CELLULAR MECHANISMS OF TUMOR CELL INVASION

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Doctor of Philosophy

by

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______________________________
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Abstract
by
Alanna E. Sedgwick

The ability of cancer cells to detach from their tumor of origin and “invade” into surrounding tissue marks a turning point in cancer progression. Invadopodia and microvesicles are cytoskeleton-rich membrane structures via which cells can proteolyze the extracellular environment to facilitate invasion, and their regulation is a focus of this dissertation. Robust motility is also integral for tumor cell invasion, and we describe how signaling of the small GTPase ARF6 coordinates these activities.

This dissertation describes how alterations in extracellular matrix compliance guide the formation of microvesicles and invadopodia to facilitate matrix proteolysis. The data presented here indicates that formation of these structures is mutually exclusive, regulated by antagonistic signaling between the small GTPases Rac1 and RhoA. In addition, we have described how Rho signaling regulates myosin light chain phosphatase during myosin contractility, required for release of microvesicles from the tumor cell surface.
Critical to ARF6-mediated regulation of tumor cell invasion are the activities of the GTPase activating proteins (GAPs) and exchange factors (GEFs) that control its nucleotide status. Activation of ARF6 downstream of Wnt5a stimulates invasive ability via the Wnt/β-catenin canonical pathway, and is opposed by signaling through SLIT2-ROBO1, which stimulates ARF6 GAPs. During epithelial cell migration, the actin-binding protein CD2AP is capable of downregulating ARF6 activity by recruiting and stabilizing the GAPs ArfGAP3 and ASAP at the cell surface.

To further delineate the contribution of ARF6 to cell invasion, we developed new reagents that target its downregulation, including a lentiviral vector for ARF6 silencing. This vector has demonstrated very effective knockdown of ARF6 levels, however, additional work will be necessary to address off-target effects of the virus before it can be utilized for studies of tumor cell invasion. We also tested the hypothesis that Src-mediated phosphorylation of the ARF6 GAP ACAP2 will result in increased ARF6-GTP levels during tumor cell invasion, and have created plasmids for the expression of ACAP2 phosphorylation mutants for these studies. Finally, we describe the use of a tumor cell spheroid assay for assessing invasive behavior, which sheds light on the negative impact that constitutive ARF6 activation has on the development of metastatic lesions.
This dissertation is dedicated to my family and friends, both two- and four-legged, who have supported and motivated me, and to those whose lives have been disrupted by cancer.
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<td>CO$_2$</td>
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<td>FBS</td>
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<td>FITC</td>
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<tr>
<td>GDI</td>
<td>Guanine Nucleotide Dissociation Inhibitor</td>
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<td>Guanine Nucleotide Exchange Factor</td>
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<tr>
<td>kD</td>
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<tr>
<td>Ng</td>
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<td>PA</td>
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<td>Raichu</td>
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<tr>
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<tr>
<td>Thr</td>
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<tr>
<td>TMV</td>
<td>Tumor-Derived Microvesicle</td>
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<tr>
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<tr>
<td>TRAIL</td>
<td>Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand</td>
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<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane-Hydrochloric Acid</td>
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<tr>
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INTRODUCTION

1.1 Cancer metastasis

In its simplest definition, the process of cancer metastasis occurs when cells break free from their tumor of origin, move through the body, and colonize at a secondary site or sites. The transition from cancer which exists in situ to the development of metastatic disease is generally an indicator of worsened prognosis\textsuperscript{18; 27}. Using breast cancer as a prevalent example, the five-year survival expectancy is 100% for women diagnosed with localized disease, while it is only 21% when diagnosed after distant metastasis\textsuperscript{65}. In the case of melanoma, the five-year survival expectancy for those diagnosed with localized disease is over 98%, while it is approximately 15% for those with distant metastasis\textsuperscript{190}. Such statistics underscore the need for a deepened understanding of the processes regulating this activity, in an effort to develop improved diagnostics and therapeutics.

During the course of metastasis, a cell or group of cells leave the tumor of origin and invade into the surrounding tissue\textsuperscript{51}. From there, cells breach the basement membrane of the tissue, and in the case of distant metastasis, migrate through the extracellular environment to either the blood or lymphatic system. Cells can then intravasate into the blood or lymphatic vessel and must survive in circulation, and then
adhere to and extravasate out of the vessel, although some cells in the lymphatic system will establish themselves in lymph nodes\textsuperscript{142; 237}. From this point, the cells can colonize to establish a secondary tumor\textsuperscript{142; 218}. There is often a correlation between the tissue in which the tumor originated and the secondary sites which are preferentially colonized. In many cases this reflects the downstream flow of blood or lymphatic fluid, with cells colonizing at a location that the circulation sweeps them to, although some tumors, such as prostate cancer, prefer less obvious targets such as bone\textsuperscript{18; 27; 139}. This phenomenon not yet fully understood and there are a variety of hypotheses for why this occurs, from the potential that certain tissues provide a better matrix for the displaced cells, to the possibility of recruitment signals for the displaced cells\textsuperscript{139}. Underpinning the process of metastasis is the ability of cells to leave the primary tumor and invade into the extracellular environment, which involves a complex orchestration of cellular behaviors.

1.2 Tumor cell invasion

Within a tumor, cells may exist as non-invasive, which will remain confined within the tumor’s boundaries, or they may acquire the ability to invade into the surrounding environment. The majority of normal cells do not possess invasive ability, beyond what is required during the processes of development. However, during the process of cancer transformation, this capability may be acquired (or re-acquired)\textsuperscript{124}. This ability of cells to invade into and traverse the extracellular environment is a prerequisite for tumor cell dissemination and metastasis. A deregulation of cell-cell and
cell-matrix interactions, along with the acquired ability to proteolyze and move through the extracellular environment\textsuperscript{196, 236}, underlie one of the most unfavorable aspects of cancer. This can be accomplished by individual cells, which have abrogated their use of cell-cell adhesion molecules by transcriptional and post-transcriptional mechanisms\textsuperscript{43, 51; 146, 148; 206; 213}, or by groups of cells moving collectively \textsuperscript{51, 53}. This ability involves the deregulation of many normal cellular behaviors, and depends upon the formation of structures which degrade the extracellular matrix. The formation of invadopodia, utilized for local proteolysis, and microvesicles, which can be used to degrade and condition the extracellular environment at greater distances, are key structures whose formation is reliant on signaling through small GTPases of the RAS superfamily\textsuperscript{41, 130, 134; 196, 205}. Signaling through these pathways is required for processes such as remodeling cellular architecture to form protrusions and adapt to the extracellular space\textsuperscript{62, 71, 82}, and for trafficking of proteases and adhesion molecules to facilitate matrix degradation and motility, respectively\textsuperscript{42, 62, 196, 205}.

1.3 Small GTPase signaling in tumor cell invasion

Ras-related small GTPases comprise a family of monomeric GTP-binding proteins which function as molecular switches in a wide variety of cell processes. Within the RAS superfamily of small GTPases there are several subfamilies: Ras, Rho, Rab, Ran, and Arf, in addition to some currently unclassified members\textsuperscript{165}. These GTPases exist in two forms: GTP-bound and active, or GDP-bound and inactive. Activation to the GTP-bound state is catalyzed by Guanine nucleotide Exchange Factors (GEFs) which substitute GTP
for GDP, and inactivation is catalyzed by GTPase Activating Proteins (GAPs), which promote GTP hydrolysis\textsuperscript{24, 205}. In general, GTPases are active and signaling when membrane-associated, and soluble in the cytoplasm when GDP-bound and inactive\textsuperscript{24, 32}. Guanine nucleotide Dissociation Inhibitors (GDIs) play a role in maintaining inactivity for the Ras, Rho, and Rab members, whose large hydrophobic domains make it difficult for them to partition into the cytoplasm unassisted\textsuperscript{24, 32}.

The ADP-ribosylation factor (ARF) subfamily consists of three classes: Class I is comprised of ARFs 1-3, class II is ARFs 4 and 5, and class III is ARF6\textsuperscript{42}. The ARF proteins have diverse functions in the cell, with ARF6 regulating endosomal membrane trafficking via clathrin-dependent and independent mechanisms, and peripheral actin rearrangements\textsuperscript{42}. The association between ARF6 and tumor cell invasion and metastasis has been well documented, and involves a complex array of downstream effectors\textsuperscript{160, 178}. For instance, studies have revealed a direct correlation between increased levels of ARF6 protein, as well as increased expression of the ARF6 GEF GEP100, with increased invasiveness in mammary cancers\textsuperscript{170}. ARF6 has been found to be required during glioma cell invasion in the brain\textsuperscript{71}. Suppression of ARF6 expression has been shown to inhibit glioma cell invasion both \textit{in vitro} and \textit{in ex vivo} brain slices, and produces less invasive tumors \textit{in vivo} in mice brains\textsuperscript{71}. ARF6 stimulates glioma cell motility through the formation of a complex with Rac1 and the IQ-domain GTPase-activating protein 1, IQGAP1, for the formation of protrusions from the plasma membrane\textsuperscript{71}. Furthermore, the brain-exclusive ARF6 GEF EFA6A has been shown to
promote cell motility and invasion through ARF6/MEK/ERK signaling cascades\textsuperscript{106}. The influence of ARF6 on tumor cell invasion has also been demonstrated in an \textit{in vivo} study from our lab, looking at melanoma tumor growth in nude mice subcutaneously injected with cells from the invasive LOX line expressing constitutively-active ARF6\textsuperscript{133}. While showing decreased tumor size, the cells displayed a much higher level of invasiveness compared to the control, with a greater number of cells breaching the tumor border and infiltrating the surrounding tissue. Cells expressing a dominant-negative ARF6 mutant showed a decrease in invasive capacity\textsuperscript{133}.

The RHO subfamily of GTPases consists of many members, including multiple Rho, Rac, and Rnd members, as well as Cdc42\textsuperscript{165; 205}. This subfamily impinges on many important cellular processes, particularly as they relate to modulation of the cytoskeleton and motility\textsuperscript{63; 205}. High levels of RhoA and Rho-associated protein kinase (ROCK) expression have been correlated with tumor metastasis\textsuperscript{85}, and an increase in the expression of certain Rho GEFs has been shown to modulate the lung metastasis of breast cancer cells\textsuperscript{28}. Rho signaling is important for the formation of protrusions in the invading cell\textsuperscript{198}, and RhoA has also been demonstrated to perform important functions in the regulation of stress fiber formation, focal adhesions, and in the ability of cells to move in a rounded, “amoeboid” manner\textsuperscript{25; 176}. Key to this activity is the involvement of Rho signaling in the regulation of myosin activity\textsuperscript{86}. \textit{In vitro}, ROCK, which lies directly downstream of Rho, has been shown to directly phosphorylate myosin light chain, facilitating its activation\textsuperscript{208}. An additional role for Rho signaling in myosin activation lies
in the ability of ROCK to phosphorylate and inactivate myosin light chain phosphatase, which facilitates sustained myosin contractility.\(^9\); \(^{10}\); \(^{19}\). Signaling through Rac1 is indispensable for many processes requiring reorganization of the actin cytoskeleton, including the formation of cell protrusions required for motility, such as lamellipodia and membrane ruffles.\(^{26};^{40};^{103};^{140};^{154};^{205}\). Rac1 has also been shown to be indispensable for the use of mesenchymal-type motility in tumor cells.\(^{176}\). Rac1 plays an important role in orchestrating invasive behavior, with Rac1 inhibition resulting in decreased invadopodia formation and invasiveness \textit{in vitro}\(^{26};^{154}\), and reduced tumor growth and invasiveness \textit{in vivo}\(^{103}\).

1.4 Invadopodia

Degradation of the extracellular environment via proteolysis is an important process in tumor cell invasion, and invadopodia are a well-characterized structure mediating this process (Figure 1.1)\(^{114};^{134};^{227}\). First identified in the 1980s as SRC-mediated cell protrusions which direct pericellular proteolysis, similar to the podosomes formed by some cells during normal tissue remodeling, these actin-based structures at the ventral surface of the cell have been implicated to play an integral role in the invasive behavior of tumor cells.\(^{134};^{225}\). In contrast with podosomes, invadopodia are tumor cell-specific and are generally longer-lived and found beneath the nucleus, as opposed to near the leading edge of the cell\(^{34};^{134}\). \textit{In vivo} intravital studies have shown the dynamic formation of these invasive protrusions.\(^{35}\). A variety of stimuli can induce invadopodia formation, such as the growth factors VEGF\(^{113}\), PDGF\(^{44}\), EGF\(^{233}\), and TGF-
β$^{154}$, and signaling downstream from integrin-matrix interactions$^{12; 15}$. Integrins also impact the recruitment of certain proteases to invadopodia$^{7; 128}$, with a wide variety of proteases being utilized for matrix degradation. At present, this includes the zinc-dependent metalloproteinases MMP-2, MMP-9, and MMP-14$^{6; 23}$, the cysteine cathepsins$^{125}$, and the serine proteases seprase and uPA$^{7; 22}$. Their formation is dependent on proteins necessary for membrane protrusion and actin remodeling, such as the Arp2/3 complex, N-WASP, cofillin, and cortactin$^{134; 233}$. Cortactin has also been shown to play an important role in regulating protease secretion$^{29; 30}$, further demonstrating the cross-talk that exists in regulating invadopodia. Multiple GTPases are involved in invadopodia formation, with key roles in regulating the actin architecture, including Rac$^{126; 103; 154}$, Cdc42$^{233}$ and ARF6$^{26; 64; 68; 203}$.

1.5 Microvesicles

Cell-derived microvesicles (MVs) are relatively newly-characterized structures that have garnered attention for the role they play in both normal cell physiology and disease. Once discounted as merely cell debris, it is now thought that MVs could provide a multitude of utilities for cells, with great relevance during disease progression$^{76; 144}$. These vesicles are selectively loaded with cargo from the cell (Figure 1.1) and are formed by outward blebbing and fission from the plasma membrane, pinched free by acto-myosin contraction$^{132}$. Tumor cell-derived microvesicles (TMVs) have been shown to possess unique membrane characteristics, with phosphatidylserine (PS) externalized$^{131}$, in contrast to its normal position in the inner leaflet of non-apoptotic
cells. They are heterogeneous in size and range from 200 nm to greater than 1 μm in diameter. They have been characterized to contain a wide variety of molecules, including nucleic acids, various proteins including functional transmembrane receptors, and proteases. Microvesicles provide an interesting target for the development of screening and diagnostic assays, as they are often found in bodily fluids, and their quantity and composition may be modulated in a wide variety of diseases, including preeclampsia, diabetes, neurologic disease, and cancer. The potential for transmission of oncogenic material from tumor cells by microvesicles is of great interest in the context of cancer treatment. In a brain tumor model, it was shown that aggressive glioma cells expressing the EGFR mutant EGFRvIII could transfer the oncogenic receptor to neighboring cells via microvesicles, inducing them to express and shed the receptor as well, with a corresponding transformation to a more aggressive phenotype. Similarly, it has been shown that microvesicles carrying tissue transglutaminase derived from breast and glioma cells were capable of transforming neighboring fibroblasts. Nucleic acids are also frequently released in microvesicles for the modulation of target cells, ranging from miRNA to cDNA. Tumor cell-derived microvesicles may be utilized for the evasion of immune attack by the inhibition of an immune response initiation by macrophages, suppression of lymphocyte response, and inducing apoptosis in neighboring immune cells by transfer of molecules such as FasL and TRAIL. The proteolytic content of TMVs is central to their role in invasion, and the protease content of TMVs has been demonstrated to correlate with the
invasiveness of the cells they are derived from\textsuperscript{57}. A variety of proteases have been localized to TMVs, including MMP-2, MMP-9, MMP-14, seprase, and uPA\textsuperscript{57; 128; 132}. Tumor cell-derived microvesicles have also been indicated in a variety of other processes, from the promotion of angiogenesis\textsuperscript{100} to use as a vessel for the expulsion of chemotherapeutics\textsuperscript{188}. ARF6 activation is required for the release of invasive microvesicles from the cell\textsuperscript{132}. Examination of three mammary lines, which range from non-invasive to highly invasive, reveals that the number of ARF6-positive microvesicles shed correlates with cell invasiveness (Figure 1.2).

1.6 Project rationale

While the process of tumor cell invasion has been a well-studied field for many years, it is a constantly evolving area. Despite the deep body of knowledge in this subject, cancer remains a very prominent global health concern. It is recognized now, more than ever, that the plasticity of tumor cells is a large problem for the targeting of therapeutics and in disease which has become refractory to treatment. The first aim of this dissertation addresses that plasticity, focusing on characterizing the matrix conditions which favor the formation of invadopodia versus microvesicles during tumor cell invasion, and examining the mutually competitive RHO signaling that regulates their formation. Next, in Chapter 3, we further characterize the regulation of myosin activity during the formation of microvesicles, and describe a newly elucidated pathway downstream of Rho, involving the inactivation of myosin light chain phosphatase. In Chapter 4, we outline signaling pathways downstream of Wnt5a which regulate the
activation of ARF6 through the use of GTPase activating proteins and exchange factors in the processes of cell invasion and migration. Finally, we describe the creation of additional tools which have been developed to regulate ARF6 activity in cells, as well as monitor its role in tumor cell invasion.
1.7 Figures

Figure 1.1 Schematic illustration of tumor cell-derived microvesicle and invadopodia architecture. TMVs and invadopodia possess unique characteristics and are comprised of a complex and diverse array of molecules.
Figure 1.2 Quantity of ARF6-positive microvesicles released correlates with invasiveness. Microvesicles were isolated from equal numbers of non-invasive, untransformed MCF-10A mammary cells; mildly invasive MCF-7 mammary adenocarcinoma cells; and highly invasive MDA-MB-231 mammary adenocarcinoma cells and then lysed, subjected to SDS-PAGE, and probed for ARF6. The cells they were isolated from were lysed, subjected to SDS-PAGE, and probed for α-tubulin to ascertain that microvesicles were isolated from an equivalent numbers of cells.
CHAPTER 2: 
ROLE OF MATRIX COMPLIANCE AND RHO SIGNALING IN DETERMINING MODE OF INVASION

2.1 Introduction

The journey from a tumor to a metastatic site in a distant region of the body presents a multitude of challenges to a tumor cell. The cell must survive the turbulent environment in lymphatic or blood circulation and evade immune attack. A variety of extracellular matrices must be traversed, comprised of collagens, fibronectins, and laminins, that range from a dense and compressed meshwork to loose and soft tissues. It has been demonstrated that the properties of the extracellular matrix and cell-matrix interactions greatly impact cell behavior and tumor progression. The matrix provides a scaffold for cell movement, and the engagement of matrix receptors is critical to provide the cues for intracellular signal transduction pathways that regulate migratory and invasive behavior. The matrix microenvironment of tumors is usually highly modified and atypical, with abnormal deposition and crosslinking, reflected in altered stiffness and the linearization of collagen fibers surrounding a tumor, which may facilitate directional migration through the extracellular environment. These characteristics are what allow most tumors to be palpated as a firm nodule in otherwise
complaint tissue. There is continual remodeling at these sites, with elevated amounts of matrix deposition and proteolysis as tumors shape their microenvironment and cells move into the surrounding area. Development of assays which model variations in the extracellular environment are important to advance the understanding of invasive cell behavior.

