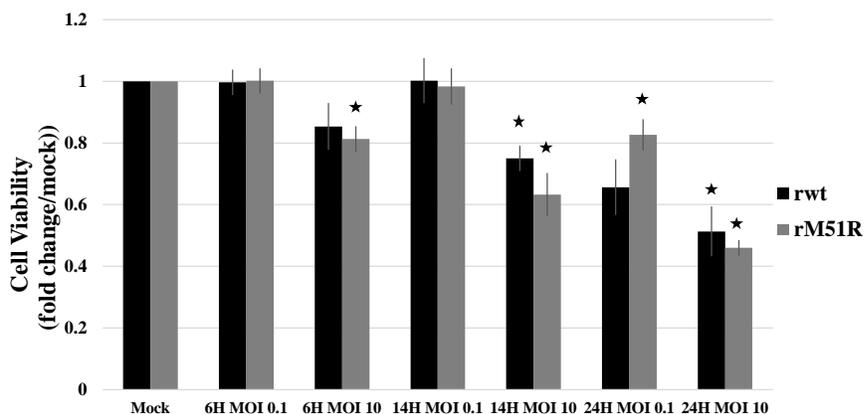


Fig.1. Src NIH 3T3 cells are susceptible to killing by VSV at a high MOI. Cells were infected with rwt and rM51R-M viruses at 6, 14, and 24 hours at MOIs of 0.1 and 10 pfu/cell and subjected to an MTT assay to determine cell viability. Data was normalized as a percentage of mock cells (uninfected) and are shown as the means \pm standard error for 3-5 experiments. Statistical analysis was carried out using unpaired Student's t-test. A star represents statistical differences ($P < 0.05$) as compared to cells that were mock-infected.

3.2 VSV infection inhibits the wound-healing ability of NIH 3T3 cells *in vitro*

The ability of cancer cells to migrate is a key feature in the early metastatic cascade. While 527 cells are relatively susceptible to VSV infection, we sought to assess whether VSV infection could alter the ability of these invasive cells to migrate using a wound healing assay. Cells grown in 6 well dishes were scratched with a pipette tip and infected with rwt or rM51R-M virus at MOIs of 0.1, 1.0, and 10.0 pfu/cell. The width of the scratch was measured over time as an indication of the degree of migration of cells. Our results indicate that the gap closure in mock-infected cells was approximately 57% over the course of 24 hours (Fig. 2A, 2B). For rwt infection at MOI 0.1 and 10.0 pfu/cell, no significant decrease in cell migration was observed compared to mock-infected cells during the 24 hour time course (Fig. 2B). However, the migration of cells infected with rwt infection at an MOI 1.0 pfu/cell significantly decreased with under 30 percent of the wound being filled (Fig. 2B). For rM51R-M virus infection at MOI 0.1 and 1.0 pfu/cell, no significant decrease in cell migration was observed (Fig. 2C). However, rM51R-M virus inhibited cell migration almost completely at an MOI 10.0 pfu/cell (Fig. 2A, 2C). This could be due to the ability of rM51R-M to induce antiproliferative, antiviral responses in these cells, and/or promote apoptosis. These results indicate that VSV infection of invasive cancer cell lines impairs their ability to migrate *in vitro*, perhaps due to impairment of proliferation. Furthermore, data show a differential response in the migration capacity of cells infected with rwt versus rM51R-M viruses.

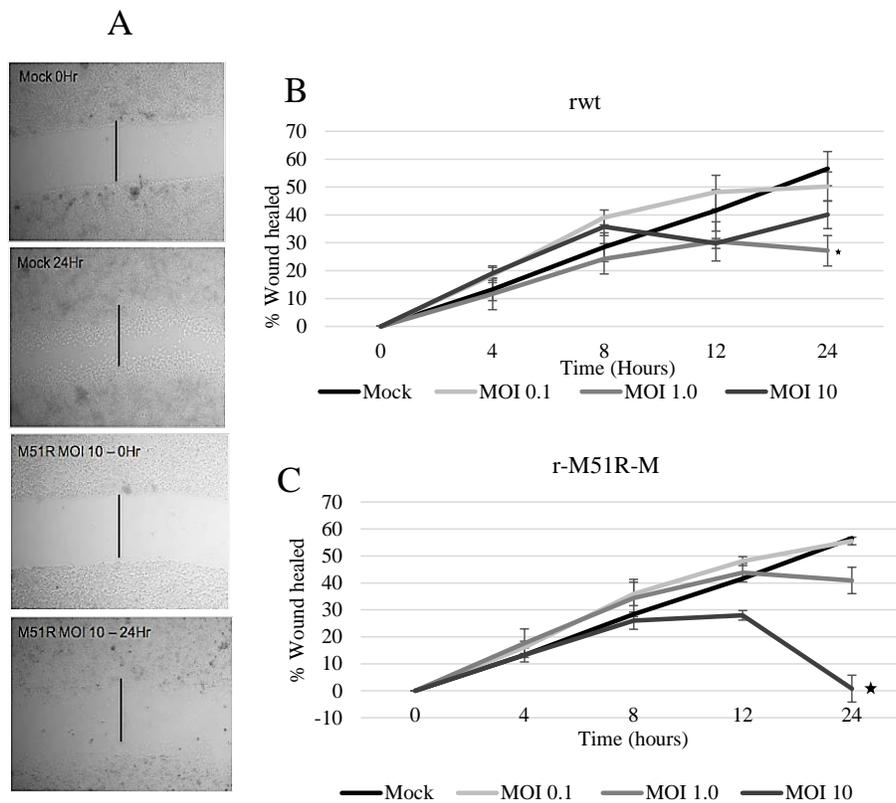


Fig.2. VSV inhibits the ability of cells to migrate. Src NIH 3T3 cells were grown to 90% confluency and scratched using a 1-100ul pipette tip (A). Cells were then infected with rwt (B) and rM51R-M (C) virus for 24 hours at MOI of 0.1, 1.0 and 10 pfu/ml. Cell migration was monitored every four hours following infection via microscopy and the wound width was measured. Data was normalized as a percentage of the width of the scratch at 0 hours and is shown as the mean of three experiments +/- standard error. Statistical analysis was performed using the Student's t-test, a star represents statistical significance ($P = <0.05$) as compared to cells not infected.

3.3 VSV inhibits the ability of 527 cells to degrade gelatin matrix in vitro

In order for cancer cells to effectively metastasize, they must not only acquire the ability to migrate, but must degrade and remodel the ECM as well as the tough connective tissue of blood vessels for intravasation and extravasation. It has been shown in previous studies that Src NIH 3T3 cells form robust invadopodia structures and readily degrade gelatin matrix (Seals et al., 2005). To determine if the ability of VSV to inhibit 527 cell migration was correlated with 527 cell ECM degradative potential, cells were seeded onto gelatin-coated coverslips and infected with VSV to assess overall degradation (Fig. 3). Our results show that infection of cells with rwt virus decreased their capacity to degrade the gelatin matrix. This was most apparent when cells were infected with virus for 14 hours at an MOI 0.1 pfu/cell (Fig. 3A, 3B). We showed previously that at this time point, cells infected with rwt virus at an MOI of 0.1pfu/cell showed no significant change in cell viability. Therefore, these results suggest that the effects VSV infection on the ability of 527 cells to degrade the gelatin matrix are separable from its ability to induce apoptosis.