The importance of RHO signaling in the plasticity of cell motility to accommodate varied matrices is an area of study that has garnered much interest in recent years. ARF6 signaling modulates Rac1 and RhoA activity in many cellular processes, and an antagonistic relationship between signaling through Rac1 and RhoA has been long established, with high levels of Rho activity associated with a decrease in Rac, and vice versa. This coordination of Rac and Rho activity is very important in regulating the formation and disassembly of stress fibers, focal adhesions, and membrane ruffles in the process of motility. Both Rac1 and RhoA have been implicated with important roles in tumor cell invasion, demonstrated both in vitro and in vivo. Given the importance of these signaling pathways in regulating the development of membrane protrusions, myosin contractility, and integrin signaling, we sought to investigate their roles in the regulation of invadopodia- and microvesicle-mediated invasion, and how this may occur downstream of ECM cues.
2.2 Materials and Methods

2.2.1 Cell culture, transfection, and reagents

LOX and LOX stably expressing ARF6(Q67L) and ARF6(T27N) (generation of which has been previously described\textsuperscript{133}) were grown in RPMI and PC-3 in F-12K, all purchased from Gibco, with 2 mM l-glutamine (Gibco), and penicillin/streptomycin (Thermo Scientific). All media was supplemented with 10% FBS from Hyclone, with the exception of the LOX mutants, which were grown in tetracycline-free FBS (Clontech), in the presence of G418 (Gibco) and hygromycin (Invitrogen). FBS in media to be used for microvesicle isolations was pre-cleared of microvesicles and exosomes as detailed in “Microvesicle Isolation” below. Cells were maintained in a 37°C humidified incubator with 5% CO\textsubscript{2}. Transfection of LOX lines was accomplished with GeneExpresso (Excellgen), following the manufacturer’s suggested protocol. NSC23766 was used at 50 μM and Y-27632 at 10 μM, and were purchased from Calbiochem. DAPI and rhodamine-phalloidin were purchased from Molecular Probes. The following antibodies were utilized for these studies: mouse anti-T7 (Novagen), rat anti-\(\beta_1\) integrin clone AIIB2 (Iowa DSHB), mouse anti-RhoA (Cytoskeleton), mouse anti-\(\alpha\)-tubulin (Sigma), rabbit anti-cortactin (Cell Signaling), mouse anti-Rac1 (BD Transduction), mouse anti-paxillin (BD Transduction), and mouse anti-HA (Covance).
2.2.2 Invasion assays

FITC-conjugated gelatin and gelatin-coated coverslips were prepared as described previously, using 18 mm round glass coverslips (Fisherbrand). Approximately 30 µm of gelatin was utilized for thick, soft coverslips, and approximately 1 µm for thin, firm coverslips. Cells were seeded onto coated coverslips and grown for 16 hours prior to fixation and staining. All inhibitor treatments were initiated after the cells had been allowed to adhere for one hour after seeding. Statistical analysis of invasion data was accomplished utilizing Microsoft Excel and GraphPad Prism software.

For 2-D experiments utilizing collagen, cells were grown on type I bovine collagen (PureCol from Advanced Biomatrix) using the manufacturer’s suggested protocol for 3-D gel preparation, in 35 mm glass-bottom dishes (MatTek), which had been coated with poly-l-lysine. Cells were fixed and stained as detailed below, and imaged using a Nikon A1R microscope equipped for confocal reflection microscopy.

The spheroid invasion assay was adapted from methods described by Smalley et al. Cells were induced to form spheroid aggregates by plating 5,000 cells per well in complete media onto 1.5% agar in a 96-well dish. Aggregates were allowed to form over the course of 72 hours, at which point they were harvested using a large bore p1000 tip and suspended in a type I collagen mixture (PureCol from Advanced Biomatrix) prepared as per the manufacturer’s specifications for a 3-D gel, using 10x RPMI medium. This was overlaid onto a base of collagen in a 24-well dish, prepared by placing 300 µL of collagen mixture made using 10x PBS as per the manufacturer’s
specifications for a 3-D gel, which was polymerized by a 1 hour incubation at 37°C. After
the top layer of collagen was polymerized, it was overlaid with complete RPMI medium.
Spheroid aggregates were imaged live using a Zeiss Observer Z1 microscope at 5x and
10x magnification. Calcein-AM was purchased from Molecular Probes and staining was
performed as per the manufacturer’s suggested protocol.

2.2.3 Immunofluorescence staining and microscopy

For immunofluorescence staining, cells were fixed and stained as described
previously\textsuperscript{14}, and mounted using Prolong Gold Antifade mounting medium (Invitrogen).
Imaging was performed using a BioRad MRC-1024 laser scanning confocal microscope.
Image J (NIH) was utilized to optimize image brightness and clarity, and for
pseudocoloring the black-and-white images.

Live videos were obtained using an Andor Revolution spinning disk confocal
microscope equipped with a Tokai Hit environmental chamber to maintain a humidified
37°C and 5% CO\textsubscript{2} environment. Cells were grown in 35 mm dishes with glass coverslip
bottoms (MatTek) in phenol-free media (Hyclone), and images were obtained every 30
seconds over the course of several hours. Image J (NIH) was utilized to optimize image
brightness and clarity, and for pseudocoloring the black-and-white images.
2.2.4 Western blotting and activation assays

Where indicated, cells were grown on coverslips coated with a thin or thick layer of gelatin for 16 hours prior to lysis. Otherwise, cells were grown on tissue culture plastic. For western blotting, cells were lysed in RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholic acid, 0.1 % SDS, 50 mM Tris-HCl pH 7.5) containing mammalian protease inhibitor cocktail (Sigma), protein was quantified when required (BCA assay from Thermo Scientific) and samples were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with the indicated antibodies as per the manufacturer’s instructions. PAK activation assays were carried out as previously described. Briefly, cells were lysed and cleared of membranous material via brief centrifugation, and subsequently incubated with PAK-PBD-GST beads with agitation for 1 hour. Samples were run via SDS-PAGE and processed as above. The kit for the Rhotekin-RBD pulldown assay was purchased from Cytoskeleton, and performed as per the manufacturer’s suggested protocol.

2.2.5 Microvesicle isolation

Isolation of tumor cell-derived microvesicles and exosomes was performed as previously described, with minor modifications: following the initial spins, microvesicles were isolated by centrifugation at 10,000 x g for 1 hour, and were then washed with PBS and repelleted 3 times prior to lysis into RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholic acid, 0.1 % SDS, 50 mM Tris-HCl pH 7.5) containing
mammalian protease inhibitor cocktail (Sigma). Protein levels were quantified via BCA assay (Pierce) and lysates subjected to western analysis as above.

2.3 Results

2.3.1 Extracellular matrix guides choice of invasive structures

In an effort to better elucidate how the matrix impacts invasive behavior, invasive cells were plated onto fluorescently-labeled, denatured collagen (gelatin) of varying thickness. Placing cells on glass coverslips coated with a thin fluorescent matrix is a well-established means by which to visualize invasive structures and matrix proteolysis\textsuperscript{203}, as demonstrated by the loss of fluorescent signal, the pattern of which can be used to infer how the matrix was degraded. In addition to the thin layer of matrix which has been used to evaluate invadopodia, we demonstrate a novel use of this assay, in which we utilize gelatin to prepare a thick and compliant surface, which allows cells to become completely embedded. Advantageously, this assay also allows for secure retention of released TMVs. These studies were carried-out primarily using LOX melanoma cells, including those which stably express constitutively-active ARF6, ARF6(Q67L), which are useful due to their heightened invasiveness and formation of both invadopodia and microvesicles, which has been well characterized\textsuperscript{132; 133; 203}. To demonstrate that these findings were universal, other tumor cells lines such as PC-3 prostate adenocarcinoma were also used where indicated in the figure legends.
Interestingly, we found that when cells were plated onto a thick, compliant matrix, the vast majority adopted a rounded and blebbing morphology as they cleared their way through paths, and they shed TMVs abundantly, which were captured in their wake (Figure 2.1 A). Since these cells display a rich pool of β₁ integrin at the cell surface, an anti-β₁ integrin antibody was used to mark the plasma membrane and released TMVs for immunofluorescence studies. We can examine behavior in an xz or yz plane, and are unable to find any evidence of invadopodia protruding from the ventral surface of cells in this amoeboid morphology (Figure 2.1 B).

Advantageously, the ability to capture microvesicles within the matrix provides a good model system for visualizing their contents. For example, it has been demonstrated that TMVs often contain nucleic acids, and this assay facilitates the observation of nuclear material being packaged inside (Figure 2.2). Live imaging of cells which are transiently transfected to express the red fluorescent protein mCherry cytoplasmically shows the way by which cells are able to protrude blebs forward into the matrix, with the cell then moving forward into that area (Figure 2.3). Areas of intense blebbing often give way to released TMVs, and some cells appear to “drill” elongated, blebbing protrusions into the matrix ahead of themselves. Subsequent cells are often seen following in tracks made by cells which came before them, demonstrating how this behavior could be very beneficial to cells moving in vivo. When examining fixed cells in a fibrillar collagen environment, they can be seen depositing TMVs along collagen fibrils, while filopodia at the leading edge of the cell appear to align
and pull upon the fibrils (Figure 2.4). To recreate a more tumor-like environment, LOX were induced to form spheroid aggregates as in an assay first described by Smalley et al. in which tumor-like aggregates of cells are embedded in a collagen matrix, and the behavior of cells invading the surrounding ECM can be monitored. Within only 24 hours, an extensive number of cells can be seen leaving the surface of the aggregate, and this behavior continues over subsequent days (Figure 2.5). The majority of these cells are in a rounded morphology, and are surrounded by a large number of released vesicles (Figure 2.6).

When plated onto a thin substrate, the majority of cells adopt a flattened and spread morphology, and form invadopodia to degrade the matrix (Figure 2.7 A). Invadopodia use can be surmised from the visualization of actin protrusions from the ventral cell surface which stain for the invadopodia marker cortactin (Figure 2.7 B). The presence of pinpoint puncta of matrix degradation underneath cells, as well as adjacent to cells which have moved away, also indicates the use of invadopodia. While a limited number of these cells do form membrane blebs, the number of microvesicles released from the cell surface is very minor in comparison to cells in the thicker environment (Figure 2.8). Live imaging of cells in this condition shows flat cells “crawling” across the surface, often leaving small puncta of proteolyzed matrix from invadopodia (Figure 2.9).
2.3.2 Inhibition of Rac1 in cells on a compliant matrix amplifies microvesicle formation

Rac1 activity has been implicated in tumor cell invasion, and existing work has demonstrated a necessity for Rac1 activity in invadopodia formation\textsuperscript{26; 103; 154}. With this in mind, we sought to analyze the necessity for Rac1 activity in the formation of invasive microvesicles. Noting the importance of a yielding matrix to encourage microvesicle formation, the effects of inhibiting Rac1 activity in microvesicle formation were analyzed by treating cells with the Rac1 inhibitor NSC23766, which interferes with Rac1 activation, or via transient transfection with the dominant-negative mutant of Rac1, Rac1(T17N), in cells growing in a compliant, thick matrix. Interestingly, cells show a dramatic increase in microvesicle shedding when Rac1 activity is inhibited by either means (Figure 2.10). Whereas control cells often display modest numbers of microvesicles shed around themselves and in their degraded paths, Rac1 inhibited cells frequently exhibit a dramatic increase in the number of TMVs released from the cell surface, most markedly seen in those treated with the potent small molecule inhibitor. Inhibitor treated cells frequently displayed a decrease in filamentous actin, demonstrated by a decrease in phalloidin staining (Figure 2.10), which is not surprising, given the important role for Rac1 in actin polymerization. To confirm that the increased vesicle release was not due to apoptosis, cells were stained for cleaved-caspase 3, which showed that the cells were not positive for the active caspase, however their vesicles often were (Figure 2.11). The implications of active caspase in these vesicles is not yet known, but it is interesting to note that the activation of caspase-3 has been shown to
be required for the externalization of phosphatidylserine to the outer leaflet of the plasma membrane in apoptotic cells\textsuperscript{119}. A recent manuscript in \textit{Science} has demonstrated a role for the protein Xkr8, which is activated by cleaved caspase-3, in the process of apoptotic PS externalization\textsuperscript{200}. In addition, caspase 3 has been shown to activate ROCK activity, leading to MLC phosphorylation, in the formation of apoptotic blebs\textsuperscript{183}. The possibility that caspase activity is also regulating PS externalization and/or contributing to MLC activation in microvesicle biogenesis are interesting questions for future investigation.

2.3.3 Rac1 inhibition is not sufficient to induce microvesicle formation on a firm matrix

To assess if Rac1 inhibition was sufficient to induce microvesicle formation in cells cultured on a firm matrix which is not conducive to TMV formation, the Rac1 inhibitor NSC23766 and dominant-negative Rac1 mutant were again utilized. Interestingly, with cells cultured on a firm matrix, an inhibition of Rac1 activity induces only a mild effect on gross morphology. The cells appear flat and well spread, as in the controls. On closer examination, it is evident that the cells exhibit a decrease in invadopodia (Figure 2.12 A&B), corroborating the published literature which highlights the importance of Rac1 for invadopodia formation\textsuperscript{26; 103; 154}. The expression of Rac1(T17N) induces the formation of small membrane blebs across the cell surface, however these are not released as microvesicles (Figure 2.13).
2.3.4 Rac1 inhibition promotes focal contact formation

When visualizing an invasion assay of cells cultured on a fluorescent matrix, it is important to be able to differentiate between areas of invadopodia-mediated proteolysis, and other artefactual means of matrix damage, such as that which may be incurred during the preparation of the slips themselves. Another important consideration is whether the degradation seen is due to invadopodia proteolyzing the matrix, or due to focal adhesions pulling up the matrix. At first glance the patterns may appear similar, however upon closer examination, certain features become apparent. Areas of matrix which are degraded by proteolysis appear dark against the fluorescent background, with no bright areas seen at the periphery of the degradation. In the case of focal contact-mediated damage, the opposite is true. A dark area of matrix damage is noted, however the periphery of the area is often ragged, and generally bright areas are seen adjacent to the dark spots, where the cell has pulled the displaced matrix over the surrounding area, as shown in the figures below. Often times the matrix which is pulled-back appears to be attached to the periphery of the cell.

In the case of cells treated with the Rac1 inhibitor, degradation of the thin gelatin matrix was sometimes observed, however closer examination revealed patterns which often did not correlate with invadopodia. Investigation of focal contacts was performed by immunofluorescence microscopy, and the inhibitor treated cells demonstrated the clustering of paxillin into the formation of robust, elongated focal contacts at the cell periphery (Figure 2.14). The areas of degradation are often bordered
by an area of bright matrix, indicating that it is doubled-over, and filaments of the matrix are often seen pulled back to the focal contact at the edge of the cell. Whereas untreated cells display diffuse, small paxillin puncta across the ventral surface, inhibitor treated cells display highly clustered, large, mature focal contacts at the periphery.

2.3.5 Rac1 inhibits microvesicle formation

Increased Rac1 activation, modeled by transient transfection with Rac1(G12V), leads to a markedly altered phenotype when placed into a thick, deformable matrix. Instead of being rounded as in the control cells, those expressing the active Rac are flattened and many are elongated, often displaying thin filipodial protrusions (Figure 2.15). Strikingly, their ability to form microvesicles is abrogated. Whereas the control cells release TMVs to facilitate their invasion, the cells expressing increased active Rac1 are unable to form blebs at the cell surface and do not release microvesicles. Some cells are able to migrate through the matrix in this elongated state, while many instead appear to be immobile.

2.3.6 Rac1 activation is increased in cells invading a firm matrix

To investigate if increased Rac1 activity could be confirmed biochemically in cells invading a firm matrix as opposed to a thick and yielding one, a PAK effector pulldown assay was utilized to assess the activation of Rac1 in cells in these environments. Indeed, this assay reveals that cells display an elevated amount of Rac1-GTP when growing on a firm substrate versus a compliant one (Figure 2.16), again pointing to the importance of
this signaling pathway in cells utilizing invadopodia to degrade the surrounding extracellular matrix.

2.3.7 RhoA/ROCK signaling promotes microvesicle formation downstream of ARF6

Taking into account the mutually competitive signaling that is characterized between Rac and Rho\textsuperscript{21;167}, we sought to investigate the requirement for Rho activity in microvesicle formation. We inquired if the increased microvesicle formation seen with Rac1 inhibition was dependent on Rho activity, and confirmed this by blocking NSC23766-induced TMV formation with concomitant ROCK inhibition (Figure 2.17). We next sought to investigate if the phenotype demonstrated by cells over-expressing active Rho recapitulated that which is seen with Rac1 suppression. Indeed, we observed that upon transient transfection with the constitutively-active RhoA mutant, RhoA(G14V), cells release a dramatically increased number of microvesicles when in a thick matrix (Figure 2.18). These cells exhibit greatly amplified plasma membrane blebbing and deposit extensive amounts of TMVs as they navigate through the matrix. In stark contrast, in cells in which microvesicle formation is upregulated via the over-expression of constitutively-active ARF6\textsuperscript{132}, inhibition of ROCK, which lies directly downstream of Rho, abrogates their ability to adopt an amoeboid morphology, and microvesicle formation is blocked(Figure 2.19). Instead, the cells adopt a flatter and often elongated morphology, and some also form filopodia. Some of the cells migrate through the ECM in this mesenchymal shape, while a large number appear to be immobile. This effect closely resembles that which is seen with Rac1(G12V). To confirm
that this Rho-mediated microvesicle formation was downstream of ARF6, LOX stably expressing dominant-negative ARF6, which are defective in microvesicle shedding\textsuperscript{132}, were transiently transfected with RhoA(G14V). These cells display the same marked increase in microvesicle formation as those cells expressing wild type ARF6 (Figure 2.20). Evaluation of LOX which stably over-express active ARF6 reveals that RhoA-GTP levels are elevated in comparison to the parental cell line (Figure 2.21). These data indicate that the Rho activity which fosters microvesicle formation lies downstream of ARF6.

2.3.8 RhoA is enriched in microvesicles

To further investigate the link of RhoA with microvesicles, western analysis of media conditioned by LOX-ARF6(Q67L) was performed and compared to the total cell lysate. The media was subjected to serial centrifugation to isolate microvesicle- and exosome-enriched fractions. Recently published work indicates that RhoA is not found on shed vesicles\textsuperscript{104}, however due to the vesicle isolation process utilized in that study which appears to select smaller exosome-sized particles, it is not possible to assess if that work was investigating the ARF6-positive microvesicle population examined here. We found that microvesicles are highly enriched in RhoA when compared with the total cell lysate, whereas the exosome fraction is barren (Figure 2.22). ARF6 was used as a marker to confirm the identity of the microvesicle fraction\textsuperscript{132}, and CD63 was used as a marker of exosomes\textsuperscript{186}.
2.3.9 RhoA/ROCK signaling suppresses invadopodia formation

To investigate the role of RhoA/ROCK signaling in the formation of invadopodia, we again utilized the ROCK inhibitor Y-27632 and transient transfection with the constitutively-active RhoA mutant, RhoA(G14V). Interestingly, when cells on a thin matrix are treated with the ROCK inhibitor, their use of invadopodia is up-regulated (Figure 2.23 A), suggesting that basal ROCK activity suppresses their formation. The cells display elevated numbers of invadopodia in comparison with controls (Figure 2.23 B), and the matrix is decorated with small, punctuate, proteolyzed spots. When cells expressing RhoA(G14V) are plated onto a thin matrix, the cells retain the flattened and spread morphology of the control cells, while forming extensive plasma membrane blebs. In this thin and firm matrix environment, however, these are not shed from the cell as microvesicles (Figure 2.24). Notably, this is very similar to the phenotype seen when Rac1(T17N) is expressed. Taken together, these findings suggest an antagonistic relationship between Rac1 and RhoA in tumor cell invasion. Decreased Rac1 activity results in decreased invadopodia formation in a thin matrix, with cells resembling those expressing active RhoA. In a thick matrix, decreased Rac1 activity causes a large increase in microvesicle formation, again resembling cells expressing active RhoA. Decreased ROCK activity in cells invading a thick matrix closely resembles the effects of increased Rac1 activation.
2.3.10 Figures

Figure 2.1 A Invasive cells release abundant microvesicles in a thick gelatin matrix. LOX and LOX-ARF6(Q67L) cells were grown on a thick FITC-gelatin matrix for 16 hours prior to fixing and staining for filamentous actin (blue) and β₁ integrin (red).

Figure 2.1 B Cells embedded in a thick gelatin matrix do not form invadopodia. LOX-ARF6(Q67L) cells were grown on a thick FITC-gelatin matrix for 16 hours prior to fixing and staining for filamentous actin (blue) and β₁ integrin (red), shown in an xz plane.
Figure 2.2 Thick gelatin matrix allows visualization of nuclear material loading into TMVs. LOX-ARF6(Q67L) were grown on a thick layer of FITC-gelatin for 16 hours prior to fixing and staining for nuclear material with DAPI (cyan), and β1 integrin to label the plasma membrane (red). Gelatin is omitted from merged image for clarity.
Figure 2.3 Invading cells clear tracks through thick matrix during invasion. LOX-ARF6(Q67L) transiently transfected to express cytoplasmic mCherry fluorescent protein were grown on thick FITC-gelatin coated coverslip-bottomed 35 mm dishes and imaged live using confocal fluorescence microscopy over the course of several hours.
Figure 2.4 Cells release TMVs in a fibrillar collagen environment. LOX-ARF6(Q67L) cells were grown on a type I collagen matrix for 16 hours prior to fixation and staining for $\beta_1$ integrin, shown in green. Collagen was imaged using confocal reflection, and is pseudocolored red.
Figure 2.5 LOX cells rapidly disseminate from a collagen-embedded spheroid aggregate. LOX cells induced to form spheroid aggregates were embedded in a type I collagen matrix and imaged live by phase contrast microscopy at plating and 24, 48, and 72 hours later.
Figure 2.6 Cells disseminating from a spheroid aggregate adopt a rounded morphology and release TMVs. Spheroid aggregates of LOX cells embedded in type I collagen were stained with Calcein-AM and imaged live after 24 hours in culture. The image of the intact spheroid on the left is taken at 10x magnification. The image on the right is focused to show extracellular vesicles, and is a 2.3x zoom of the region of the sphere indicated by the box.
Figure 2.7 Cells form invadopodia when placed on a thin matrix. A) LOX and LOX-ARF6(Q67L) were grown on a thin FITC-gelatin matrix and incubated for 16 hours prior to fixing and staining for filamentous actin (red) and cortactin (blue). Cells are shown in an xy plane. B) LOX-ARF6(Q67L) were grown on a thin FITC-gelatin matrix for 16 hours prior to fixing and staining for filamentous actin (red) and cortactin (blue). Cells are shown in an xz plane.
Figure 2.8 Microvesicle release is upregulated in a thick matrix.
Microvesicles released from LOX-ARF6(Q67L) were quantified by microscopy. Fields were chosen at random, moving systematically through the coverslip, isolated cells were identified, and the number of $\beta_1$ integrin positive microvesicles clearly detached from the cell, within one cell’s distance from the periphery, were counted by focusing up and down through the gelatin. 140 cells were counted per condition, compiled from 3 independent experiments. Error bars represent the standard error of the mean. *$p<0.0001$. 

![Bar graph showing average number of TMVs shed per cell for thin and thick matrix conditions.](image)
Figure 2.9 Cells proteolyze punctuate foci on thin matrix. LOX-ARF6(Q67L) transiently transfected to express cytoplasmic mCherry fluorescent protein were grown on thin FITC-gelatin coated coverslip-bottomed 35 mm dishes and imaged live using confocal fluorescence microscopy over the course of several hours.
Figure 2.10 Rac1 inhibition increases microvesicle shedding. LOX cells, which were transiently transfected with T7-Rac1(T17N) or treated with 50 μM NSC23766, were grown on a thick layer of FITC-gelatin for 16 hours. Cells were fixed and stained for β1 integrin (grey) and filamentous actin (red, top) or the T7 tag (red, bottom). Integrin is shown in blue in the merged images for clarity.
Figure 2.11 NSC23766 treatment does not induce apoptosis; TMVs may contain cleaved caspase 3. LOX-ARF6(Q67L) were grown on a thick layer of FITC-gelatin and treated with 50 µM NSC23766 and incubated for 16 hours prior to fixing and staining for β1 integrin and cleaved caspase-3.
Figure 2.11 Cells treated with Rac1 inhibitor exhibit decreased invadopodia on a thin matrix. A) LOX cells were grown on a thin FITC-gelatin matrix, treated with 50 μM NSC23766, and incubated for 16 hours prior to fixing and staining for β1 integrin (red).
Figure 2.11 Cells treated with Rac1 inhibitor exhibit decreased invadopodia on a thin matrix. B) LOX cells were grown on a thin FITC-gelatin matrix, treated with 50 μM NSC23766, and incubated 16 hours prior to fixing and staining for β1 integrin. Cells were counted and those with “invadopodia spots” underneath were noted, and calculated as the “Percentage of Cells Over Degradation”. 100 cells were counted per condition, compiled from 3 independent experiments. Error bars indicate standard error of proportion. *p<0.05.
Figure 2.12 Over-expression of Rac1(T17N) induces the formation of small plasma membrane blebs. LOX cells transiently transfected with T7-Rac1(T17N) were grown on thin FITC-gelatin for 16 hours prior to staining for the T7 tag (blue) and β1 integrin (red).
Figure 2.13 Cells treated with Rac1 inhibitor display increased focal contact formation. LOX cells were grown on thin FITC-gelatin, treated with 50 μM NSC23766, and incubated for 16 hours prior to fixing and staining. Cells are stained for paxillin (red) as a marker of focal adhesions.
Figure 2.14 TMV formation is abrogated in cells expressing Rac1(G12V). LOX-ARF6(Q67L) transiently transfected with T7-Rac1(G12V) were grown on a thick layer of FITC-gelatin for 16 hours prior to fixing and staining for $\beta_1$ integrin (red) and the T7 tag (blue).
**Figure 2.15 Relative level of active Rac1 is increased in cells invading a thin matrix.** LOX-ARF6(Q67L) were grown for 16 hours on a thin or thick gelatin matrix prior to lysis and PAK effector pulldown assay to assess levels of active Rac1. A sample of the total cell lysate was blotted for total Rac1.
Figure 2.16 ROCK is required for Rac1 suppression-induced TMV formation. LOX-ARF6(Q67L) were grown on a thick FITC-gelatin matrix and co-treated with 50 µM NSC23766 and 10 µM Y-27632 to inhibit both Rac1 and ROCK activity for 16 hours prior to fixing and staining for β1 integrin (red).
Figure 2.17 Cells expressing active RhoA display increased TMV formation. LOX transiently transfected with T7-tagged RhoA(G14V) were grown on a thick layer of FITC-gelatin for 16 hours prior to fixing and staining for β1 integrin (red) and the T7 tag (blue).
Figure 2.18 ROCK inhibition abrogates TMV formation in a thick matrix, downstream of ARF6. LOX-ARF6(Q67L) were grown on a thick layer of FITC-gelatin and treated with 10 μM Y-27632 for 16 hours prior to fixing and staining for β₁ integrin (red). The lower panel shows the cells in an xz plane.
Figure 2.19 RhoA promotes TMV formation downstream of ARF6. LOX cells which stably express dominant-negative ARF6, ARF6(T27N), were transiently transfected to express constitutively-active RhoA and grown on a thick FITC-gelatin matrix for 16 hours prior to fixing and staining for the T7 tag on RhoA(cyan) and $\beta_1$ integrin (red).
Figure 2.20 RhoA is activated downstream of ARF6. LOX and LOX stably over-expressing ARF6(Q67L) were assessed for levels of RhoA-GTP with a Rhotekin-RBD effector assay to pull down active RhoA, and subsequent western analysis. Cell lysates were also probed for total levels of RhoA, HA to confirm the identity of the HA-tagged ARF6 mutant, and α-tubulin as a loading control.
**Figure 2.21 RhoA is enriched in microvesicles; absent in exosomes.**

Microvesicle-conditioned media was harvested from LOX-ARF6(Q67L) prior to serial centrifugation to yield microvesicle and exosome pellets. The pellets were lysed, protein amounts quantified, and equal amounts of protein used for western blotting. ARF6 is used as a marker for TMVs and CD63 for exosomes.
Figure 2.22 ROCK inhibition increases invadopodia-mediated invasion. A) PC-3 cells were grown on a thin FITC-gelatin matrix, treated with 10 μM Y-27632, and incubated for 16 hours prior to fixing and staining for β1 integrin (blue) and filamentous actin (red). B) Cells were prepared as above, counted, and those with “invadopodia” spots underneath were noted and calculated as the “Percentage of Cells Over Degradation”. 100 cells were counted per condition, compiled from 3 independent experiments. Error bars indicate standard error of proportion. *p<0.05.
Figure 2.23 Cells expressing active RhoA form blebs but do not release TMVs on a thin matrix. LOX transiently transfected with T7-tagged RhoA(G14V) were grown on a thin layer of FITC-gelatin and incubated for 16 hours prior to fixing and staining for β₁ integrin (red) and the T7 tag (blue).
2.4 Discussion

Here we have described a mechanism by which cells can alter their use of invadopodia or microvesicles to facilitate invasion, based upon the compliance of the matrix they are invading. Existing work has demonstrated the importance of ECM stiffness in guiding tumor cell invasion and modulating signal transduction downstream of matrix receptors\textsuperscript{46, 101}. The importance of signaling through Rac1 and RhoA is well accepted for the processes of cell invasion, as is the mutual antagonism between the two pathways\textsuperscript{21, 167}. Just as it has been demonstrated that cells are able to toggle between the use of Rac-driven mesenchymal-type motility and Rho-driven amoeboid motility\textsuperscript{176}, we have shown that cells are able to interconvert between the use of invadopodia and microvesicles in response to ECM cues, and this is also driven by antagonistic signaling through Rac and Rho.

These data provide insight into tumor cell behavior and suggest a new mechanism of invasive plasticity, in which cells may be able to toggle back-and-forth between a mesenchymal mode of motility, with a corresponding use of invadopodia to proteolyze firm matrices directly adjacent to the cell, and the adoption of an amoeboid motility and the release of microvesicles for distal discharge of proteases when a more compliant environment is encountered. The \textit{in vitro} matrix conditions used here are intended to provide a context of the environments encountered \textit{in vivo}, such as dense connective tissue versus a soft adipose matrix. We have demonstrated that Rho signaling promotes the formation of microvesicles when appropriate cues from a
compliant matrix are present. Without those ECM cues, elevated signaling through Rho simply results in membrane blebbing. An abrogation of Rho signaling by inhibiting ROCK can block this behavior, and instead elicits the adoption of a mesenchymal morphology, and an upregulation of invadopodia formation when the cells are on a thin matrix. The modulation of Rac1 can mimic this behavior, with an increase in Rac1 activation abrogating the formation of microvesicles in a compliant ECM, and Rac1 inhibition resulting in a marked upregulation of TMV formation in this environment, which requires ROCK activity. Again, alterations in intracellular signaling alone are insufficient to elicit this behavior, and the microvesicle upregulation is not seen in cells which are on a firm substrate. Interestingly, in the presence of Rac1 inhibition on a firm matrix, an increase in the formation of mature focal contacts is noted, again indicating an upregulation of Rho and ROCK signaling\(^2\). In addition, we have illustrated that this Rho-driven regulation of microvesicle formation lies downstream of ARF6, as the inhibition of Rho kinase in cells expressing constitutively-active ARF6 is sufficient to completely abrogate TMV formation, and the over-expression of active RhoA in cells in which TMV formation is blocked by the expression of dominant-negative ARF6 is sufficient to induce prolific microvesicle release.

Future directions for these studies may include investigation of Rac and Rho interacting proteins that could bridge these signaling cascades. It has been shown that knockdown of proteins which affect signaling through Rac1, such as NEDD9, WAVE2, or DOCK3\(^1\), impairs the ability to move using a mesenchymal mode of motility and
induces an amoeboid type of cell movement. Conversely, ROCK phosphorylation of FilGAP\textsuperscript{171} and Rho signaling to the RacGAP ARHGAP22\textsuperscript{176} suppress mesenchymal movement by antagonizing Rac activation. Investigating these proteins and other related Rac and Rho GAPs and GEFs may help elucidate the competitive signaling involved in invadopodia and microvesicle biogenesis. Additionally, the utilization of fluorescent probes to localize active Rac1 and RhoA in living cells could prove to be very helpful, by allowing visualization of the activity of these proteins, both spatially and temporally, within the context of invading different environments. There are some such Fluorescence Resonance Energy Transfer (FRET) probes currently available\textsuperscript{77; 136}, and these are found as unimolecular or bimolecular systems, in which the fluorescence is inherent to the GTPase itself, or this energy transfer occurs between a GTPase and an associated activating protein, with fluorophores on both. Ras And Interacting Chimeric Unit (Raichu) probes have become a popular tool as a unimolecular small GTPase activation probe\textsuperscript{77; 136}. Unfortunately their use has been limited by inherent drawbacks of FRET systems, such as artificially altering interaction kinetics by interfering with RhoGDIs\textsuperscript{153}, the possibility that the over-expressed FRET GTPase may act as a dominant-negative protein\textsuperscript{136}, and imaging issues due to low photostability and high phototoxicity\textsuperscript{77; 136}. Further advancements in these probes could prove to be very useful in assessing the signaling in invasive cells.
CHAPTER 3:
PARALLEL PATHWAYS REGULATE MYOSIN ACTIVATION IN MICROVESICLE RELEASE

3.1 Introduction

Unlike exosomes, which are released into the extracellular environment via exocytosis, microvesicle blebs must be actively pinched free from the plasma membrane, facilitated by myosin machinery localized at the base of the vesicles. Myosins are ubiquitously expressed, ATP-dependent actin-binding motor proteins, and those of the myosin II class, also known as the “conventional myosins”, are the primary form of myosin found in skeletal and smooth muscle. Non-muscle myosin II (NM II) (which, incidentally, is also found in muscle), is primarily active in physiology at a cellular level, such as cell-cell and cell-matrix adhesion, alterations in cell shape, motility, and cytokinesis. NM II is composed of two heavy chains, each with a globular head and an α-helical tail, which dimerize with each other; two essential light chains that stabilize the heavy chains; and two regulatory light chains (MLC). Unlike other myosins, only myosin II can associate in a bipolar conformation, facilitating the binding of actin filaments of opposite polarity, to generate contraction. NM II is activated to an unfolded, actin-binding conformation by phosphorylation at Thr and Ser of MLC,
mediated by myosin light chain kinase (MLCK)\textsuperscript{173} and ROCK\textsuperscript{3,111,208,223}. Inhibitory phosphorylation of MLC at Ser\textsuperscript{1}, Ser\textsuperscript{2}, and Thr\textsuperscript{9} is mediated by protein kinase C (PKC)\textsuperscript{223}.