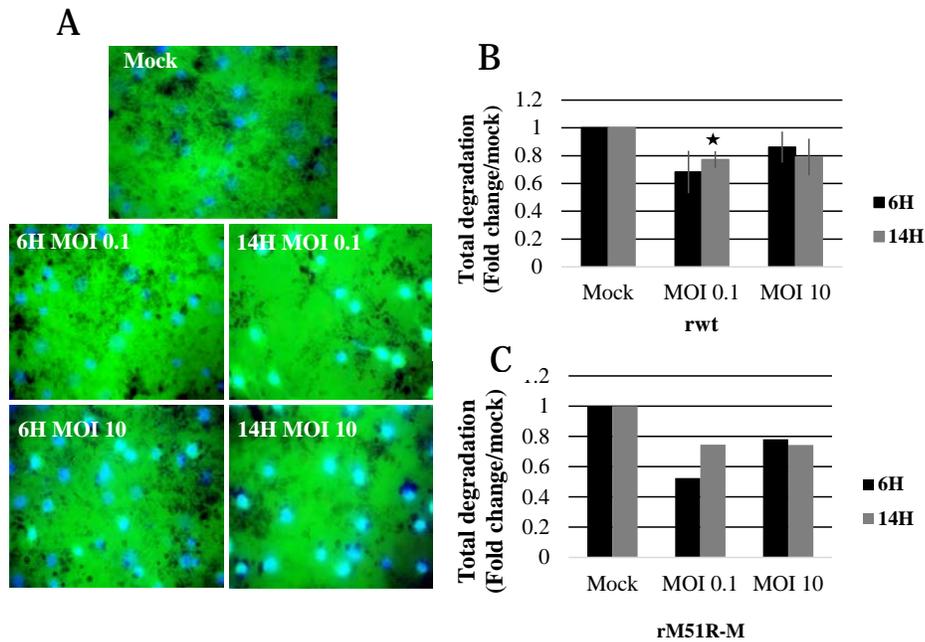


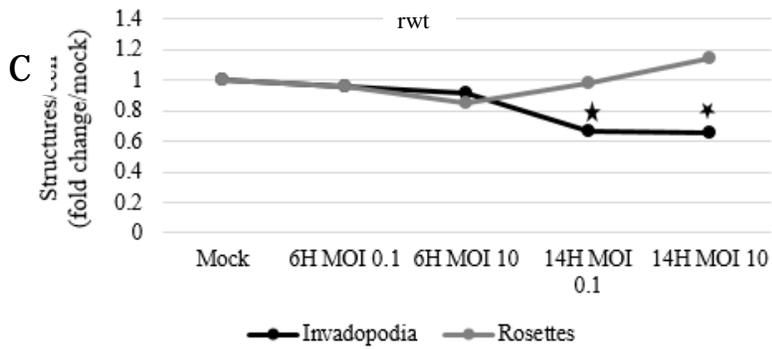
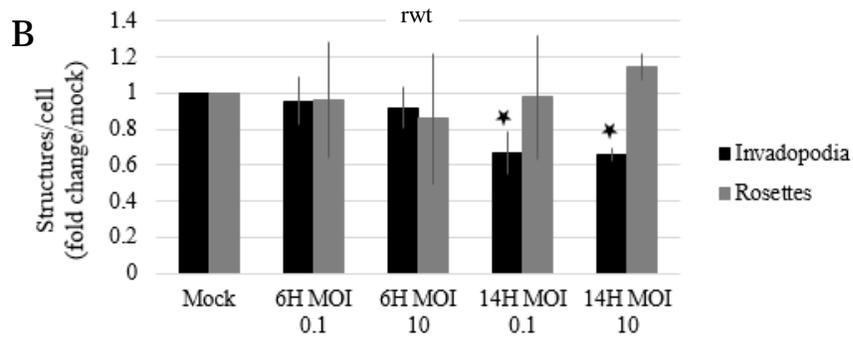
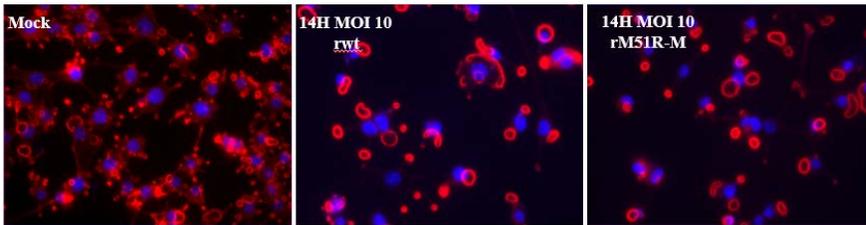
Fig. 3. Infection of cells with VSV decreases their ability to degrade extracellular matrix. Coverslips were mounted onto microscope slides and the total area degraded (pixels) was measured for each condition (A) using fluorescence microscopy. 527 cells on coverslips were infected with rwt (B) and rM51R-M (C)(n=2) virus for 6 and 14 hours at MOIs of 0.1 and 10 pfu/cell. Data was normalized as a percentage of mock cells and is shown as the means \pm standard error for 3-5 experiments. Statistical analysis was carried out using unpaired Student's t-test. A star represents statistical differences ($p < 0.05$) as compared to cells that were not infected.

3.4 VSV inhibits invadopodia formation while increasing the number of rosette structures

In recent years, actin-rich subcellular protrusions known as invadopodia have been shown to be critical for ECM degradation (Linder, 2007). Invadopodia consist of an actin-rich core surrounded by a number of important protein components, including cytoskeletal modulators, adhesion proteins, scaffolding proteins, and signaling molecules. Invasive cancer cells can also form invadopodia superstructures called rosettes. Rosettes have been studied most extensively in osteoclasts where they form specialized sealing zones to potentiate bone resorption (Destaing et al., 2003). However, in 527 cells, the mechanism of the organization of podosomes into such superstructures remains obscure and their role is not fully understood. It is known that many viruses may alter the host cell's cytoskeletal network in order to potentiate the viral replication cycle. Given that invadopodia structures have rich actin cores, we hypothesized VSV infection would alter these structures due to 1: the ability of rwt virus ability to shut down host gene expression and 2: both invadopodia structures and VSV's need for some of the same necessary proteins for successful formation and entry/exit respectively. Furthermore, results in Figure 3 show that degradation of the extracellular matrix is reduced in cells infected with VSV. In order to investigate how VSV infection may alter the formation of these structures, 527 cells were seeded onto glass coverslips and infected with either rwt or rM51R-M virus for 6 or 14 hours at MOIs of 0.1 and 10

pfu/cell. The coverslips were then stained, processed and viewed using fluorescent microscopy (Fig. 4A.) to determine the number of individual invadopods as well as rosettes. Invadopods are visualized as punctate, actin rich structures, while the rosettes can be distinguished by the formation of circular superstructures. For rwt virus infection, no significant changes in structures were observed at 6 hours post infection at either MOI (Fig 4B, C). However, at 14 hours post infection, a significant decrease in invadopodia structures was observed (30% decrease compared to mock-infected). In contrast, there was no change in the number of rosette structures upon virus infection as compared to mock-treated cells. For rM51R-M infected cells, significant decreases in invadopodia structures were observed at 6 and 14 hours post infection at an MOI 10 pfu/cell (Fig.4 D, E). Interestingly, an increased trend was observed in the number of rosette structures upon infection with rM51R-M virus. rM51R-M virus was slightly more effective than rwt virus at inhibiting the formation of invadopodia. These results show a promising correlation between viral-mediated inhibition of matrix degradation and number of invadopodia. These results also show that VSV infection inhibits the ability of cells to form invadopodia structures while maintaining and increasing the number of rosette structures. The decrease in invadopodia structures during viral infection may be due to altered expression of proteins known to be critical for invadopodia assembly and maturation. Importantly, the observed increase in rosette structures may be helpful in shedding light into the possible mechanisms by which VSV infection inhibits the invasiveness of 527 cells *in vitro*.

A



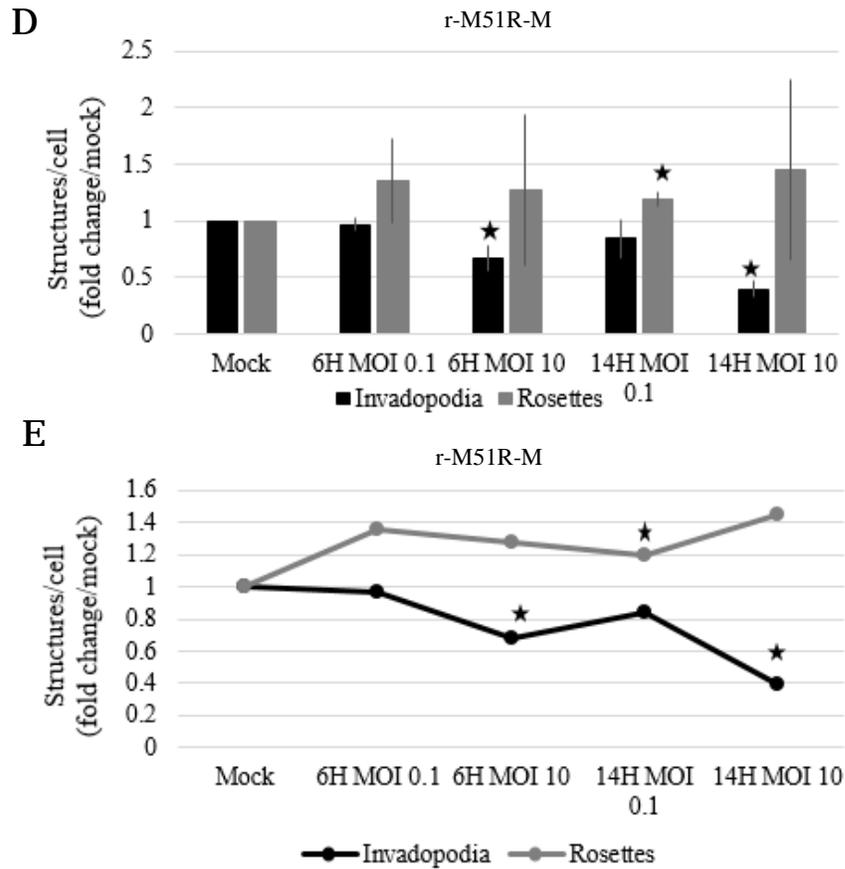


Fig. 4. Formation of invadopodia and rosette structures upon infection with VSV. Coverslips were mounted onto microscope slides and using fluorescent microscopy (A), the number of rosettes and invadopodia structures were counted. 527 cells grown on coverslips were infected with rwt (B, C) or rM51R-M (D, E) virus for 6 and 14 hours at MOIs of 0.1 and 10 pfu/cell. Data was normalized as a percentage of mock cells and is shown as the means \pm standard error for 3-5 experiments. Statistical analysis was carried out using unpaired Student's t-test. A star represents statistical differences ($p < 0.05$) as compared to mock-infected cells.

3.5 VSV infection causes decreased Tks5 expression while increasing Cortactin expression

The role of Tks5 in invadopodia was originally discovered using a Src substrate screening assay and further characterized based on its location in invadopodia in Src transformed fibroblasts (Seals et al., 2005). It has been shown that Tks5 is required for both invadopodia formation and invasion in many different cancer cell lines, as Tks5 knockdown showed reduced matrix degradation and invasion (Eckert et al., 2011). Cortactin is a cytoskeletal protein that when phosphorylated can activate the Arp 2/3 complex to promote invadopodia formation. Since cortactin regulates many actin-based cellular processes, including invadopodia formation and dynamics, its role in tumor cell invasion and metastasis is well documented. For example, it has been shown that overexpression of cortactin in NIH 3T3 cells increased invasion (Patel et al., 1998). Therefore, we wanted to investigate whether the decrease in invadopodia structures upon infection with VSV correlated with a decrease in the expression levels of Tks5 and cortactin in 527 cells. In order to assess the level of Tks5 and cortactin, 527 cells were seeded into 6-well dishes and infected with rwt virus for 6 and 14 hours at MOIs of 0.1 and 10.0 pfu/cell. The expression of Tks5 and cortactin was analyzed via western blot analysis. The data in Figure 5A show a significant decrease in Tks5 expression when cells were infected with rwt virus for 14 hours at an MOI of 10.0 pfu/cell as compared to mock infected cells. In contrast, a robust increase in cortactin expression was found under the same conditions. The decrease in Tks5 expression correlates with the decrease in invadopodia structures as shown in Figure 4. However, the robust increase in cortactin may be facilitating the production of rosette structures in infected cells. These results

indicate that the expression of proteins necessary for invadopodia form and function are altered during the course of viral infection and may relate to a decrease in overall function.