Myosin light chain phosphatase (MLCP) functions as a counterbalance to MLCK, acting to dephosphorylate and inactivate MLC. MLCP is a heterotrimeric enzyme which is comprised of three subunits: a small, approximately 20 kD subunit of unknown function; a protein phosphatase 1 catalytic (PP1c) subunit, which is approximately 37 kD; and a large, 110-130 kD myosin-binding regulatory subunit referred to as the myosin phosphatase target subunit (MYPT1)\textsuperscript{90,109,195}. MYPT1 contains two well-characterized inhibitory phosphorylation sites, at Thr\textsuperscript{696} and Thr\textsuperscript{853}(90;109;195) which inhibit MLCP from targeting and binding to myosin. Inhibitory phosphorylation of MYPT1 is performed by ROCK\textsuperscript{90,109,195}, and ERK has been shown to regulate the phosphorylation at Thr\textsuperscript{853}(229).

An alternate mechanism to suppress MLCP activity is via the PP1c subunit. This catalytic subunit can be inhibited by CPI-17 which has been activated by phosphorylation at Thr\textsuperscript{38}, which is performed by ROCK or PKC\textsuperscript{17,97,231}. Interestingly, CPI-17 can function as an oncoprotein by inhibiting MYPT1-PP1c, which is required to dephosphorylate and activate the tumor suppressor Merlin, and its over-expression is associated with cellular transformation\textsuperscript{81}.

Previous studies from our lab have demonstrated the importance of acto-myosin contractility in the release of microvesicles from the surface of the cell\textsuperscript{132}. In an ARF6-dependent process, activation of MLC via myosin light chain kinase has been shown to mediate vesicle abscission from the plasma membrane. ARF6 activates phospholipase D
(PLD) to form phosphatidic acid (PA), which, in turn, is required for activation of extracellular regulated kinase I/II (ERK) and its localization to the cell surface. Previous work has suggested that this occurs via PA recruitment of activated c-Raf to the plasma membrane\textsuperscript{210}, which assists in forming a scaffold for the localization and signaling of Ras, Raf, MEK, and ERK at the plasma membrane\textsuperscript{4}. Activated ERK then phosphorylates MLCK, which facilitates myosin light chain activity via phosphorylation at Thr\textsuperscript{18} and Ser\textsuperscript{19}, stimulating contractility and the fission of vesicles from the membrane\textsuperscript{132}. Myosin light chain phosphatase (MLCP) functions to counter the activity of MLCK\textsuperscript{90; 109; 195}, and its contribution to the process of microvesicle formation is investigated here.

3.2 Materials and Methods

3.2.1 Cell culture and transfection

Cell culture and transfection were performed as outlined in Chapter 2.

3.2.2 FITC-gelatin invasion assay

FITC-gelatin invasion assays were performed as outlined in Chapter 2.

Cells were grown on gelatin-coated coverslips for 16 hours prior to fixation and staining. All inhibitor treatments were initiated after the cells had been allowed to adhere for one hour after plating. NSC23766 was used at 50 μM, ML-7 at 10 μM, and Y-27632 at 10 μM, and all were purchased from Calbiochem. U0126 was used at a 20 μM concentration and purchased from LC Laboratories.
3.2.3 Immunofluorescence staining and microscopy

Immunofluorescence staining was performed as described in Chapter 2. Imaging was performed using a BioRad MRC-1024 laser scanning confocal microscope. Image J (NIH) was utilized to optimize image brightness and clarity, and for pseudocoloring the black-and-white images. Cells were stained using rat anti-β1 integrin clone AIIB2 (Iowa DSHB) and phalloidin conjugates, which were purchased from Molecular Probes.

3.2.4 Western blotting

Cells were grown on coverslips coated with a thick layer of gelatin for 16 hours prior to lysis. Cells were lysed in RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholic acid, 0.1 % SDS, 50 mM Tris-HCl pH 7.5) containing mammalian protease inhibitor cocktail (Sigma), and the samples were then subjected to SDS-PAGE, transferred to PVDF membrane, and probed with the indicated antibodies as per the manufacturer’s instructions. Lysis buffer prepared for analysis of phosphorylated proteins was supplemented with 500 μM pervanadate. The antibodies used were mouse anti-α-tubulin (Sigma), rabbit anti-total ERK, mouse anti-pERK, rabbit anti-total MLC, mouse anti-pMLC\textsuperscript{Thr18,Ser19}, and rabbit anti-MYPT1/anti-MYPT1\textsuperscript{Thr696}/anti-MYPT1\textsuperscript{Thr853}, all of which were purchased from Cell Signaling.
3.3 Results

3.3.1 Multiple pathways of MLC activation in microvesicle release

We sought to investigate the regulation of MLC activity in the increased microvesicle formation seen due to Rac1 inhibition or constitutive RhoA activation. Rho and ROCK play key roles in MLC activation\textsuperscript{3, 86, 208, 223}, which prompted this line of investigation. Cells growing in a compliant matrix were treated with the Rac1 inhibitor NSC23766 or transiently transfected with the constitutively-active RhoA(G14V), and then evaluated by western blotting for the presence of active MLC. Indeed, an increase in pMLC\textsuperscript{18/19} is seen in both instances (Figure 3.1), supporting the hypothesis that MLC activation downstream of Rho was regulating the increased microvesicle formation.

To begin to unravel the pathway regulating MLC activation, cells were also evaluated for active ERK, known to be involved in the activation of MLC via MLCK\textsuperscript{95, 132} and also by the inhibition of myosin light chain phosphatase\textsuperscript{229}. An increase in ERK phosphorylation was observed with Rac1 suppression and with constitutive RhoA activation, which could be abrogated by inhibition of MEK(Figure 3.1), which lies immediately upstream of ERK. We found that microvesicle shedding, upregulated by either Rho activation or Rac1 suppression, is dependent on ERK activity. In either case, MEK inhibition abrogates microvesicle release, and instead, the plasma membrane is studded with blebs (Figure 3.2, Figure 3.3). This indicates that ERK activation lies downstream of Rho in microvesicle formation, which supports published observations for the interaction of these proteins in the cases of cytoskeletal rearrangement and cell-
matrix interaction\textsuperscript{89, 99, 151}. Interestingly, concomitant inhibition of MLCK and Rac1 does not prevent TMV release (Figure 3.3), which suggests that a mechanism outside of MLCK contributes to the increased MLC phosphorylation seen in this model of amplified microvesicle shedding.

3.3.2 Myosin light chain phosphatase is inhibited with amplified TMV release

We next sought to investigate if inhibition of myosin light chain phosphatase was modulating the increased MLC activity in amoeboid cells. As mentioned previously, the concerted activities of MLCK and MLCP are required for regulating myosin contractility\textsuperscript{90, 109}. Indeed, with microvesicle formation augmented by Rac1 suppression, an increase in phosphorylation is seen at Thr\textsuperscript{696} and Thr\textsuperscript{853} of MYPT1 (Figure 3.4), the characterized inhibitory sites, thereby facilitating sustained MLC activity. To investigate if MYPT1 inhibition correlated with ARF6 nucleotide status, and therefore, microvesicle release, we investigated MYPT1 phosphorylation in LOX over-expressing a dominant-negative ARF6 mutant, as well as a constitutively active mutant. As expected, we see increased MYPT phosphorylation with the expression of active ARF6, and a further increase with TMV release amplified by Rac1 suppression (Figure 3.5). These data support the hypothesis that preventing the inactivation of MLC by MLCP modulates an increase in its activity.
3.3.3 Figures

Figure 3.1 Rac1 suppression and RhoA activation elicit MLC and ERK phosphorylation. LOX cells were left untransfected or transfected with T7-tagged RhoA(G14V), treated with 50 µM NSC23766 (NSC), 20 µM U0126 (U), or a combination thereof, and grown on a thick gelatin matrix for 16 hours prior to lysis. Western analysis was performed to detect levels of phospho- and total MLC, phospho- and total ERK, and the T7 tag on RhoA(G14V). α-Tubulin is included as a loading control.
Figure 3.2 ERK activation is required for TMV shedding stimulated by active RhoA. Control LOX cells or those transiently transfected with RhoA(G14V) were grown on a thick FITC-gelatin matrix and treated with DMSO vehicle or U0126 at 20 µM and incubated for 16 hours prior to fixing and staining for the T7 tag on RhoA (not shown, for clarity), and β₁ integrin (red).
Figure 3.3 ERK activity is required for microvesicle formation stimulated by Rac1 inhibition, but MLCK is not. LOX-ARF6(Q67L) were grown on a thick FITC-gelatin matrix and treated with 50 µM NSC23766 + 20 µM U0126 or 50 µM NSC23766 + 10 µM ML-7 for 16 hours prior to fixing and staining for filamentous actin (blue) and β1 integrin (red).
Figure 3.4 Suppression of Rac1 activation leads to inhibitory phosphorylation of MYPT1. LOX-ARF6(Q67L) were grown on a thick gelatin matrix for 16 hours in the presence of 50 μM NSC23766 or 10 μM Y-27632 prior to lysis and western analysis for phospho- and total MYPT1. α-Tubulin is included as a loading control.
Figure 3.5 Inhibitory phosphorylation of MYPT1 is increased with ARF6 activation and amplified TMV release. LOX, LOX stably expressing ARF6(T27N), LOX stably-expressing ARF6(Q67L), and LOX-ARF6(Q67L) treated with 50 µM NSC23766 (NSC) were grown on a thick gelatin matrix for 16 hours prior to lysis and western analysis for phospho- and total MYPT1. α-Tubulin is included as a loading control.
Figure 3.6 Proposed mechanisms for MLC regulation in microvesicle formation. Schematic diagram depicting the pathways that facilitate MLC activation for microvesicle formation and release, via ROCK signaling downstream of ARF6. The putative role for CPI-17 in MLC-regulated TMV release is indicated by the dashed lines, as is the direct phosphorylation of MLC by ROCK.
3.4 Discussion

Here we have described a novel mechanism for the regulation of myosin activity in the formation of microvesicles. Existing literature has delineated the importance of acto-myosin contractility in the fission of microvesicles into the extracellular milieu, and described a signaling cascade which facilitates this contractility, with the activation of MLC via MLCK, downstream of ARF6. We have shown an additional mechanism to facilitate sustained MLC activation, by the suppression of myosin light chain phosphatase. In addition, we have outlined the redundancy which exists in the signaling cascade downstream of ARF6 to facilitate MLC activation via Rho kinase (Figure 3.6).

The data presented here demonstrate that the amplified TMV formation initiated by Rac1 suppression or Rho activation lies downstream of ARF6, as the expression of a dominant-negative ARF6 mutant is unable to inhibit microvesicle formation in these conditions. However, utilizing a system of Rac1 inhibition in cells invading a compliant matrix which facilitates greatly amplified microvesicle formation, we observed that simply inhibiting MLCK was insufficient to block microvesicle formation. With this in mind, we investigated the possibility for parallel regulation of MLC via myosin light chain phosphatase, inhibition of which facilitates sustained MLC activation.

Indeed, in this model of stimulated microvesicle formation, substantially increased phosphorylation is observed at both Thr and Thr of the regulatory MYPT1 subunit of myosin light chain phosphatase. This is particularly interesting in light of the
fact that ROCK is capable of phosphorylating MLCP at these inhibitory sites\textsuperscript{90; 109; 195}.

These data coordinate well with our studies which show an antagonistic relationship between Rac1 and Rho in microvesicle formation, suggesting that the suppression of Rac1 facilitates an increase in ROCK activation downstream of RhoA. ROCK, in turn, inactivates myosin light chain phosphatase by the inhibitory phosphorylation of the MYPT1 subunit. The upregulation of ROCK activity can also foster MLC activation by its phosphorylation of CPI-17\textsuperscript{17; 97; 231}, as well as the direct phosphorylation of MLC\textsuperscript{208}. These data demonstrate how the suppression of Rac1 activity in invasive cells can promote sustained MLC activation to facilitate microvesicle abscission from the cell surface and promote movement through a compliant matrix. We also show that MLCP inhibition correlates with ARF6 activation status, further establishing that MLCP inhibition occurs downstream of ARF6 as an added mechanism by which ARF6 facilitates increased MLC activity, in addition to its role in MLCK activation.

To further understand the signaling pathway regulating MLC activation downstream of ARF6, we investigated ERK activity, as ERK has been shown to impact myosin activation through both MLCK and MLCP\textsuperscript{95; 132; 138; 195; 229}. We found that ERK is activated upon Rac1 suppression or constitutive Rho activation, and is required for microvesicle formation. We observed a blockade of TMV release upon concomitant MEK inhibition, however inhibiting MLCK activity did not quell TMV release. These data suggest that ERK activation is upstream of both MLCP and MLCK in TMV release.
The work described here highlights the redundancy which exists in regulating myosin activation in the release of microvesicles, all of which lies downstream of ARF6 → Rho → ROCK signaling. On one arm, an increase in myosin activation can be achieved through direct phosphorylation by ROCK\textsuperscript{208}, or the activation of MLCK moderated by ERK\textsuperscript{95, 132, 138, 195, 229}. On the other arm, a decrease in myosin dephosphorylation may be mediated by an inhibition of the MYPT1 subunit of myosin phosphatase, also via ERK, in addition to the inhibition of MLCP initiated via CPI-17\textsuperscript{17, 97, 231}. The redundancy in these pathways underscores how the activation of a signaling cascade can amplify a signal to achieve a downstream function; in this case, to support tumor cell microvesicle release.
CHAPTER 4: MULTIPLE PATHWAYS REGULATE ARF6 ACTIVITY IN CELL MIGRATION AND INVASION

4.1 Introduction

Signaling pathways which regulate cell motility in normal physiological development and tissue remodeling are often exploited in tumor cell invasion. Robust migratory ability is required for tumor cell invasion as cells move from their tumor of origin to colonize a secondary site. The processes of invasion and migration are tightly linked, with both requiring similar modulations of the cytoskeleton for the formation of peripheral protrusions which facilitate cell translocation, matrix modification, and extracellular proteolysis. Both invasion and migration are heavily influenced by signaling through ARF6. The importance of ARF6 for the cell migration required in normal development is clearly demonstrated in a mouse knockout model, which shows embryonic lethality\textsuperscript{201}. The livers of these animals are improperly formed, with cells unable to migrate normally from the primordial hepatic epithelial sheet to form a functional hepatic cord\textsuperscript{201}. Without robust migratory ability, tumor cells are unable to invade and metastasize. The importance of ARF6 for the migration of tumor cells is well-established, and ARF6 knockdown or the expression of a dominant-negative ARF6 mutant interferes with this ability\textsuperscript{72,123}, while cells expressing constitutively-active ARF6
or over-expressed ARF6 GEFs show increased migration and invasion\textsuperscript{71, 133, 175}. Multiple pathways are utilized to activate and suppress ARF6 activity in order to regulate migratory and invasive behaviors, likely in a cell- or tissue-specific manner. This regulation of ARF6 may be facilitated by recruiting GTPase Activating Proteins (GAPs), which inactivate ARF6 to its GDP-bound form and thereby decreasing migratory and invasive ability, or Guanine Nucleotide Exchange Factors (GEFs), which facilitate ARF6 activation.

One potential regulator of ARF6 activity is the membrane scaffold protein CD2AP, which has been characterized with important roles in actin reorganization as it relates to the formation of cellular protrusions and migration. \textit{In vivo}, CD2AP is essential for proper renal function, required for the normal motility of podocytes, the highly differentiated and specialized cells of the glomerulus which filter excess water and waste from the blood\textsuperscript{152}, as well as their ability to extend the interdigitated foot processes which are required for this filtration\textsuperscript{94, 239}. CD2AP knockout in a mouse model is associated with the development of fatal renal failure and nephrotic syndrome within approximately four weeks, and this can be ameliorated by the exogenous expression of CD2AP in podocytes\textsuperscript{60}. Similarly, in humans, CD2AP dysfunction is associated with a spectrum of renal diseases\textsuperscript{87, 112, 226}. Key to its function is its impact on actin reorganization at the cell periphery, required for migration and foot downprocess extension\textsuperscript{94, 239}. In tumor cells, CD2AP has been found to associate with cortactin, suggesting a possible role in invasion(Lynch et al. 2003, 21805-21813). Much of the work
investigating CD2AP in epithelial tissues has been performed using a Drosophila model, looking at its fly homolog, Cindr. Cindr is required for the migration of cells within epithelium, necessary for the patterning of complex tissues such as the eye\textsuperscript{83}.