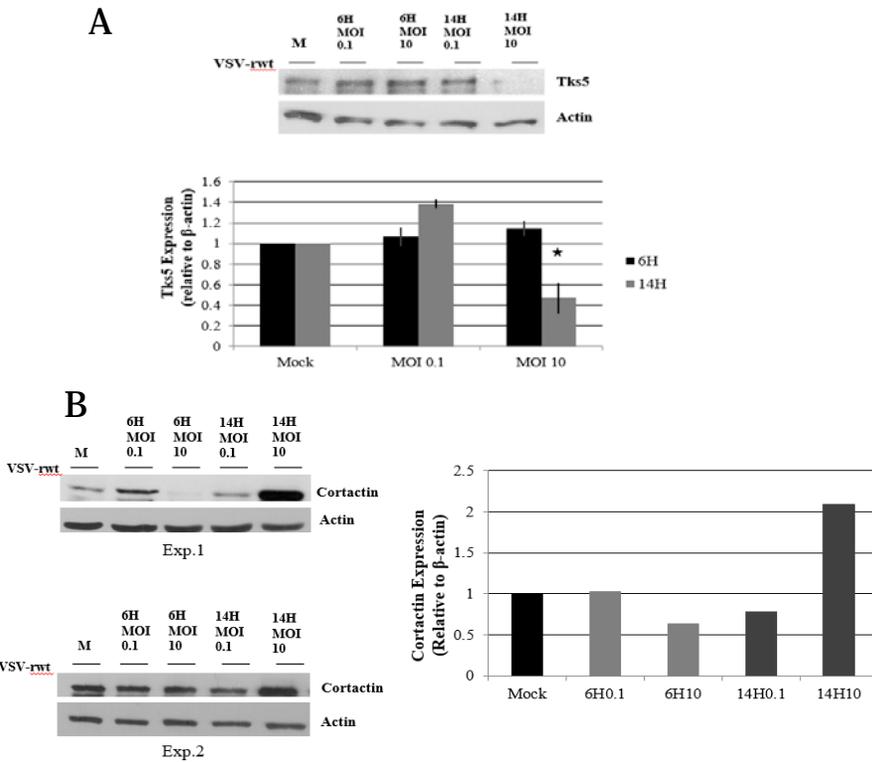


Fig.5. Differential expression of Tks5 and cortactin in response to rwt virus. Cells were infected with rwt virus for 6 and 14 hours at MOIs of 0.1 and 10 pfu/cell. Lysates were collected at 14 hours post infection and subjected to Western Blot analysis with antibodies to Tks5 (A) and Cortactin (B). Representative images from bands subjected to development on photographic film. Levels of Tks5 and Cortactin were normalized to levels of β -actin in each lane and expressed relative to mock samples. Data represent the mean \pm standard error for 2-3 experiments. Statistical analysis were conducted using the Student's T test. A star represents statistical differences in protein levels as compared to the mock infected group.

4. Discussion

Metastatic cancers are responsible for nearly 90% of all cancer related deaths.

Although there are several cancer treatment options in practice, these are often outdated, non-targeted treatments that typically are non-effective and are correlated with highly adverse side effects. Not only do common cancer treatments provide little efficacy, they are also not tailored towards late stage metastatic cancer. Therefore, the development of oncolytic VSV, with specific anti-metastatic mechanisms could facilitate safer and more effective treatment options for those with late stage metastatic cancers.

In this study, we determined whether VSV could alter several steps in the metastatic process, including the formation of invadopodia structures and corresponding matrix degradation, and expression of several proteins known to be required for invasion. The data presented here indicate that VSV strains can effectively inhibit the metastatic potential of highly invasive cancer cells *in vitro*. We found that upon viral infection, the number of invadopodia structures per cell were significantly reduced (Fig. 4). Although it is possible some viral-induced cell death could contribute to the observed decrease in invadopodia, our cell viability results (Fig. 1) indicate that at 14 hours post infection, minimal cell death has occurred, suggesting that the observed effect is mediated by more direct viral control of invadopodia structures. Although no previous studies have been carried out concerning the effects of VSV infection on cancer cell invadopodia formation, it is known that different viruses, including human immunodeficiency virus (HIV), hepatitis B virus (HBV) and human papilloma virus (HPV), manipulate the host cells' cytoskeletal network in order to potentiate viral infection (Chen et al., 2002).

Indeed, the cytoskeletal network is critical for intracellular transport, including the transport of viral components. More specifically, many of these viruses target the RHO family of small GTPases in order for subversion of actin based structures to better potentiate viral infection. Tan et al. (2008) showed that that HBV-replicating HepG2 cells exhibited morphological changes and had the appearance of membrane rufflings and lamellipodia-like structures, similar to the observed, ring-like structures in our study (Fig. 3). Further research into VSV interaction with RHO family GTPases will help determine if these are similar phenomena.

The mechanisms by which VSV affects host cytoskeletal network is limited to the understanding of viral entry (Lara-Pezzi et al., 2001). Studies have shown that VSV virions enter cells via clathrin-coated pit-mediated endocytosis which is enhanced by the formation of actin filaments due to VSV's elongated structure (Cureton et al., 2009). VSV engages in a "viral surfing" phenomenon, where proteins located on the virion surface bind with high specificity and affinity to cellular receptors that are associated with actin filaments just beneath the cell surface. The virion movement (surfing) towards entry sites is mediated by internal myosin motors, which are located in the cortical actin or at the base of filopodia and which contract the actin filaments. In effect, the movement of the actin cytoskeleton drags the cellular receptor-virion complexes to sites of high endocytic activity. It could be possible that VSV enters the host cells at areas of high actin turnover, such as the observed actin rich, rosette-like structures (Medeiros et al., 2006)

Our results indicate that while the number of invadopodia structures decreased, the number of rosette like structures increased upon infection of cells with VSV. It has previously been shown that the rosette-like structures on Src- NIH 3T3 cells correlate with matrix degradation (Seals et al., 2005). Interestingly, the rosette-like structures we observed in our study (Fig. 3) did not contribute to matrix degradation during virus infection (data not shown). Therefore, it is possible that following virus infection, these rosette structures function in a manner similar to that observed by circular dorsal ruffles (CDRs). CDRs are dynamic, yet transient actin protrusions on the apical surface of cells that serve critical functions in receptor internalization and cell migration (Hoon et al., 2012). Recent studies have revealed that viruses can induce the formation of CDRs for their entry processes (Hetzeneker et al., 2016). For example, HCMV induced CDRs for micropinocytosis-mediated viral entry. Although it is agreed on that VSV enters cells via clathrin mediated endocytosis, there is debate as to whether VSV may enter using multiple mechanisms, depending on cell type. Alternatively, it is possible that the ability of VSV to induce CDRs is independent of its viral entry mechanisms. Ultimately, while we have observed decreased numbers of invadopodia and increased numbers of rosette like structures, the mechanisms are unknown. We may assume that the decrease in actin rich invadopodia structures is associated with an increase in actin rich rosettes-like structures, but the function of these rosette-like structures upon VSV infection has yet to be elucidated.