Another pathway which may impact ARF6 activation in invasive behaviors is the SLIT-ROBO signaling axis. The SLIT-ROBO pathway has been well-characterized to regulate a wide variety of cell behaviors, including axon guidance, dendritic branching, organ development, and cell migration\textsuperscript{10}. There are multiple binding motifs on the cytoplasmic tail of ROBO for adaptor proteins which modulate actin reorganization\textsuperscript{67; 193}. Four members of the SLIT family, SLITs 1-4, have been identified in vertebrates, along with three Roundabout (ROBO) family proteins, ROBOs 1-3. The SLIT glycoprotein serves as a ligand for the ROBO receptor, and their interaction modulates signaling in important developmental and pathological conditions, including cancer. SLIT-ROBO signaling has demonstrated a pivotal role in cancer progression, and these proteins have been shown to have the ability to act as tumor suppressors and oncogenes in different contexts, preventing or promoting migration, cell adhesion, proliferation, and angiogenesis\textsuperscript{10}. An antagonistic relationship with ARF6 has been demonstrated, with signaling downstream of ROBO1 serving to recruit ARF6 GAPs to decrease ARF activation in an angiogenesis model\textsuperscript{84}.

In collaborative efforts, we sought to examine how the regulation of ARF6 activation, mediated by CD2AP and SLIT-ROBO, impacted cell migration and invasion. In part of a study investigating the role of Cindr-mediated regulation of ARF6 in the cell
migration and patterning of the fly eye, we elucidated a role for CD2AP on ARF6 activation and cell motility in a mammalian model system. Additionally, we investigated the role of SLIT2-ROBO4 signaling in tumor cell invasion, helping identify Wnt/β-catenin canonical signaling taking place downstream of Wnt5a, and describing competitive signaling between Wnt and SLIT-ROBO which serve to regulate invasive behavior in melanoma cells, through ARF6.

4.2 Materials and Methods

4.2.1 Cell culture, transfection, and reagents

LOX lines were grown in DMEM (Gibco) supplemented with 10% FBS (Hyclone), 2 mM l-glutamine (Gibco), 1 mM sodium pyruvate (Hyclone), and penicillin/streptomycin (Thermo Scientific). MDCK I were grown in DMEM (Gibco) supplemented with 10% FBS (Hyclone), 2 mM l-glutamine (Gibco), and penicillin/streptomycin. LOX stably expressing β-catenin(S33Y) were generated and maintained in the presence of G418 selection, as described. Cells were maintained in a 37°C humidified incubator with 5% CO₂. Transfection of MDCK was performed using Lipofectamine 2000, following the manufacturer’s suggested protocol. GFP-tagged CD2AP was kindly provided by Ross Cagan. For SLIT2 experiments, cells were treated with 20 nM salt-extracted SLIT2 in growth medium. SecinH3 (Calbiochem and Albany Molecular Research) was added to the growth medium at 30 µM.
4.2.2 Scratch-wound assay

MDCK I cells were transfected with a control GFP-expressing plasmid or GFP-tagged CD2AP and grown to confluence on tissue culture plastic in 6-well dishes. At 24 h post-transfection, cells were “wounded” with a p10 micropipettor tip, washed once with serum-free media, then returned to complete media and imaged via phase contrast microscopy with a Zeiss Observer Z1 microscope immediately thereafter, and 9.5 hours after wounding, to monitor migration. The bottom of the wells was marked with a grid in order to facilitate monitoring the same field over time. To calculate the distance cells traveled during healing, the wound edges were mapped at 0 hours at again at 9.5 hours using Image J software (NIH), with the percentage of healing calculated as the final wound area divided by the initial wound area. At 10 hours post-wounding, the cells were lysed for western analysis.

4.2.3 Western blotting and ARF6 activation assay

The MT-2 assay was performed as described previously\textsuperscript{13; 181}. Briefly, cells were lysed and cleared of membranous material via brief centrifugation, and subsequently incubated with MT-2-GST beads with agitation for 1 hour. Samples were run via SDS-PAGE, transferred to PVDF membrane and probed for ARF6. A sample of the total cell lysate was run alongside to evaluate the level of total ARF6, and α-tubulin was probed as a loading control. Densitometry of western blots was performed using Image J software (NIH).
4.2.4 Invasion assays, immunofluorescence staining and reagents

FITC-conjugated gelatin-coated coverslips were prepared as described previously\textsuperscript{68;69}. Cells were seeded onto coated slips and grown for 16 hours prior to fixing and staining, as described previously\textsuperscript{14}, and were mounted using Prolong Gold antifade mounting medium (Invitrogen). SLIT2 and SecinH3 treatment was initiated at the time of plating on gelatin. Rhodamine-phalloidin was purchased from Molecular Probes. Imaging was performed using a BioRad MRC-1024 laser scanning confocal microscope. Image J (NIH) was utilized to optimize image brightness and clarity, and for pseudocoloring the black-and-white images.

4.3 Results

4.3.1 CD2AP over-expression decreases epithelial cell motility

In light of studies describing a role for CD2AP in actin rearrangement and the formation of membrane protrusions, we sought to investigate the effects of CD2AP over-expression in mammalian epithelium, using the Madin-Darby Canine Kidney I (MDCK I) cell line, commonly used to study migration and tumor cell epithelial-to-mesenchymal transition. Cells were transiently transfected to over-express GFP-tagged CD2AP and grown into a confluent monolayer to enable polarization and the establishment of mature cell-cell contacts prior to “wounding” with a pipette tip to remove a region of the monolayer. The plates were marked to enable repeated
monitoring of the same area, and the cells were monitored live via phase contrast microscopy as they mobilized to repopulate the cleared area.

At 9.5 hours post-wounding, a significant defect in wound healing was noted in the CD2AP transfectants. Control cells had efficiently migrated to close approximately 47% of the cleared area, while the CD2AP transfectants lagged at only 29% closure (Figure 4.1 A, 4.1 B). This delay does not appear to be due to a decrease in cell proliferation, as the CD2AP transfected cells attained confluence at the same rate as the controls, and equivalent amounts of protein were isolated in cell lysates (Figure 4.2 A).

4.3.2 CD2AP decreases ARF6 activation in migrating cells

CD2AP is characterized to regulate cell migration and actin remodeling\textsuperscript{135,239}, and has been shown to colocalize with actin in cells expressing active ARF6 and\textsuperscript{177}. In order to investigate if CD2AP was altering ARF6 activity in the diminished migration we observed, ARF6 activation in both migrating and non-migrating cells over-expressing CD2AP was assessed. In order to accomplish this, cell monolayers were either left intact or wounded and allowed to initiate migration, as in the scratch-wound assay above. The cells were then lysed and analyzed via an MT-2 effector assay to pull down active ARF6. Very interestingly, ARF6 activation was diminished in the migrating cells over-expressing CD2AP, but not in the intact monolayer. At 10 hours post-wounding there was nearly a 40% decrease in ARF6-GTP in the CD2AP transfectants as compared to control cells, whereas there was no significant difference observed in the non-migrating cells (Figure
4.2 A, Figure 4.2 B). This suggests that indeed, ARF6 activation is modulated downstream of CD2AP in cell migration.

4.3.3 SLIT2 decreases invadopodia-mediated invasion

Another pathway which is well-characterized in cell migration and invasion is the SLIT-ROBO pathway. Initially characterized for an important role in axon guidance during embryogenesis, SLIT-ROBO signaling has since demonstrated a role in tumor progression, serving to regulate cell migration, cell adhesion, proliferation, and angiogenesis. The glycoprotein SLIT2 is the best studied of the ROBO ligands, with its expression frequently modulated in tumor cells. Because it has been demonstrated that SLIT2 serves to decrease ARF6 activation by the recruitment of ARF GAPs in endothelium, we sought to investigate if SLIT2 would modulate the invasive ability of melanoma cells. To assess the effects of SLIT-ROBO signaling in melanoma invasion, cells were plated on a thin fluorescent gelatin matrix and treated with SLIT2 protein to activate the pathway. After 16 hours, the number of cells that were colocalized over areas of matrix degradation were counted, and invadopodia-mediated invasion was found to be significantly inhibited in SLIT2 treated cells (Figure 4.3). Whereas greater than 70% of the control cells displayed matrix degradation beneath them, less than 10% of the SLIT2 treated cells exhibited degradation. As described in Grossmann et.al. 2013, this was ROBO-dependent, as SLIT2 treatment of ROBO silenced cells did not alter invasive ability. ARF6 activation was assessed in these cells, and indeed, it was demonstrated that ARF6 activation was diminished in cells treated
with SLIT2. To analyze if ARF6 inhibition with the small molecule compound SecinH3, which antagonizes the cytohesin family of ARF GEFs, recapitulated the SLIT2 treatment phenotype, we treated cells with SecinH3 and analyzed invasion by plating cells on a fluorescent matrix. Indeed, we again see diminished invasive ability, with less than 10% of the cells invading the matrix (Figure 4.3). This suggests that the SLIT-ROBO downregulation of ARF6 activity is serving as an invasive antagonist, perhaps through the cytohesin family of ARF6 GEFs.

Due to the established role for ARF6 in regulating the recycling of cadherin and β-catenin to adherens junctions, how the suppression of ARF6 activation mediated by SLIT2 or SecinH3 was impacting the localization of β-catenin was investigated, as described in Grossman et al. 2013. It was found that ARF6 suppression in these instances fortifies the population of β-catenin bound to cadherin at the cell surface, and decreases the cytoplasmic and nuclear pools. We further explore the impact of ARF6 in cell invasion by the development of new tools for ARF6 silencing and downregulating ARF6 activation, in Chapter 5.

4.3.4 Stabilized β-catenin increases melanoma invasion

In order to help elucidate if the reduction in intracellular β-catenin was responsible for the decreased invasion modulated by SLIT-ROBO, the Wnt pathway was explored. β-catenin is a multi-functional protein, with a presence at the plasma membrane to link cell-cell contacts to the actin cytoskeleton in complex with E-cadherin, and a role in signal transduction downstream of canonical Wnt activation, whereupon it
translocates to the nucleus to serve as a co-activator of transcription factors of the TCF/LEF family\(^\text{214}\). Multiple pathways converge to direct \(\beta\)-catenin activity. In addition to the destabilization of adherens junctions and the catenin internalization initiated by ARF6 activation\(^\text{148}\), canonical Wnt signaling serves to disrupt the formation of the \(\beta\)-catenin destruction complex which targets cytoplasmic \(\beta\)-catenin to the proteasome for degradation\(^\text{31}\). This stabilizes the cytoplasmic pool of \(\beta\)-catenin which can then translocate to the nucleus to activate transcription. There are 19 members of the Wnt family identified in humans, which are secreted ligands for Frizzled family receptors in canonical and non-canonical pathways\(^\text{161}\). Wnts can also signal by means which are independent of \(\beta\)-catenin, and this non-canonical signaling is characterized as Jun N-Terminal Kinase (JNK) or calcium mediated, important in cell polarity and motility\(^\text{161}\). The ability of Wnt signaling to activate ARF6 in canonical signaling has been demonstrated\(^\text{92}\), and sustained ARF6 activation has been demonstrated to disrupt adherens junctions\(^\text{148}\), promoting the internalization of \(\beta\)-catenin, which could promote signaling through the canonical Wnt pathway. The pro-oncogenic effects of Wnt signaling are well-established\(^\text{158}\), and with this in mind, we sought to elucidate if canonical Wnt signaling was responsible for the effects on invasion we observed when SLIT-ROBO signaling was impaired.

To accomplish this, we first looked at the effects of over-expression of stabilized \(\beta\)-catenin on cell invasion, using a LOX cell line stably expressing the \(\beta\)-catenin mutant, \(\beta\)-catenin(S33Y). In the absence of a Wnt stimulus, cytoplasmic \(\beta\)-catenin is degraded by
a multi-protein destruction complex. \( \beta \)-catenin(S33Y) lacks the serine phosphorylation site for GSK-3\( \beta \), a member of the destruction complex, required to mediate proteasomal degradation. This facilitates an increased pool of nuclear protein and makes the mutant a useful model for this pathway.

LOX stably expressing the \( \beta \)-catenin(S33Y) mutant were grown onto a thin layer of FITC-gelatin overnight, to monitor invasion. Approximately 20% more cells which were expressing stabilized \( \beta \)-catenin displayed invadopodia activity, than did the parental cell line, showing extensive punctate proteolysis of the matrix (Figure 4.4). The cells created abundant paths of degradation, indicating that they also possess robust migratory ability. This data supports a role for nuclear \( \beta \)-catenin in cell invasion, and supports studies which have found that transcriptionally active \( \beta \)-catenin increases both melanoma migration and invasion. In additional studies, it was elucidated that the canonical pathway driving invasion was activated by Wnt5a, the first identification for this Wnt in canonical signaling regulating cell invasion, and demonstrated that Wnt was regulating ARF6 through the ARF6 GEF GEP100, which has previously been shown to promote invasion and metastasis.
4.3.5 Figures

Figure 4.1 CD2AP slows epithelial cell migration. A) MDCK I cells transiently transfected to over-express GFP (control) or GFP-CD2AP were grown to confluence and wounded with a pipette tip to remove a portion of the monolayer. The same area of cells was imaged immediately after wounding and 9.5 hours later to monitor the ability of cells to repopulate the cleared area.
Figure 4.1 CD2AP slows epithelial cell migration. B) MDCK I cells transiently transfected to over-express GFP (control) or GFP-CD2AP were grown to confluence and wounded with a pipette tip to remove a portion of the monolayer. The same area of cells was imaged immediately after wounding and 9.5 hours later to monitor the ability of cells to repopulate the cleared area. The area of the wound was measured using Image J software at 0 hours and again at 9.5 hours, and the amount of wound covered was calculated as a percentage.
Figure 4.2 CD2AP decreases ARF6 activation in migrating cells. A) MDCK I cells transiently transfected to over-express GFP (control) or GFP-CD2AP were grown to confluence and wounded with a pipette tip to remove a portion of the monolayer. After 10 hours the cells were lysed, and lysates subjected to MT-2 pulldown and SDS-PAGE to assess levels of ARF6-GTP. Additional lysate was run to assess levels of total ARF6 and an α-tubulin loading control. Equivalent volumes of lysate were loaded. B) Densitometry of western blots was performed using Image J software.
Figure 4.3 SLIT2 and SecinH3 decrease invadopodia-mediated invasion. LOX melanoma cells were grown on a thin layer of FITC-gelatin and treated with vehicle (control), 20 nM SLIT2, or 30 µM SecinH3 and incubated for 16 hours prior to fixing and staining for filamentous actin (red).
Figure 4.4 Stabilized β-catenin increases melanoma invasion. LOX cells and LOX stably over-expressing β-catenin(S33Y) were grown on a thin layer of FITC-gelatin for 16 hours prior to fixing and staining for filamentous actin (red).
4.4 Discussion

Here we have described how the recruitment of ARF6 activating proteins and exchange factors can be exploited to regulate cell motility and invasion. We have demonstrated a role for the membrane scaffold protein CD2AP in regulating epithelial migration by downregulating ARF6 activation by the recruitment of ArfGAPs. In a Drosophila model, our collaborators went on to elucidate that this was occurring by the recruitment and anchoring of the Drosophila ARF6 GAPs ArfGAP3 and ASAP at the plasma membrane, where they can inactivate ARF6 to stabilize the actin cytoskeleton and adherens junctions. Efforts to decrease ArfGAP3 and ASAP levels in our mammalian system proved toxic to the cells, so these studies were not completed. Due to the well-established role for CD2AP in the formation of membrane protrusions in podocytes, as well as a demonstrated ability to bind cortactin, a key component of invadopodia, further studies investigating a role for CD2AP in tumor cell invasion are merited. In addition, it remains to be elucidated if ArfGAP3 and ASAP1 are the ArfGAPs functioning to regulate ARF6 downstream of CD2AP in the mammalian system, and further studies are warranted to investigate the GAPs involved to further understand the mechanisms of this pathway.

In addition, we have illustrated a mechanism in which ARF6 acts as a pivot point in the regulation of melanoma invasion. SLIT2-ROBO1 signaling recruits ARF6 GAPs, resulting in decreased ARF6 activation and a decreased pool of cytoplasmic and nuclear β-catenin, with a corresponding attenuation of invasive ability. Conversely, Wnt5a
binding can stimulate ARF6 activity via the ARF6 GEF GEP100, facilitating the release of β-catenin from the plasma membrane to augment the cytoplasmic and nuclear pools, amplifying canonical Wnt signaling and invasive ability.

Together, these examples of ARF6 modulation underscore the great complexity with which it is controlled, and the varied array of proteins involved in regulating its behavior. Like many of the small GTPases, ARF6 functions as a molecular switch in a wide range of behaviors within individual cells. This includes clathrin-dependent and -independent endocytosis, endosomal trafficking within the cell, cell adhesion, and peripheral actin rearrangement\(^42\). The ability to partition this activity spatially and temporally is enabled by a multitude of diverse effectors, and the loss of proper functioning in any of these pathways which leads to aberrant ARF6 activation may facilitate migratory and invasive abilities, promoting tumor progression.
CHAPTER 5:
DEVELOPMENT OF REAGENTS FOR ASSESSING THE ROLE OF ARF6 IN TUMOR CELL INVASION

5.1 Introduction

Inhibiting ARF6 activity in tumor cell lines by the expression of dominant-negative ARF6 has been shown to diminish invasive ability\textsuperscript{132; 203}. In vivo, suppression of ARF6 activity, either by the expression of the dominant-negative ARF6 mutant, ARF6(T27N)\textsuperscript{133} or ARF6 silencing\textsuperscript{71} was shown to retard invasion by melanoma and glioma cells, respectively. In contrast, persistent ARF6 activation has been shown to result in a marked increase in invasiveness\textsuperscript{133}. ERK is a key modulator of invasive behavior, impinging on many important signaling pathways related to motility, invasion, and cell shape\textsuperscript{221; 222; 228}. The importance of ERK signaling in ARF6-mediated invasion has been well-established, with constitutive activation of ARF6 leading to heightened ERK activation, and dominant-negative ARF6 expression resulting in a suppression of ERK activity\textsuperscript{203}. In addition, inhibition of ERK signaling abrogates the invasive ability catalyzed by increased ARF6 activation\textsuperscript{203}. Due to the pivotal role for ARF6 in tumor cell invasion, we are interested in developing further reagents to facilitate its analysis and elucidation of the downstream signaling that it impacts. In this chapter, we describe the
creation of a viral vector for ARF6 silencing, as well as plasmid constructs to evaluate the function of the ARF6 GAP ACAP2.