Cortactin, a cytoplasmic regulator of several cortical actin structures, has been shown to mediate invadopodia and CDR formation by forming a complex with large endocytic GTPase dynamin 2 (Dyn2). Krueger et al. (2003) showed that many viruses interact with

RHO family of small GTPases, which are also key regulators of CDRs (Ladwein and Rottner, 2008). Interestingly, Tks5 is required to form invadopodia structures associated with invasive cells but is not required for the formation of CDRs (Seals et al., 2005). We observed an increase in cortactin levels upon infection with VSV and a corresponding decrease in Tks5 expression. Therefore, these data further indicate that VSV may be enhancing the expression of factors responsible for the formation of CDRs and decreasing invadopodia forming factors in 527 cells. The ultimate effect may be the recruitment of actin for the formation of alternate structures such as CDRs and the reduced capacity of cells to form invadopodia, subsequently reducing the cell's ability to degrade the extracellular matrix (Fig. 5).

In this study, we observed that both rwt and rM51R-M viruses significantly inhibited the ability of cells to migrate into a wound by 24 hours post infection (Fig. 2). For rwt virus infection, the decrease in wound closure can likely be attributed to viral-mediated cell death, since wt strains of VSV are effective inducers of apoptotic mechanisms. This was also the case in our 527 cell viability experiments, as we observed that by 24 hours post infection with rwt virus, approximately 40% of cells were killed (Fig. 1). Interestingly, rM51R-M virus was more effective than rwt virus at preventing wound healing. This is most likely due to the antiproliferative effects induced by rM51R-M virus due to stimulation of the type I IFN (IFN α/β) response by this virus (Stout et al., 1993). In support of this hypothesis, previous studies have shown that wound edge reunion was significantly inhibited by IFN α/β . Therefore, type I IFN inducing pathways lead to an enhancement of the antiviral response while inducing expression of factors that inhibit cell growth and proliferation. We also observed a greater decrease in

viability when cells were infected with rM51R-M virus as compared to rwt virus. The combination of antiproliferative responses and induction of cell death mechanisms by rM51R-M virus may account for the greater ability of this virus to prevent wound closure as compared to rwt virus. Furthermore, the differences in migration do not significantly differ before 24 hours post infection. This represents a late point in the virus life cycle and is associated with the induction of cell death mechanisms. Therefore, this suggests that the further inhibition of wound closure at 24 hours was in fact due to the enhancement of viral-mediated cell death.

A promising observation we found was that VSV infection significantly decreased overall cancer cell-mediated matrix degradation (Fig. 3). Interestingly, we found that both rwt and rM51R-M viruses decreased overall degradation of the matrix which correlates with the reduction in invadopodia structures. Since both strains of the virus decreased the cell's ability to degrade matrix, the effects of VSV on inhibition of matrix degradation were independent of their ability to inhibit host gene expression and mediate antiviral responses. The decrease in degradation was also not due to virus-induced cytopathology as our cell viability data show that at 14 hours post infection at MOI 0.1, no significant cell death was present. It is known that matrix metalloproteinases (MMPs) are responsible for ECM degradation and remodeling (Saarialho-Kere et al., 1992). MMP activity has also been shown to localize to CDRs, so unfortunately while the decrease in overall degradation is promising from a therapeutic point of view, this result does not shed light on how VSV may be modulating rosette-like structures. Although very limited work has been done to address viral effects on cancer cell-mediated matrix degradation, some studies have shown that MMP levels from human macrophages are

dramatically reduced when infected with human cytomegalovirus (HCMV) (Strååt et al. 2009). The mechanism/s by which the virus inhibits these enzymes remains unclear, although it may be postulated that MMP vesicle recycling may be interrupted or be utilized by viruses to transport viral proteins within the cytoplasm. In support of this notion, we did not observe complete inhibition of degradation, suggesting that some MMP activity was still present. Strååt et al. (2009) demonstrated that MMP levels were affected by immediate-early or early viral gene products, but not at later stages of infection. This may suggest that MMP-mediated degradation was inhibited at the early stages of VSV infection, but recovered function later in the viral life cycle. Furthermore, this may explain why we observed similar levels of degradation at different time points post infection.

We have shown that VSV infection not only kills invasive cancer cells (Fig. 1), but also alters the ability of 527 cells to migrate and form invadopodia structures (Fig. 2,4), leading to an overall decrease in cancer cell mediated matrix degradation *in vitro* (Fig. 3). VSV may represent a safer, more targeted treatment option for late stage cancers as compared to radiation, surgery, and chemotherapies. This opens the possibility of VSV being used to not only locally lyse tumor cells, but also to inhibit the ability of tumor cells to form invasive structures, thus decreasing the metastatic potential of the cancer. Ongoing research needs to be done regarding the mechanisms by which VSV inhibits the formation of invadopodia and subsequent degradation. The mechanisms by which VSV induces increased rosette-like structures and how VSV benefits from their induction may be critical in understanding how this virus manipulates the host cell cytoskeletal network, and how to better tailor VSV as an oncolytic agent.

References

- Ahmed, M., Cramer, S.D., Lyles, D.S., 2004.** Sensitivity of prostate tumors to wild type and M protein mutant vesicular stomatitis viruses. *Virology*, 330(1), 34-49.
- Ahmed, M., McKenzie, M.O., Puckett, S., Hojnacki, M., Poliquin, L., Lyles, D.S., 2003.** Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis. *Journal of Virology*, 77(8), 4646-4657.
- Atherton, M.J., Lichty, B.D., 2013.** Evolution of oncolytic viruses: novel strategies for cancer treatment. *Immunotherapy*, 5(11), 1191-1206.
- Bartlett, D.L., Liu, Z., Sathaiyah, M., Ravindranathan, R., Guo, Z., He, Y., Guo, Z.S., 2013.** Oncolytic viruses as therapeutic cancer vaccines. *Molecular Cancer*, 12(1), 103.
- Black, B.L., Rhodes, R.B., McKenzie, M., Lyles, D.S., 1993.** The role of vesicular stomatitis virus matrix protein in inhibition of host-directed gene expression is genetically separable from its function in virus assembly. *Journal of Virology*, 67(8), 4814-4821.
- Blouw, B., Seals, D.F., Pass, I., Diaz, B., Courtneidge, S.A., 2008.** A role for the podosome/invadopodia scaffold protein Tks5 in tumor growth in vivo. *European journal of cell biology*, 87(8), 555-567.
- Chen, D., Wang, M., Zhou, S., Zhou, Q., 2002.** HIV-1 Tat targets microtubules to induce apoptosis, a process promoted by the pro-apoptotic Bcl-2 relative Bim. *The EMBO journal*, 21(24), 6801-6810.
- Cureton, D.K., Massol, R.H., Saffarian, S., Kirchhausen, T.L., Whelan, S.P., 2009.** Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization. *PLoS Pathogens*, 5(4), p.e1000394.
- Destaing, O., Saltel, F., Géminard, J.C., Jurdic, P., Bard, F., 2003.** Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. *Molecular Biology of the Cell*, 14(2), 407-416.
- Eckert, M.A., Lwin, T.M., Chang, A.T., Kim, J., Danis, E., Ohno-Machado, L., Yang, J., 2011.** Twist1-induced invadopodia formation promotes tumor metastasis. *Cancer Cell*, 19(3), 372-386.
- Einhorn, S., Strander, H., 1993.** Interferon treatment of human malignancies-a short review. *Medical Oncology*, 10(1), 25-29.

- Hanahan, D., Weinberg, R.A., 2011.** Hallmarks of cancer: the next generation. *Cell*, 144(5), 646-674.
- Hastie, E., Grdzlishvili, V.Z., 2012.** Vesicular stomatitis virus as a flexible platform for oncolytic virotherapy against cancer. *Journal of General Virology*, 93(12), 2529-2545.
- Hetzenecker, S., Helenius, A., Krzyzaniak, M.A., 2016.** HCMV induces macropinocytosis for host cell entry in fibroblasts. *Traffic*, 17(4), 351-368.
- Hoon, J.L., Wong, W.K., Koh, C.G., 2012.** Functions and regulation of circular dorsal ruffles. *Molecular and Cellular Biology*, 32(21), 4246-4257.
- Krueger, E.W., Orth, J.D., Cao, H., McNiven, M.A., 2003.** A dynamin–cortactin–Arp2/3 complex mediates actin reorganization in growth factor-stimulated cells. *Molecular Biology of the Cell*, 14(3), 1085-1096.
- Kopecky, S.A., Willingham, M.C., Lyles, D.S., 2001.** Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus. *Journal of Virology*, 75(24), 12169-12181.
- Ladwein, M., Rottner, K., 2008.** On the Rho'd: The regulation of membrane protrusions by Rho-GTPases. *FEBS Letters*, 582(14), 2066-2074.
- Lara-Pezzi, E., Serrador, J.M., Montoya, M.C., Zamora, D., Yáñez-Mó, M., Carretero, M., Furthmayr, H., Sánchez-Madrid, F., López-Cabrera, M., 2001.** The hepatitis B virus X protein (HBx) induces a migratory phenotype in a CD44-dependent manner: possible role of HBx in invasion and metastasis. *Hepatology*, 33(5), 1270-1281.
- Linder, S., 2007.** The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends in Cell Biology*, 17(3), 107-117.
- Lun, X., Senger, D.L., Alain, T., Oprea, A., Parato, K., Stojdl, D., Lichty, B., Power, A., Johnston, R.N., Hamilton, M., Parney, I., 2006.** Effects of intravenously administered recombinant vesicular stomatitis virus (VSV Δ M51) on multifocal and invasive gliomas. *Journal of the National Cancer Institute*, 98(21), 1546-1557.
- Martin, K.H., Hayes, K.E., Walk, E.L., Ammer, A.G., Markwell, S.M., Weed, S.A., 2012.** Quantitative measurement of invadopodia-mediated extracellular matrix proteolysis in single and multicellular contexts. *Journal of Visualized Experiments: JoVE*, (66).
- Medeiros, N.A., Burnette, D.T., Forscher, P., 2006.** Myosin II functions in actin-bundle turnover in neuronal growth cones. *Nature Cell Biology*, 8(3), 216-226.
- Mehlen, P., Puisieux, A., 2006.** Metastasis: a question of life or death. *Nature Reviews Cancer*, 6(6), 449-458.

- Patel, A.S., Schechter, G.L., Wasilenko, W.J., Somers, K.D., 1998.** Overexpression of EMS1/cortactin in NIH3T3 fibroblasts causes increased cell motility and invasion in vitro. *Oncogene*, 16(25).
- Saarialho-Kere, U.K., Chang, E.S., Welgus, H.G. and Parks, W.C., 1992.** Distinct localization of collagenase and tissue inhibitor of metalloproteinases expression in wound healing associated with ulcerative pyogenic granuloma. *Journal of Clinical Investigation*, 90(5), 1952.
- Seals, D.F., Azucena, E.F., Pass, I., Tesfay, L., Gordon, R., Woodrow, M., Resau, J.H., Courtneidge, S.A., 2005.** The adaptor protein Tks5/Fish is required for podosome formation and function, and for the protease-driven invasion of cancer cells. *Cancer Cell*, 7(2), 155-165.
- Stojdl, D.F., Lichty, B.D., Paterson, J.M., Power, A.T., Knowles, S., Marius, R., Reynard, J., Poliquin, L., Atkins, H., Brown, E.G., Durbin, R.K., 2003.** VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell*, 4(4), 263-275.
- Stout, A.J., Gresser, I., Thompson, W.D., 1993.** Inhibition of wound healing in mice by local interferon alpha/beta injection. *International journal of experimental pathology*, 74(1), 79.
- Strååt, K., de Klark, R., Gredmark-Russ, S., Eriksson, P., Söderberg-Nauclér, C., 2009.** Infection with human cytomegalovirus alters the MMP-9/TIMP-1 balance in human macrophages. *Journal of Virology*, 83(2), 830-835.
- Tan, T.L., Fang, N., Neo, T.L., Singh, P., Zhang, J., Zhou, R., Koh, C.G., Chan, V., Lim, S.G., Chen, W.N., 2008.** Rac1 GTPase is activated by hepatitis B virus replication— involvement of HBX. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 1783(3), 360-374.
- Whitlow, Z.W., Connor, J.H., Lyles, D.S., 2006.** Preferential translation of vesicular stomatitis virus mRNAs is conferred by transcription from the viral genome. *Journal of Virology*, 80(23), 11733-11742.
- Wong, L.H., Krauer, K.G., Hatzinisiriou, I., Estcourt, M.J., Hersey, P., Tam, N.D., Edmondson, S., Devenish, R.J., Ralph, S.J., 1997.** Interferon-resistant human melanoma cells are deficient in ISGF3 components, STAT1, STAT2, and p48-ISGF3 γ . *Journal of Biological Chemistry*, 272(45), 28779-28785.

Chapter 3

Summary and Future Experiments

We demonstrated within this project that upon infection with VSV, 527 cells showed a decrease in invadopodia structures per cell, which correlated with both a decrease in Tks5 and a decrease in matrix degradation. In addition, we found that following infection with VSV, 527 cells showed an increase in the number of rosettes per cell, which correlated with an increase in cortactin expression, but did not correlate with matrix degradation. We are left with the underlying questions, how is the virus impeding the function of invadopodia structures, and why? Furthermore, what aspect of the viral infection leads to the increase in rosette structures, and what is their function? These are the questions that need further research to elucidate.

The decrease in degradation can be attributed to invadopodia loss of form/structure, and may be associated with the decrease in Tks5. So without Tks5, there are fewer invadopodia, which in turn means less MMP activity to degrade matrix. Further research needs to be done looking at the levels of necessary MMP levels post infection in order to confidently say that the decrease in matrix degradation was indeed attributed to MMP inhibition.