The development of RNA interference (RNAi) gene silencing technology has revolutionized cell biology since its elucidation by Andrew Fire and Craig Mello in the 1990s\textsuperscript{48}. Exploiting a naturally occurring viral defense in cells\textsuperscript{80; 107; 118}, the use of RNAi by the introduction of siRNA oligonucleotides or plasmids encoding short hairpin RNA (shRNA) sequences can be used to artificially knock down the expression of selected proteins in the cell, to help elucidate their function. Both siRNA oligos and shRNA-containing plasmids can be used to attain the same silencing effect, with shRNA requiring a bit more processing by the RNAi machinery of the cell.

In the cell, initial shRNA processing is performed by Dicer, which recognizes and cleaves the hairpin loop to leave short double-stranded siRNAs. From this point, siRNA is loaded into the RNAi-induced silencing complex (RISC) and unwound, to form an activated RISC. This activated RISC can then target the complementary mRNA, and the RNase-like enzyme Argonaute will then initiate degradation, thereby preventing the translation of the corresponding protein\textsuperscript{73; 122}.

One problem frequently encountered when investigating the effects of mutant protein expression or gene silencing, both \textit{in vivo} and \textit{in vitro}, is the low transfection efficiency and/or high cell death of many cell lines with standard transfection methods, such as the use of lipophilic reagents and electroporation\textsuperscript{91}. The LOX melanoma line used frequently for the study of tumor cell invasion is particularly difficult to transfec
for tissue culture studies, with optimized lipophilic transfection providing 30-40% transfection efficiency at best. For many studies, such as biochemical assays, this is inadequate. A promising means to circumvent this issue is by the use of viral vectors for gene transfer. Retroviral vectors have been utilized for gene expression and silencing for many years, and often offer benefits over standard transfection methods, including higher efficiency of gene transfer, low toxicity, and amenability to use in vivo\textsuperscript{150};\textsuperscript{172}. Of the retroviruses, lentiviridae provide many key benefits, including the ability to transduce both dividing and non-diving cells, usually with very high transduction efficiency; wide-range of species which are amenable to transduction; as well as improved rate of integration into the host genome, making selection of stable lines more efficient\textsuperscript{150};\textsuperscript{172}. HIV-based vector systems have become popular for these uses, giving reliable and efficient transduction, and the pLKO.1 system is a very popular system for shRNA expression. The genes required for virus production are divided into three separate plasmids for safety reasons, separating transfer, packaging, and envelope components\textsuperscript{166}. The virus that is produced is replication-deficient, and the separation of these components on different plasmids minimizes the opportunity for spontaneous recombination which could produce a replication-competent virus.

In addition to the development of reagents which involve direct modulation of ARF6 activation or expression, the development of reagents which can indirectly alter the activation of ARF6 may also be useful for analyzing the effects of ARF6 in tumor cell invasion and other cellular processes. One such avenue is by the use of GAPs or GEFs,
which can be over-expressed to decrease or increase the levels of active ARF6 in a system, respectively\textsuperscript{24, 205}. One proposed mechanism by which a protein such as ARF6 can have wide-ranging functions in the cell is by interacting with different sets of GAPs or GEFS for separate processes, to spatially regulate activation. Two such GAPs of interest are ACAP1 and ACAP2, also known as centaurins beta1 and 2, which have shown a higher specificity for ARF6 than for the other ARFs\textsuperscript{78}. At present, the function of the ACAPs has not been fully elucidated, however there is data to support involvement of both proteins in endosomal trafficking\textsuperscript{96, 105}. A role for ACAP1 in cell migration has been demonstrated, wherein ACAP1 regulates the trafficking of β\textsubscript{1} integrin\textsuperscript{105}. ACAP2 has been demonstrated to play an important role in NGF-induced neurite outgrowth, involved in regulating ARF6 activity in a Rab35-positive endosome population\textsuperscript{96}. In HeLa cells, over-expression of either protein has been shown to abrogate the formation of ARF6-dependent protrusions\textsuperscript{78}. As described below, ACAP2 was found to be expressed in LOX melanoma cells, however ACAP1 was not. A unique structural characteristic of ACAP2 is the presence of a Src phosphorylation site at tyrosine 742. This was particularly interesting in light of the fact that ARF6 activity is initiated downstream of active Src, promoting the internalization of adherens junctions and cell migration\textsuperscript{148}. Aberrant Src activation is strongly associated with oncogenic transformation, and this is accomplished by modulating cell migration, adhesion, survival, and proliferation\textsuperscript{50}. Src activation plays a key role in the formation of invadopodia, phosphorylating cortactin and Tks5 to modulate rearrangements of the actin cytoskeleton, and regulating the
recruitment of proteases to degrade the surrounding matrix\textsuperscript{29, 88, 182, 199}. With this in mind, ACAP2 presents an interesting target in understanding tumor cell invasion driven by ARF6.

5.2 Materials and Methods

5.2.1 Generation and use of lentivirus

pLKO.1 (Addgene plasmid 8453, originally obtained from Bob Weinberg), pMD2.G (Addgene plasmid 12259, originally obtained from Didier Trono), and psPAX2 (Addgene plasmid 12260, originally obtained from Didier Trono) plasmids for lentivirus production were purchased from Addgene. To create GFP-expressing “empty vector” virus and the two GFP-expressing ARF6 shRNA-containing viruses, the puromycin cassette in pLKO.1 was replaced with EGFP. Virus particles carrying shRNA targeting ARF6 were prepared and stored according to the distributor’s protocol.

For infection, cells were seeded at sub-confluence and grown for one day prior to infection. Virus was used at half strength, diluted with the target cells’ normal culture medium, supplemented with polybrene at 8 μg/mL. At 24 hours after transfection the media was changed and cells were incubated for an additional two days prior to use. BSL2 practices were adhered to for all virus work.

5.2.2 Cell culture and transfection

Cell culture and transfection were accomplished as outlined in Chapter 2. For non-viral expression of ARF6 shRNA, a pSUPER expression plasmid was utilized.
5.2.3 Cell staining and microscopy

Cells were fixed and stained as described previously\textsuperscript{14}, using rhodamine-phalloidin purchased from Molecular Probes. Imaging was performed using a Zeiss Observer Z1 fluorescent microscope. Image J (NIH) was utilized to optimize image brightness and clarity, and for pseudocoloring the black-and-white images.

5.2.4 Western blotting and activation assays

Cells were lysed in RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholic acid, 0.1 % SDS, 50 mM Tris-HCl pH 7.5) containing mammalian protease inhibitor cocktail (Sigma). Samples were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with the indicated antibodies as per the manufacturer’s instructions. Lysis buffer prepared for analysis of phosphorylated proteins was supplemented with 500 µM pervanadate. Antibodies used were mouse anti-ARF6 (as previously described)\textsuperscript{179}, mouse anti-α-tubulin (Sigma), rabbit anti-total ERK (Cell Signaling), mouse anti-pERK (Cell Signaling), mouse anti-Rac1 (BD Transduction), and rabbit anti-RhoA (Santa Cruz). MT-2, PAK, and RBD activation assays were performed as described previously\textsuperscript{13; 181}.

5.2.5 Invasion assays

The two-dimensional FITC-gelatin invasion assays and spheroid aggregate invasion assays were performed as outlined in Chapter 2.
5.2.6 Reverse transcriptase PCR to assess expression of ACAP1 and ACAP2

RNA was isolated from cells using the Qiagen RNeasy kit, and cDNA was synthesized using the Quanta qScript cDNA SuperMix, according to the manufacturer’s recommended amplification conditions. Unless otherwise noted, PCR was performed using Promega GoTaq Green Master Mix, with the following cycling conditions: 95°C denature for 30 sec, 68°C anneal for 30 seconds, and 72°C extension for 45 seconds. Beta-actin was run as a positive control of amplification, for each experiment. Products were run on a 1% agarose gel.

Primers for PCR detection of ACAP1:

Forward- 5’-CCTTCAGTCAGGCTCGCCTTGATGAC-3’
Reverse- 5’-CCTCGAATCTCAGGCAGCTTGGTCAG-3’

Forward- 5’-GGGAAGGGAGCCTGGGGGAG-3’
Reverse- 5’-GCCTCCTTCTCCTGCGGGGA-3’

Forward- 5’-AGCTGCTCCCGGCAGAGAA-3’
Reverse- 5’TCCGGTCTGCTGACGCAGGC-3’

Primers for PCR detection of ACAP2:

Forward- 5’-CATCTCCATCCACAGGAAGCCTAGATTCTG-3’
Reverse- 5’-TGAACCTTGGGCAGATGCTCTGACTTGGTC-3’

Primers for PCR detection of beta-actin:

Forward- 5’-ACTGGGACGACATGGAGAAG-3’
Reverse- 5’CGTGGGTAGTTCGTAGCTCAGTC-3’

5.2.7 Site-directed mutagenesis for development of ACAP2 constructs

Wild-type ACAP1 and ACAP2<sup>K394R</sup> plasmids were kindly provided by Paul Randazzo. The PhosphoSitePlus database was utilized to identify phosphorylation sites
on ACAP\textsuperscript{20}. The QuikChange Lightning site-directed mutagenesis kit (Agilent) was utilized to mutate the 394 residue of ACAP2 to the wild-type lysine, and the tyrosine at amino acid 742 to phenylalanine for the non-phosphorylatable mutant, or glutamate for the phosphomimetic mutant. The recommended cycling conditions were used. The manufacturer’s software was utilized for primer design, and the primer sequences utilized for mutagenesis are as follows:

**Primers for mutagenesis of tyrosine 742 of ACAP2 to phenylalanine:**

Forward: 5'-TGCGGGAATCAGAAGGACTTTTTGGACAGCCAG-3'
Reverse: 5'-CTGGCTGTCCAAAAAGTCCTTCTGATTCCCGCA-3'

**Primers for mutagenesis of tyrosine 742 of ACAP2 to glutamate:**

Forward: 5'-CGGGAATCAGAAGGACTTTGAAGGACAGCCAGGTGATG-3'
Reverse: 5'-CATCACCTGGCTGTCCTTCAAGTCTTCTGATTCCCG-3'

**Primers for mutagenesis of arginine 394 of ACAP2 to lysine:**

Forward: 5'-CTGGAAATGAGTCCAAAGAGAAATTATTGGAAGAGGATGC-3'
Reverse: 5'-GCACCTTCTCTTTCAATAATTCTTCTTTGGACTCATTTCCAG-3'

5.3 Results

5.3.1 Lentiviral vector containing shRNA targeting ARF6 is capable of high transduction efficiency and efficient depletion of ARF6

The pLKO.1 lentiviral shRNA expression system\textsuperscript{166} was utilized to create a vector for silencing ARF6 expression. The transfer vector encodes an shRNA targeting the gene of interest, and also contains a cassette to convey puromycin resistance to enable the
selection of transduced cells. In order to visualize the transduced cells, we created an additional transfer vector in which the puromycin cassette has been replaced by GFP, which is expressed cytoplasmically (Figure 5.1). We created an “empty vector” virus to use as an infection control, which contains GFP and a stuffer fragment in place of an shRNA cassette, alongside two separate lentiviral shRNA constructs targeting ARF6. Virus #1 carries a short hairpin sequence targeting nucleotides 76-98 of the ARF6 coding sequence, which has demonstrated effectiveness, and virus #2 targets nucleotides 348-368. The virus was used at half-strength, with hexadimethrine bromide (“polybrene”), a cationic polymer, added to facilitate interaction between the negatively-charged virus and cell membrane. Using this method, the transduction efficiency was consistently close to 100%, for all viruses (Figure 5.2). In addition, no overt toxicity was noted. Just as importantly, the ARF6 knockdown achieved in each experiment was substantial, with undetectable protein levels at 72 hours post-transduction with virus carrying either of the shARF6 virus constructs (Figure 5.3 A, Figure 5.3 B). These results verified lentiviral transduction as an efficient and effective means for ARF6 knockdown in the LOX melanoma cell line.

5.3.2 Lentivirally-mediated knockdown of ARF6 induces increased invasiveness in LOX

With the integral role for ARF6 in tumor cell invasion well documented, it was anticipated that silencing ARF6 expression would yield similar results as over-expression of the dominant-negative ARF6 mutant, ARF6(T27N), which is a significant inhibition of invasion. It was surprising then, that while the effects were inconsistent, a marked
increase in invasive capacity was observed. LOX cells transduced with either of the shARF6-carrying viruses exhibited very high levels of invadopodia-mediated cell invasion on FITC-gelatin (Figure 5.4). The cells proteolyzed large paths across thin gelatin matrices, indicating robust invadopodia formation and motility. In addition, in a thick and compliant matrix, microvesicle formation is robust (Figure 5.5). These results were unexpected, with the integral role for ARF6 in tumor cell invasion well-characterized. Existing literature clearly explicates a reduction in invasive capacity in cells in which ARF6 expression has been silenced\textsuperscript{59; 64; 71}. These findings elicited further examination of the activation of pathways related to cell invasion, in this system. Since the #1 shRNA sequence had been used successfully in our lab for studies of other cell behaviors, this construct was selected for subsequent studies.

5.3.3 ARF6 silencing results in ERK activation

Interestingly, when ARF6 was silenced using the lentiviral vector, ERK activation was markedly elevated (Figure 5.6). While ERK activation corroborates with the increased invasiveness shown by these cells\textsuperscript{164; 221; 222}, this data is in contrast with the previously established relationship between ARF6 and ERK\textsuperscript{203}. This impelled the investigation of related invasion pathways, to further elucidate the mechanisms involved in the increased invasion observed.

5.3.4 Lentiviral infection may produce artefactual pathway activation

While lentiviral infection presents many benefits for gene transfer or silencing, there are caveats to using this system. The act of simply infecting a cell with a virus can,
in some cases, elicit a response from the cell\textsuperscript{49; 91}, regardless of any gene the virus may be transferring. Accordingly, it has been demonstrated that RhoA activation can be induced by the binding of HIV-1 to the cell\textsuperscript{39}, and activation of Rac1 has also been demonstrated following HIV binding, resulting in actin rearrangement in the cell\textsuperscript{79; 159}. Indeed, in our system, we see that RhoA-GTP and Rac1-GTP levels increase following infection with the shARF6 virus and with the empty vector virus, as compared to uninfected cells (Figure 5.7). This suggests that viral infection alone elicits activation of pathways that influence the cell invasion process. This is problematic, as the off-target modulation of these pathways makes it difficult to clearly delineate the effects of ARF6 silencing on cell behavior. Direct transfer of the shRNA plasmids via liposomal transfection does not result in the dramatic increase in invasiveness seen with lentiviral transduction (Figure 5.8). For this reason, further optimization of the lentiviral-based reagents is required before they are used to investigate effects on cell invasion.

5.3.5 Development of ACAP2 reagents for modulation of ARF6 activity

Initial experiments were performed to assess whether ACAP1 and ACAP2 were expressed in the LOX melanoma cell line that we have well characterized for studies of cell invasion. To this end, we have used reverse transcriptase PCR to amplify these proteins, as outlined in the materials and methods section. We were able to successfully amplify ACAP2 in this cell line (Figure 5.9 A), however we could not detect ACAP1 (Figure 5.9 B), and this was verified with repeated experiments utilizing different primers and amplification conditions, with primers that were confirmed to be functional.
using cells transfected to express ACAP1. These results suggested that ACAP1 is not expressed in LOX, and for this reason, ACAP2 was selected for further evaluation.

As described above, ACAP2 contains a number of phosphorylation sites which may regulate its activity, including a putative Src phosphorylation site at Tyr\textsuperscript{742}. With the prominent role of Src in tumor cell invasion and invadopodia formation\textsuperscript{61, 74, 88, 157}, we evaluated a role for phosphorylation of this site in cell invasion. To this end, phosphomimetic (mimicking Src phosphorylation) and non-phosphorylatable (which cannot interact with Src) mutants were created by site-directed mutagenesis, substituting glutamate and phenylalanine, respectively (Figure 5.10). In addition, to provide a control for the GAP activity of ACAP2, a mutant with a lysine to arginine mutation at residue 394 was utilized (Figure 5.10). This site lies at the boundary of the GAP domain, and, as described below, this mutant was confirmed to be defective in inactivating ARF6 (Figure 5.11).

MT-2 effector pulldown assays were performed to assess levels of active ARF6\textsuperscript{181}, and confirm the ability of ACAP2 to reduce the level of ARF6-GTP (Figure 5.11). Transfection with the ACAP2(K394R) mutant consistently resulted in a mild increase in ARF6-GTP levels, consistent with the loss of a functional GAP (Figure 5.11). One difficulty with these experiments has been the low transfection efficiency of the plasmids, which makes biochemical studies difficult, and may account for the limited effect noted on ARF6 activation. Unfortunately, the transfection of the Tyr\textsuperscript{742} mutants is too low for use in biochemical assays. Interestingly, using an MT-2 effector pulldown assay, we can see
that ACAP2 is precipitated from cell lysates with ARF6-GTP (Figure 5.12), suggesting a sustained interaction between these proteins. An additional experiment was performed to look at ACAP1 in cells transfected to express the protein, and a similar pulldown interaction is observed (Figure 5.12).