In terms of the rosette structures we observed post infection, it is known that Tks5 is always localized and required in, and for the production of, invadopodia structures. In similar structures, like CDRs and other membrane ruffings, Tks5 is not required. Localization experiments need to be done with Tks5 in order to see if upon infection

with VSV and, if Tks5 is localized to those rosette structures. If Tks5 remained localized to the rosette structures, we can assume that they didn't lose their fundamental makeup, but that the viral infection in some manner inhibited MMP activity. However, if Tks5 was not localized to the rosette structures, we could assume the virus affected Tks5, or some upstream activators of Tks5. This would also suggest that the rosette structures seen post infection were more membrane ruffle-like, rather than invadopodia-like. Both of these are promising results, as they suggest a viral-mediated decrease in metastatic potential. However, to further investigate the ability of VSV to inhibit metastatic potential, Boyden chamber invasion assays need to be performed. This assay consists of two chambers separated by a filter coated with different extracellular matrix components. The cell suspension is placed in the top chamber and incubated in the presence of test media containing specific chemoattractants in the bottom chamber. Cells migrate from the top chamber through the coated filter pores to the bottom of the filter. This would assess how VSV affects the cells' ability to chemotactically invade through a matrix. If inhibition was observed after infection, we may then state that VSV inhibits overall matrix degradation, migration, and collectively, invasion.

Excitingly, the questions we investigated within this project have not, as of yet, been explored until now. A drawback to investigating a new area is the lack of published research with which to support and direct our own experiments. Therefore, the mechanisms by which VSV infection impacts cancer cells metastatic processes, specifically the formation of invadopodia structures and downstream effects are unknown. Invadopodia assembly and maturation, as well as disassembly, is an extremely complex and dynamic system, involving many signaling pathways with countless critical

proteins. In that regard, we did investigate the levels of some known critical proteins following infection with VSV. As we have stated previously, Tks5 is necessary for the development of invadopodia structures. We found that at 14 hours post infection with rwt at an MOI 10.0 pfu/cell, the expression of Tks5 was significantly decreased. It is possible that the ability of rwt virus to shut down host gene expression is the reason for the decrease. To better understand what the mechanism of inhibition may be, the levels of Tks5 need to be assessed after infection with rM51R-M virus. If we then observed similar decreases in expression, we could assume the decrease is not attributed to the ability of rwt virus to shut down host gene expression. Furthermore, we could then address whether VSV was blocking Tks5 phosphorylation by Src, or in some way sequestering Tks5 away from the invadopodia structures, perhaps through interruption of vesicle trafficking.

We are also interested in assessing levels of cortactin upon infection of cells with VSV. Cortactin is a critical player in the development of not only invadosomal structures, but any structure requiring actin, as cortactin functions alongside the actin nucleating *Actin-Related Proteins* Arp2/3 complex, facilitating and stabilizing nucleation sites for actin branching. Interestingly, we observed a robust increase in cortactin expression 14 hour post infection with rwt virus at an MOI 10. This result correlates with the increase in actin rich rosette structures we observe following infection. However, how rwt infection promotes cortactin expression is unclear. Further experiments need to address the changes in levels of cortactin in cells infected with rM51R-M virus in order to elucidate whether these effects are attributed to the ability of rwt virus to shut down host gene expression. If we observe no differences in cortactin

expression, we may assume that rwt virus is inhibiting regulators of cortactin, such as tyrosine and serine/threonine kinases. Interestingly, it has been shown that VSV P protein requires phosphorylation by cellular casein kinase 2 (CKII) for transcriptional activity (Barik and Banerjee, 1992). It could be possible that CKII mediated viral transcription decreases the function of CKII in regulating the phosphorylation of cortactin.

It is known that cortactin is required for VSV entry into the cell, as actin machinery is needed for viral clathrin-mediated endocytosis. Cureton et al. (2009) showed that VSV depends on actin machinery for complete internalization. Additionally, they found that during growth of virus-containing pits, the levels of actin, Arp3, and cortactin increased steadily (Cureton et al., 2009). This activity may be correlated with the observed increase in cortactin expression, however, this seems unlikely as we observed the increased cortactin expression and rosettes during later stages of infection.

It may be possible to accentuate the effects of VSV on cancer cells. It has been shown previously that curcumin, a natural and principal curcuminoid in turmeric, actually promotes the oncolytic capacity of VSV for the treatment of prostate cancers by inhibiting STAT1, an essential regulator of the antiviral response pathway (Fehl and Ahmed, 2016). Other natural compounds, such as *Moringa olifera* and bromelain from pineapples, are currently being investigated in combination with VSV to assess possible synergistic effects. Although we found that VSV infection caused an overall decrease in matrix degradation, MMP activity remained prevalent. Therefore it is important to address possible combination therapies which may reduce MMP enzymes, or accentuate

the ability of VSV to inhibit them. The latter, unfortunately, would prove difficult until the mechanisms by which VSV inhibits matrix degradation are elucidated. It would be of great value to test natural derived compounds, such as curcumin, *Moringa olifera*, and bromelain with VSV to see if they enhanced the ability of VSV to inhibit matrix degradation and invadopodia formation. Another promising combination treatment could involve inserting in a rapamycin gene into the genome of rM51R-M. Rapamycin is a macrolide from the bacteria *Streptomyces hygroscopicus* (Sehgal et al., 1983). Rapamycin blocks transcriptional activation of cytokines, effectively inhibiting them. rM51R-M lacks the ability to shut down host gene expression, so expressing rapamycin may enable selective tumor lysis without the risk of host infection. Due to cost and time, a co-infection with both rM51R-M and *S. hygroscopicus* may also be effective.

Project Limitations

This project would greatly benefit from future *in vivo* studies, investigating whether VSV infection has the ability to inhibit metastasis in mouse models of cancer. Although we obtained promising results from *in vitro* experiments, there are many factors left unaccounted for without any *in vivo* results, such as dose, immune response, and toxicity. *In vivo* work needs to be done in order to progress VSV as an oncolytic agent to more clinical based application. Results from Ahmed et al. (2004) demonstrated that VSV infection decreased tumor overall volume in mice, however; VSV infection on the tumor metastatic potential has yet to be investigated. Unfortunately, an enduring problem in cancer research is the failure to reproduce encouraging preclinical therapeutic findings using transplanted or spontaneous primary tumors in mice in clinical trials of

patients with advanced metastatic disease (Kubota et al., 1994; Morikawa, 1998). Orthotopic transplantation of tumors actually enhances the possibility of distant metastatic spread, compared with ectopic transplants, and advanced multiple metastases can be obtained (Barnett and Eccles, 1984; Dawson et al., 2009), especially if the primary tumors are surgically resected. (Munoz et al., 2006). This prolongs survival and allows sufficient time for disseminated cells from the primary tumor to develop into established metastases, thus recreating the multiple sequential steps that are associated with the metastatic cascade (Barnett and Eccles, 1984; Munoz et al., 2006). So, we could orthotopically transplant human primary breast cancer cells into severe combined immunodeficient (SCID) mice, and allow tumor growth and establishment for ~3 weeks. The tumor could then be surgically resected to prevent the rapidly growing primary tumor from causing end point termination of the experiment. Several months later, after the mice show signs of lung metastasis, those cells could be collected and cultured. In theory, this enrichment process would allow for a reliable, breast to lung metastasis. The enriched cells would then be transplanted into new hosts and tagged with a marker such as luciferase 41. After allowing the tumor to establish, various treatments of both rwt and rM51R-M virus would be injected intratumorally. At varying times post infection, the location of cells could be monitored via *in vivo* bioluminescence imaging. This would indicate whether VSV infection inhibited the ability of those cells to metastasize to the lungs.

Importantly, the experiments we performed need to be replicated using rM51R-M virus. This virus represents a safer oncolytic treatment option due to its lack of pathogenicity *in vivo* (Ahmed et al., 2004). The implications of using this attenuated

virus are great, as it can be used to not only target and lyse tumor cells selectively, but recruit immune cells to the tumor site due to the virus inability to shutdown host antiviral factors. rM51R-M virus allows the expression of genes involved in antiviral responses, such as the type I interferon (IFN) gene. Ahmed et al. (2006) demonstrated that, in contrast to the rwt virus, rM51R-M virus induced the maturation of myeloid dendritic cell (mDC) populations, as indicated by an increase in the surface expression of CD40, CD80, and CD86 as well as the secretion of interleukin-12 (IL-12), IL-6, and type I IFN. In addition, mDC infected with rM51R-M virus effectively activated naïve T cells in vitro, whereas rwt virus-infected mDC were defective in antigen presentation. The study by Ahmed et al. (2006) indicated that rM51R-M virus effectively stimulates the maturation of mDC and has the potential to promote effective T-cell responses to vector-expressed antigens, activate DC at tumor sites during therapy, and aid in tumor immunosurveillance and destruction. Future treatments using rM51R-M are promising, as the virus is not only effective at tumor oncolysis and inhibition of metastatic processes, but also in the activation and maturation of immune cells to assist in tumor destruction.

Additionally, an important limitation to the project was using only one cell line. This cell line (527) was used due to its constitutively active Src, which subsequently enhances the cells' proliferation rate, survival, migration, degradation, invadopodia, and overall metastatic potential. This model cell line allowed us to better assess the effects of VSV infection on critical metastatic factors such as migration, matrix degradation, and invadopodia formation. Unfortunately, because we used only one cell line, we cannot confidently state that the effects we observed in 527 cells would also translate to other

types of cancers. For example, not all cancers have aberrant Src signaling. Presumably, the effects of VSV infection on the invasive structures of 527 cells would be similar in other cell types since invadopodia have actin rich cores, but without further research on other cell types, we cannot safely make that assertion.

In conclusion, the results from this project have yielded important insights on the interactions between VSV and invasive cancer cells. We found that VSV infection significantly inhibits the ability of invasive cancer cells to form structures which are necessary for metastasis. Our studies have contributed to the field of oncolytic virotherapy by laying out a foundation for how VSV may inhibit the metastatic process. This study further demonstrates that there may be safer, more effective late stage cancer treatments than those currently being employed.

References

- Ahmed, M., Brzoza, K.L., Hiltbold, E.M., 2006.** Matrix protein mutant of vesicular stomatitis virus stimulates maturation of myeloid dendritic cells. *Journal of Virology* 80(5):2194-205
- Ahmed, M., Cramer, S., Lyles, D.S., 2004.** Sensitivity of prostate tumors to wild-type and M protein mutant vesicular stomatitis viruses. *Journal of Virology*. 330: 34-49.
- Barik, S., Banerjee, A.K., 1992.** Phosphorylation by cellular casein kinase II is essential for transcriptional activity of vesicular stomatitis virus phosphoprotein P. *Proceedings of the National Academy of Sciences*, 89(14),6570-6574.
- Barnett, S.C., Eccles, S.A., 1984.** Studies of mammary carcinoma metastasis in a mouse model system. I: Derivation and characterization of cells with different metastatic properties during tumour progression in vivo. *Clinical and Experimental Metastasis*, 2(1), 15-36.
- Cureton, D.K., Massol, R.H., Saffarian, S., Kirchhausen, T.L., Whelan, S.P., 2009.** Vesicular stomatitis virus enters cells through vesicles incompletely coated with clathrin that depend upon actin for internalization. *PLoS Pathogens*, 5(4), e1000394.
- Dawson, M.R., Duda, D.G., Fukumura, D., Jain, R.K., 2009.** VEGFR1-activity-independent metastasis formation. *Nature*, 461(7262), E4-E4.
- Fehl, D.J., Ahmed, M., 2017.** Curcumin promotes the oncolytic capacity of vesicular stomatitis virus for the treatment of prostate cancers. *Virus Research*, 228, 14-23.
- Kubota, T., 1994.** Metastatic models of human cancer xenografted in the nude mouse: the importance of orthotopic transplantation. *Journal of Cellular Biochemistry*, 56(1), 4-8.
- Munoz, R., Man, S., Shaked, Y., Lee, C.R., Wong, J., Francia, G., Kerbel, R.S., 2006.** Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. *Cancer Research*, 66(7), 3386-3391.
- Morikawa, K., Walker, S.M., Jessup, J.M., Fidler, I.J., 1988.** In vivo selection of highly metastatic cells from surgical specimens of different primary human colon carcinomas implanted into nude mice. *Cancer Research*, 48(7), 1943-1948.
- Sehgal, S.N., H. Baker, C.P. Eng, K. Singh., C. Vezina. 1983.** Demethoxyrapamycin (AY-24,668), a new antifungal antibiotic. *J Antibiot (Tokyo)*. 36:35 1-4.

Vita

Dakota Wayne Goad was born in Mt. Airy, North Carolina, to Guthrie and Lisa Goad. He graduated from East Wilkes High School in May, 2009. The following autumn, he entered Wilkes Community College, and in January 2011 he was awarded the Associate of Arts degree. In the fall of 2011, he entered Appalachian State University for Cell and Molecular Biology, and in May 2014, was awarded the Bachelor of Science degree. In the fall of 2014, he entered the Biology Master of Science program at Appalachian State University where he accepted a teaching assistantship. The M.S. was awarded in August, 2017.

Mr. Goad is an avid mountain biker and resides in Boone, North Carolina teaching Genetics until he enters a Ph.D. program the Fall of 2018.

47.

Formatted: Font: (Default) Times New Roman, 12 pt

Formatted: Normal, Indent: Left: 0.5", No bullets or numbering