Initial data obtained following transient transfection with the ACAP2 phosphorylation mutants does not appear to show an alteration in the ability of cells to form invadopodia to invade a thin matrix (Figure 5.13) or invasive microvesicles in a thick matrix (Figure 5.14), nor does the GAP defective ACAP2(K394R) mutant (Figure 5.13, Figure 5.14). This data indicates that while ACAP2 is a functional ARF6 GAP in this cell line, it does not appear to be involved in regulating cell invasion. Further studies are required to confirm these observations as well as to fully elucidate if phosphorylation of tyrosine 742 of ACAP2 impacts its ability to inactivate ARF6.

5.3.6 Application of a spheroid assay for the study of ARF6 in cell invasion

Two-dimensional invasion assays, in which single-cell suspensions are plated onto a matrix, can provide valuable information with regard to cell behavior\textsuperscript{51, 68, 203}. However, these assays do not always accurately recapitulate the immediate tumor environment. The dense association of cells and the altered physiology of cells in the hypoxic tumor core\textsuperscript{66} are unique features of a tumor cell mass that are difficult to model in a two-dimensional assay. For this purpose, the use of “spheroid” tumor cell aggregates can be very useful. Initially developed to study drug resistance\textsuperscript{194}, the aggregate invasion assay provides a model which simulates a tumor in a type I collagen
matrix environment. In this assay it is possible to assess the ability of cells to form a solid mass, as well as their ability to proliferate and expand the aggregate, and evaluate the way in which cells detach from the tumor spheroid and invade the surrounding matrix.

To encourage the formation of aggregates, cells are first plated onto firm agar in a 96-well plate. The agar will solidify with a deep meniscus, forming a “bowl” which the cells will not adhere to. The cells will collect at the deepest point of the agar, and adhere to one another under normal circumstances to form a solid spheroid mass. After 48-72 hours, the aggregate is harvested from the surface of the agar, suspended in a type I collagen mixture, and transferred to a collagen bed where it can then be evaluated over the course of days or weeks (Figure 5.15, Figure 2.5). When non-invasive cells, such as HEK-293T, are used in this assay, a solid aggregate is formed but the aggregate remains intact when placed into a collagen matrix, and no cells are observed migrating away from the cell mass (Figure 5.16). In contrast, invasive cells will leave the aggregate and travel into the surrounding matrix (Figure 5.16). This all occurs without the need for any type of chemoattractant gradient, such as would be used in a Boyden chamber assay\textsuperscript{187}.

In accord with data from 2-D assays, the invasive behavior of the cells transduced with lentivirus expressing shRNA targeting ARF6 is increased when compared with those expressing the empty-vector virus, with cells seen invading away from the spheroid more quickly than in the controls. Within just one hour after plating, the ARF6 silenced cells are forming extensive protrusions into the collagen, whereas the
empty vector controls do not (Figure 5.17). Imaging of the spheroid beyond 48-72 hours in collagen becomes challenging, as the aggregate grows and disseminates to such a wide extent that it is difficult to visualize a majority of the cells in one field of view.

Comparable studies were utilized to assess the effects of constitutive ARF6 activation on the formation of tumor aggregates in LOX melanoma cells. Interestingly, these cells don’t form aggregates on agar. Images taken utilizing a stereoscopic microscope show the irregular clumps of cells that are present on the agar at 72 hours post-plating (Figure 5.18). Even when allowed additional time to aggregate, no solid masses are formed and any cell clumps quickly dissociate upon any attempt to remove them from the agar surface.
5.3.7 Figures

Figure 5.1 Creation of a lentiviral vector for ARF6 silencing. The 3-plasmid pLKO.1 lentivirus system was utilized for the creation of lentiviral particles expressing shRNAs targeting one of two sequences in ARF6. Each sequence has been made into a plasmid expressing puromycin resistance, to allow for selection of transduced cells, as well as into a plasmid expressing GFP. An “empty vector” control has been made for each, which expresses a 1.9 kb stuffer fragment in the place of the shRNA, alongside puromycin resistance protein or GFP.
Figure 5.2 Very high transduction efficiency is attainable with a lentiviral vector. LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid and a control stuffer sequence (“empty vector”) or one of two sequences targeting ARF6. At 72 hours post-infection, cells were fixed and stained with rhodamine-phalloidin to visualize filamentous actin (red).
Figure 5.3 ARF6 levels are severely depleted with shRNA-carrying lentivirus. A) LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence ("empty vector") or one of two sequences targeting ARF6. At 72 hours post-infection cells were lysed and analyzed by western blot for ARF6 levels. α-Tubulin is shown as a loading control. B) Densitometry of ARF6 levels, analyzed using Image J software (NIH).
Figure 5.4 Lentivirally-mediated ARF6 silencing increases invasion. LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence (“empty vector”) or one of two sequences targeting ARF6. At 72 hours post-infection, the cells were plated onto a thin FITC-gelatin matrix and grown for 16 hours prior to being fixed and stained with rhodamine-phalloidin, to visualize filamentous actin (red).
Figure 5.5 Microvesicle formation is robust in lentivirally ARF6 silenced cells. LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence (“empty vector”) or one of two sequences targeting ARF6. At 72 hours post-infection the cells were plated onto a thick FITC-gelatin matrix and grown for 16 hours prior to being fixed and stained with for β1 integrin (red).
Figure 5.6 Lentivirally-mediated ARF6 silencing elicits ERK activation.
LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence ("empty vector") or an shRNA targeting ARF6. At 72 hours post-infection the cells were lysed and evaluated for levels of ARF6, and phospho- and total ERK via western blot. α-Tubulin is shown as a loading control.
**Figure 5.7 Lentiviral infection elicits Rac1 and RhoA activation.** LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence ("empty vector") or an shRNA targeting ARF6. At 72 hours post-infection, the cells were lysed and subjected to PAK-PBD and RBD pulldowns to evaluate the level of GTP-bound Rac1 and RhoA, respectively. Lysates were also blotted for total Rac1 and RhoA.
Figure 5.8 Transient transfection with ARF6 silencing plasmid does not elicit same invasive behavior as lentiviral infection. LOX cells were transiently transfected with a plasmid carrying only GFP, or GFP with an shRNA targeting ARF6. Cells were grown on a thin layer of FITC-gelatin for 16 hours prior to fixing and staining for filamentous actin.
Figure 5.9 LOX cells express ACAP2 but do not express ACAP1. cDNA from LOX cells was either used undiluted, or diluted 1:10 or 1:100 with primers for A) ACAP2 or B) one of three sets of primers for ACAP1. Amplified fragments are expected between 350-500 kb for ACAP1. Actin was amplified as a control in all experiments. ACAP1 primers were confirmed to be working by amplifying ACAP1 in LOX cells transfected to express the protein (data not shown).
ACAP2 contains three primary structural domains: a plekstrin homology domain, an ArfGAP domain, and an ankyrin repeat domain. ACAP2 mutants were generated by site-directed mutagenesis, to create mutations at Lys394 and Tyr742.

* K394R GAP defective
* Y742F Src Phosphomimetic
* Y742E Src Non-phosphorylatable

Figure 5.10 Schematic representation of ACAP2 mutants. ACAP2 contains three primary structural domains: a plekstrin homology domain, an ArfGAP domain, and an ankyrin repeat domain. ACAP2 mutants were generated by site-directed mutagenesis, to create mutations at Lys394 and Tyr742.
Figure 5.11 ACAP2 decreases ARF6 activation; ACAP2(394R) is defective in GAP activity. HEK-293T cells were transfected with a plasmid carrying FLAG-tagged WT ACAP2 or ACAP2(394R) which contains a mutation at the boundary of the GAP domain, or a control plasmid carrying only GFP. ARF6 activation was assessed with an MT-2 effector assay to pull down ARF6-GTP, and subsequent western analysis. Total levels of ARF6 were assessed for comparison, and α-tubulin was run as a loading control. LOX which stably over-express constitutively-active ARF6, ARF6(Q67L), were run as a positive control for the MT-2 assay.
Figure 5.12 ACAP 1 and ACAP2 form a stable complex with ARF6-GTP. HEK-293T cells were transfected with a plasmid carrying FLAG-tagged ACAP1 or ACAP2, or a control plasmid carrying only GFP. ARF6 activation was assessed with an MT-2 effector assay to pull down ARF6-GTP, and subsequent western analysis. Total levels of ARF6 were assessed for comparison, and α-tubulin was run as a loading control. LOX which stably over-express constitutively-active ARF6, ARF6(Q67L), were run as a positive control for the MT-2 assay. ACAPs 1 and 2 were detected with an antibody directed against their FLAG tag.
Figure 5.13 ACAP2 does not appear to modulate invadopodia formation in LOX melanoma cells. LOX cells were transiently transfected with wild-type (WT) ACAP2, or ACAP2 carrying mutations at lysine 394 or tyrosine 742, prior to plating on a thin layer of FITC-gelatin. Cells were incubated for 16 hours prior to fixation and staining for the FLAG tag on ACAP2 (red) and filamentous actin (cyan).
Figure 5.14 Expression of ACAP2 mutants does not inhibit microvesicle formation. LOX cells were transiently transfected with FLAG-tagged wild-type ACAP2 or plasmids encoding ACAP2 with a point mutation at residues 742 or 394, a phosphorylation site for Src kinase and the boundary of the GAP domain, respectively. Cells were grown on a thick layer of FITC-gelatin for 16 hours prior to fixing and staining for the FLAG tag on the ACAP2 plasmids (red).
Figure 5.15 Schematic representation of tumor cell spheroid aggregate assay. Cells are harvested from adherent culture, counted, and plated onto firm agar in 96-well plates. Aggregates are allowed to form for 48-72 hours prior to harvest and embedment in a type I collagen matrix. Invasive cells will leave the aggregate and move out into the matrix, while non-invasive cells will simply grow in place.
Figure 5.16 Spheroid aggregate assay highlights invasive abilities. Non-invasive HEK-293T renal cells and invasive LOX melanoma cells were induced to form aggregates and subsequently transferred to type I collagen to monitor invasive behavior. Images are shown after 48 hours in collagen.
Figure 5.17 Lentivirally-mediated ARF6 silencing elicits rapid invasion from tumor cell aggregates. LOX cells were transduced with lentiviral particles carrying a GFP-expressing plasmid with a control stuffer sequence (“empty vector”), or an shRNA targeting ARF6. These cells were induced to form spheroid aggregates prior to embedding in type I collagen. Images were obtained via phase contrast microscopy at 5x magnification at 1 hour post-embedment, and a zoomed image of ¼ slice of each aggregate is shown here to enable visualization of cellular details.
Figure 5.18 Constitutive ARF6 activation inhibits formation of cell aggregates. LOX and LOX stably expressing ARF6(Q67L) were plated on firm agar to induce spheroid aggregate formation. At 72 hours post-plating, parental LOX cells have formed cohesive aggregates, while those expressing ARF6(Q67L) are loosely assembled.
5.4 Discussion

The reagents and techniques presented here have been developed to help further understand the regulation of tumor cell behavior. With the integral role for ARF6 in tumor cell invasion, continued development of methods to modulate its activity may be beneficial in further unraveling how this signaling regulates invasive behavior.

The generation of lentiviral vectors for silencing ARF6 expression presents potential to be very helpful when working with cell lines that are difficult to transfect with traditional transfection methods, as well as facilitating transduction in vivo. Preliminary data generated with these vectors has run contrary to expectations, relative to other investigations that have evaluated ARF6 silencing in cell invasion\(^59; 64; 71\). In our studies utilizing a lentiviral shRNA system, ARF6 silencing generated aberrant behavior, resulting in increased invasion. Evaluation of downstream pathways shows an increase in ERK phosphorylation, as well as activation of Rac1 and RhoA signaling. Complicating matters is the fact that transduction with the empty-vector virus alone results in Rac1 and RhoA activation above the level of untransduced cells, indicating off-target effects of viral infection. While these viral vectors are still promising for future studies, additional investigation is required to better elucidate ways to minimize non-specific, viral-mediated effects.

Reagents have been developed to investigate the role of ACAP2 in ARF6 regulation, which can be utilized for the study of tumor cell invasion. At present, ACAP2 has not been studied in this context, however, due to its impact in intracellular
trafficking, it presents an interesting target. Due to the importance of Src activity in many facets of cancer cell behavior\textsuperscript{61, 74, 157}, further investigation of the putative Src phosphorylation site on ACAP2, Tyr\textsuperscript{742}, may yield helpful data. Preliminary data demonstrates the ability of ACAP2 to function as a GAP in the LOX invasion model, and also shows sustained interactions between the ACAPs and ARF6-GTP. Future work will build on pilot studies, to investigate a role for ACAP2 in tumor cell invasion.

Finally, the use of a spheroid cell aggregate assay has emerged as a valuable tool for visualizing cell behavior in a tumor-like environment, and has shed light on previous \textit{in vivo} work from our lab, which demonstrated that LOX stably over-expressing ARF6(Q67L) are highly invasive, but tumors formed by subcutaneous injection are very small, and these cells do not colonize to form metastatic lesions when injected via the tail vein\textsuperscript{133}. In the spheroid assay we see that these cells are not able to form aggregates. This may due to the fact that ARF6(Q67L) expression alters the trafficking and internalization of adhesion molecules\textsuperscript{59, 148}, preventing these cells, which already have significantly down-regulated cell-cell contacts, from clustering into an adhesive mass. This assay is a valuable addition to the existing two-dimensional assays we currently utilize, allowing the evaluation of cell behavior and matrix invasion in a simulated tumor environment. Spheroid aggregates present an attractive tool for high throughput screening, particularly in preparation for \textit{in vivo} studies, saving the expense and labor associated with animal work and allowing the evaluation of a clonal and more consistent model system than can be achieved \textit{in vivo}.
Taken together, these data help us understand the ways in which ARF6 impacts tumor invasion and metastasis. The new reagents which have been developed and adapted for the study of the signaling which regulates tumor formation and cell invasion will be helpful as we move forward to further unravel the regulation of invasive cell behavior.
CHAPTER 6:

INVESTIGATING CHOLESTEROL-RICH EXTRACELLULAR VESICLES IN TUMOR CELL INVASION

6.1 Introduction

Abnormalities in membrane lipids and lipid rafts are areas with relevance to many disease pathologies, including cancer. Lipid rafts are membrane microdomains that are enriched in cholesterol and sphingolipids, and serve as a platform to concentrate an assortment of proteins, including a variety of transmembrane receptors, Src-family kinases, GPI-linked proteins, caveolins, flotillins, and trimeric G proteins. Lipid rafts are thought to serve as key signaling platforms for coordinating a variety of cellular processes, and a role for lipid rafts has been suggested in both microvesicles and invadopodia. Microvesicles are an inherently heterogeneous population, and depending on their cell of origin and their function, they can carry a wide range of cargo and have varied membrane composition. The role of lipids in the function of microvesicles has not been well-elucidated, but evidence is emerging which suggests an important role for lipids in microvesicle biogenesis, perhaps involved in regulating membrane curvature. The membrane of microvesicles derived from tumor cells is frequently found to be enriched in phosphatidylserine (PS), and
the membrane bilayer rearrangement required for PS externalization is thought to be important in TMV formation\textsuperscript{108; 132}. A requirement for cholesterol in microvesicle formation has also been suggested\textsuperscript{38}, however, due to the microvesicle isolation methods utilized in these studies it is difficult to discern that a fairly pure microvesicle fraction is being investigated, and not one enriched in exosomes. Similarly, a requirement for cholesterol in the formation of invadopodia has been studied, with cholesterol depletion inhibiting invadopodia formation in the conditions used\textsuperscript{20; 232}.

In the work detailed in this chapter we describe how cholesterol modulates invasive behavior. We describe a newly-characterized population of cholesterol-rich extracellular vesicles. We examine the mechanism of their formation and their contents, aimed at elucidating their function.

6.2 Materials and Methods

6.2.1 Cell culture, transfection, and cholesterol modulation

All cell media was purchased from Gibco. LOX and LOX ARF6 mutant stable lines were maintained in RPMI, MDCK I and HEK-293T in DMEM, PC-3 in F-12K, GM fibroblast lines in MEM, and SW480 in Leibovitz’s L-15, all with 2 mM l-glutamine (Gibco), and penicillin/streptomycin (Thermo Scientific). All media was supplemented with 10% FBS from Hyclone, with the exception of LOX-ARF6(Q67L) which were grown in tetracycline-free FBS (Clontech), in the presence of G418 (Gibco) and hygromycin (Invitrogen). MCF-
10A cells were maintained in DMEM/F12 (Gibco) supplemented with 5% equine serum (HyClone), EGF (20 ng/ml final, from PeproTech), hydrocortisone (500 ng/ml final, from Sigma), cholera toxin (100 ng/ml final, from Sigma), insulin (10 μg/ml final, from Sigma), and penicillin/streptomycin (Thermo Scientific). Cells were maintained in a 37°C humidified incubator with 5% CO₂, except for SW480 which were kept at 0% CO₂.

For cholesterol depletion via methyl-β-cyclodextrin, cells were plated and allowed to adhere to coverslips for 16 hours prior to treatment. MβCD was purchased from Sigma Aldrich and used at 3 mM in LOX-ARF6(Q67L), and 1 mM in SW480, dissolved in complete medium. U18666A was purchased from Calbiochem, and cells were plated and allowed to adhere for one hour prior treatment at 10 μg/mL in complete medium. Panobinostat and paclitaxel were purchased from LC Labs and used at 40 nM and 100 nM, respectively. Bevacizumab was used at 2 mg/mL. For cytoplasmic GFP expression, LOX-ARF6(Q67L) were transfected with pEGFP-C1 using GeneExpresso (Excellgen), following the manufacturer’s suggested protocol. To examine VAMP3 localization, cells were transfected with pEGFP3-VAMP3, kindly provided by Philippe Chavrier.

6.2.2 Immunofluorescence reagents, staining, and microscopy

For immunostaining, cells were processed as described previously. Filipin staining was also performed as described previously. Filipin was purchased from Sigma-Aldrich. Imaging was performed using a Zeiss Observer Z1 fluorescence microscope. AlexaFluor555 conjugated cholera toxin B was purchased from Invitrogen.
Phalloidin conjugates were purchased from Molecular Probes. The antibodies used were as follows: rat anti-\( \beta_1 \) integrin clone AIIB2 (Iowa DSHB), goat anti-ApoA1 (Abcam), mouse anti-uPAR (American Diagnostica), mouse-MT1-MMP (Millipore), mouse anti-EB1 (BD Pharmingen), rat anti-BAP31(Pierce), mouse anti-\( \alpha \)-tubulin (Sigma-Aldrich), mouse anti-vimentin (Sigma-Aldrich), rabbit anti-ARF6 (lab prepared), mouse anti-HA (Covance), mouse anti-caveolin (BD Pharmingen), mouse anti-clathrin (Calbiochem), and rabbit anti-\( \alpha \)-enolase was kindly provided by Dr. Lindsay Miles.

For scanning electron microscopy, cells were grown on 18 mm #1 coverslips, fixed using 2.5% glutaraldehyde, and post-fixed using 1% osmium tetroxide. All washes were done using cacodylate buffer. Samples were then ethanol dehydrated and dried using a critical point drier, prior to coating with 4 nm iridium. Samples were imaged using an FEI Magellan 400 scanning electron microscope.

6.2.3 Invasion assays

FITC-conjugated gelatin was prepared as described previously\(^{68, 69}\), and invasion assays were performed as outlined in Chapter 2.
6.3 Results

6.3.1 Depletion of membrane cholesterol increases invadopodia-mediated matrix degradation

A requirement for cholesterol in invadopodia formation has been suggested in the literature\(^{20}\). In these experiments, cellular cholesterol was depleted and cells were then allowed to invade for a short three hour period during cholesterol recovery\(^{20}\). We sought to adapt this experiment to the longer timepoints that we have characterized for invasion assays, and particularly as we see cellular cholesterol quickly recover in cells given serum-containing media, we wished to evaluate cells which were in a condition of cholesterol-depletion throughout the invasion time. To further probe the requirement for cholesterol in invadopodia-mediated invasion, highly invasive LOX-ARF6(Q67L) were plated onto a thin layer of FITC-gelatin and membrane cholesterol was depleted. This was accomplished by treating cells with U18666A, an amino-steroid which interferes with cholesterol synthesis and trafficking, and by treatment with methyl-beta-cyclodextrin (MβCD), a cyclic oligosaccharide which selectively extracts cholesterol from the cell. Treatment with U18666A results in the reduction of cholesterol at the membrane, with an accumulation of free cholesterol in late endosomes/lysosomes and is often used to model the phenotype of the cholesterol storage disease Niemann-Pick Type C\(^{126;240}\), while treatment with MβCD lowers total cholesterol levels. In our experiments cells were plated and allowed to fully adhere and spread, and then treated to invade in the presence of the cholesterol altering agent for 24 hours prior to fixing.
and immunostaining. Labeling with filipin III allowed for the visualization of unesterified cholesterol.

Cholesterol depletion mediated by either reagent resulted in a significant increase in invadopodia-mediated invasion (Figure 6.1). Cells with reduced membrane cholesterol exhibited significant matrix proteolysis, and due to the long continuous “trails” of invasion, it can be surmised that cellular motility is increased accordingly. This increase in invasion appears to be due to the reduction of cholesterol at the membrane, as this behavior is seen whether all cellular cholesterol is depleted with MβCD, or when cholesterol is removed from the membrane and aggregated intracellularly with U18666A treatment.

An additional interesting observation in these studies was that, when treated with a sufficient concentration of MβCD, it appears that osmotic lysis of the cells occurs due to a loss of membrane integrity and intact invadopodia are left in the gelatin matrix (Figure 6.2). This presents potential for exploitation as a methodology for the isolation of invadopodia for biochemical analysis. Mechanical methods currently exist for invadopodia isolation\textsuperscript{129}, however they are rarely implemented as they are technically difficult and the purity of the material which is harvested is dubious.

6.3.2 Cholesterol depletion does not reduce invasive microvesicle formation

To assess the requirement for cholesterol in the formation of invasive microvesicles, LOX-ARF6(Q67L) cells were plated onto a thick, deformable gelatin matrix and treated with U18666A to remove membrane cholesterol. Cells were allowed to
invade over the course of 24 hours prior to fixing and immunostaining. Anti-β₁ integrin antibody was used to label the plasma membrane and microvesicles, as this integrin is a well-characterized component of the invasive microvesicles\textsuperscript{100;132}. Filipin III staining was used to label unesterified cholesterol.

Interestingly, no observable alteration in microvesicle formation was observed with membrane cholesterol depletion. Cells appear to invade through the matrix in a manner comparable to that of the controls, and release integrin-positive microvesicles into the ECM in a similar fashion as well (Figure 6.3). Strikingly, we also observed a population of filipin-positive vesicles shed from the surface of untreated cells, which were highly enriched in cholesterol and do not contain the integrin label (Figure 6.4). This population was evaluated further.

6.3.3 Identification of a novel population of cholesterol-rich microvesicles shed from normal and tumor cells

During the course of evaluating a requirement for cholesterol in the formation of invasive tumor cell-derived microvesicles, a new population of microvesicles was noted, visible by filipin staining for unesterified cholesterol. The newly identified population of vesicles varies widely in size and on average are larger than the previously identified integrin-positive population, with some being larger than cells (Figure 6.5). These vesicles do not contain actin or β₁ integrin (Figure 6.4), both markers commonly used to identify microvesicles\textsuperscript{100;132}. In addition, these vesicles do not contain any cytoplasm, as evidenced by exclusion of cytoplasmic GFP (Figure 6.6), and most appear to have a semi-
deflated appearance, not turgid as in most integrin-positive microvesicles. Areas of “wrinkled” unesterified cholesterol are frequently observed on the cell surface, usually at the cell periphery, and appear to be an immature form of the large cholesterol vesicles (Figure 6.7). Images obtained by scanning electron microscopy appear to show these structures, and show a distinct separation between the cell membrane and the forming cholesterol vesicle on top (Figure 6.8).

To assess whether this was a tumor cell specific vesicle population, a variety of tumor and non-cancerous lines were investigated, including MDCK I, MDA-MB-231, MCF-10A, GM03123, GM05659, GM18436, GM17923, SW480, PC-3, HeLa, LOX, and LOX-ARF6(Q67L), and all of the cells examined were able to form these structures to some extent. Depletion of membrane cholesterol is sufficient to block their formation (Figure 6.3), and they rapidly return upon wash-out of the cholesterol-altering compound. In addition, supplementing the culture media with exogenous cholesterol in the form of water soluble cholesterol:methyl-beta-cyclodextrin is able to augment the formation of the extracellular vesicles, in addition to the intracellular pool (Figure 6.9).

6.3.4 Stabilized microtubules provide framework for cholesterol vesicles

Due to the lack of actin cytoskeleton stabilizing the newly-identified cholesterol vesicle population, we examined other cytoskeletal components to identify how the vesicle structure was supported. We did not find evidence of intermediate filament involvement, as shown by a lack of appreciable vimentin (Figure 6.10), however we did find a significant amount of α-tubulin. This was shown by immunostaining and also by
the expression of GFP-tagged α-tubulin (Figure 6.11 A & B). We also see robust staining for EB1, a microtubule (MT) plus-end protein (Figure 6.12).

Treatment with the MT depolymerizing compound nocodazole was not sufficient to inhibit cholesterol vesicle formation. Although the microtubules within the cell were collapsed and dissociated upon treatment, cholesterol vesicles appeared unaltered (Figure 6.13). In an effort to elucidate the mechanism of this nocodazole resistance, we looked for means by which cells can produce stabilized MTs. In neurons, dendritic microtubules are nucleated by so-called “golgi outposts”; small fragments of golgi which support MT nucleation independent of a centrosome\textsuperscript{145; 235}. Golgi outposts have been noted to persist in MT nucleation despite nocodazole treatment\textsuperscript{145}. Golgi outposts have not been characterized in non-neuronal cells, however we see that these cholesterol vesicles contain GM130 (Figure 6.14), a golgi marker, suggesting that similar MT nucleation may be at play in this case. Golgi-associated MT are often heavily acetylated, which conveys stability and nocodazole resistance\textsuperscript{45; 163}. AKAP450, a scaffolding protein and marker of golgi outposts, has been shown to regulate the acetylation of golgi-associated microtubules\textsuperscript{163}, and a requirement for AKAP450 in cholesterol vesicle formation, as well as assessing the acetylation of cholesterol vesicle MTs are interesting avenues for future exploration. Due to the importance of ARF1 in intra-golgi traffic and golgi integrity\textsuperscript{224}, we also investigated the effects of ARF1 inhibition on cholesterol vesicle formation. This was accomplished by the expression of ARF1(T31N), which induces disassembly of the golgi\textsuperscript{224}. In these preliminary experiments we did not see any
effect on cholesterol vesicle formation although there is some co-localization of ARF1 to sites of vesicle formation (Figure 6.15).

6.3.5 Microtubule stabilization induces increased cholesterol vesicle formation and promotes cholesterol efflux

In order to assess if increased microtubule stability would augment the formation of cholesterol vesicles, two compounds were utilized. The first was paclitaxel (Taxol®), which stabilizes microtubules, in part by increasing acetylation\(^{230}\). The second compound used was panobinostat, also known as LBHS89, a histone deacetylase inhibitor which is characterized to also inhibit tubulin deacetylation\(^{75}\). Interestingly, panobinostat has been characterized to reduce cholesterol accumulation in NPC mutant fibroblasts\(^{156}\), which has been attributed to increased expression of NPC1 protein.

To accomplish this we utilized GM05659 wild type fibroblasts, alongside GM03123 NPC1 mutant fibroblasts which display considerable accumulation of LE/LY cholesterol. The preliminary data for these experiments is promising, demonstrating a marked reduction in intracellular cholesterol with either method of MT stabilization (Figure 6.16, Figure 6.17). Even more interesting is that cholesterol vesicle formation is increased in cells treated with paclitaxel for 24 hours (Figure 6.18). This indicates that these structures may represent an efflux mechanism for cholesterol and raise the possibility of therapeutic use of microtubule stabilizing agents for diseases such as NPC which display abnormal cholesterol accumulation. With panobinostat treatment and longer treatment times with paclitaxel, an increase in cholesterol vesicle formation is
not observed, which at this point appears to be due to the fact that intracellular cholesterol has already been markedly lowered.

6.3.6 Characterizing the content of cholesterol-rich microvesicles

To better understand the function of these extracellular cholesterol vesicles, we have examined them for a number of proteins with relevance to endosomal trafficking, cell invasion, cholesterol metabolism, or with an affinity for lipid localization. We have characterized that this population does not contain β1 integrin, actin, nor vimentin, as outline above, nor do they endoplasmic reticulum as evidenced by a lack of BAP31 staining, nor do they contain ARF6, caveolin, clathrin, ApoA1, VAMP3, MT1-MMP, or PIP2 (Figure 6.19). By phase contrast microscopy they appear also (Figure 6.20), but are difficult to discern, presumably due to low protein content. In addition to the α-tubulin and EB1 noted above, we also see α-enolase and uPAR (Figure 6.21), in addition to cholera toxin B staining which indicates the presence of the ganglioside GM1 (Figure 6.22). GM1 and uPAR are known to localize to lipid rafts\textsuperscript{155}, and similar targeting may be at play here. Enolase activity is involved in cholesterol metabolism\textsuperscript{185}, and a requirements for its activity in the formation of these vesicles is an interesting question for future studies. Due to the established relationship between uPAR and vascular endothelial growth factor (VEGF) in angiogenic sprouting\textsuperscript{16; 212}, we also investigated if VEGF inhibition would abrogate cholesterol vesicle formation, and did not note any effect (Figure 6.23).
Due to the unique cholesterol enrichment seen at sites of cholesterol vesicle formation we investigated a role for P-glycoprotein (P-gp), which is characterized for its ability to reorganize membrane cholesterol\textsuperscript{54}. To accomplish this we utilized the potent and selective P-gp inhibitor tariquidar. As P-glycoprotein is a well-characterized efflux pump in mediating multidrug resistance, intracellular localization of the chemotherapeutic compound doxorubicin was investigated in parallel. Doxorubicin-resistance has been attributed to P-glycoprotein activity\textsuperscript{11,37}, and due to its inherent fluorescence with a 488 nm excitation, it can be imaged by fluorescence microscopy. We sought to visualize if doxorubicin was being removed from resistant cells via extracellular cholesterol vesicles. To accomplish these studies we utilized PC-3 prostate adenocarcinoma cells, including those which had been slowly conditioned to increasing concentrations of doxorubicin, to establish a moderate level of doxorubicin resistance, as previously described in the literature\textsuperscript{37}.

It does not appear that P-glycoprotein is required for the formation of cholesterol vesicles. Tariquidar treatment in high concentrations and extended treatment times did not hinder cholesterol vesicle biogenesis (Figure 6.24). Accordingly, we did not see any localization of doxorubicin to these vesicles. With sufficient treatment concentrations we could induce marked cell spreading and doxorubicin accumulation in the nucleus, without any localization to the cholesterol vesicles (Figure 6.25), indicating that they are likely not involved in drug efflux.
6.3.7 Figures

Figure 6.1 Depletion of membrane cholesterol induces increased invadopodia-mediated invasion. LOX-ARF6(Q67L) were grown on thin FITC-gelatin coverslips and treated with U18666A or methyl-β-cyclodextrin prior to fixation and staining for β1 integrin (red) and unesterified cholesterol (blue).
Figure 6.2 Potential to use methyl-β-cyclodextrin treatment for invadopodia isolation. LOX-ARF6(Q67L) were grown on a thin layer of FITC-gelatin (green) and treated with 3 mM methyl-β-cyclodextrin to deplete cellular cholesterol, prior to fixation and staining for filamentous actin (red). The top image shows remnant invadopodia in an xy plane. The two bottom images show an xz plane.
Figure 6.3 Cholesterol depletion does not reduce invasion in a soft matrix nor does it inhibit TMV formation. LOX-ARF6(Q67L) were treated with U18666A and grown on a thick layer of FITC-gelatin prior to staining for unesterified cholesterol (grey) and β₁ integrin (red).
Figure 6.4 Identification of a novel population of cholesterol-rich extracellular vesicles. LOX-ARF6(Q67L) were grown on a thick layer of FITC-gelatin prior to staining for unesterified cholesterol (grey) and $\beta_1$ integrin (red). A population of novel, cholesterol-rich extracellular vesicles is indicated by the arrows.
Figure 6.5 Cholesterol-rich extracellular vesicles vary in size. LOX-ARF6(Q67L) stained with for unesterified cholesterol (grey) and actin (green). Vesicles are indicated by the arrows in the merged image.
Figure 6.6 Cholesterol-rich extracellular vesicles do not contain cytoplasm. LOX-ARF6(Q67L) were transiently transfected to express cytoplasmic GFP, and fixed and stained for unesterified cholesterol (grey) and β1 integrin (red). Cholesterol is shown in blue in the merged image, for clarity.
Figure 6.7 Stages of cholesterol-rich extracellular vesicle biogenesis. A) PC-3 cells were imaged at what appear to be progressive stages of cholesterol vesicle formation, prior to release (from left-to-right). The cells are stained for β₁ integrin (red) and unesterified cholesterol (green). The arrows indicate the forming vesicles. B) An example of many cholesterol vesicles forming at the periphery of a HeLa cell.
Figure 6.8 Scanning electron micrographs appear to show cholesterol-rich vesicle formation. SW480 cells were processed for scanning electron microscopy, and images are shown at 5,000x magnification on the left and 20,000x on the right. The red outlined areas highlight the forming vesicles (left), and the unusual membrane texture under the vesicles (right).
Figure 6.9 Provision of excess exogenous cholesterol augments the formation of cholesterol-rich extracellular vesicles. SW480 were treated with 1 mM cholesterol:methyl-β-cyclodextrin in serum-containing media to augment cholesterol levels in the media for 24 hours prior to fixation and staining for unesterified cholesterol using filipin (green).
Figure 6.10 Cholesterol-rich extracellular vesicles do not contain actin or vimentin. SW480 stained for unesterified cholesterol (grey), vimentin (green), and filamentous actin (red).
Figure 6.11 Cholesterol-rich extracellular vesicles contain α-tubulin. A) SW480 cells fixed and stained for unesterified cholesterol (grey), α-tubulin (green), and filamentous actin (red). Cholesterol is shown in blue in the merged image for clarity.
Figure 6.11 Cholesterol-rich extracellular vesicles contain α-tubulin. B) SW480 cells were transfected to express GFP-tagged α-tubulin, and were fixed and stained for unesterified cholesterol (grey), and β₁ integrin (red). Cholesterol is shown in blue in the merged image for clarity.
Figure 6.12 Microtubule plus-end protein EB1 localizes to cholesterol-rich extracellular vesicles. SW480 cells stained for unesterified cholesterol (grey), EB1 (green), and filamentous actin (red). Cholesterol is shown in blue in the merged image for clarity.
Figure 6.13 Nocodazole treatment does not inhibit the formation of cholesterol-rich extracellular vesicles. SW480 cells were treated with 10 μM nocodazole for 16 hours prior to fixation and staining for unesterified cholesterol (grey), α-tubulin (green), and filamentous actin (red). Cholesterol is shown in blue in the merged image for clarity.
Figure 6.14 GM130 Localizes to sites of cholesterol-rich extracellular vesicle formation. SW480 cells stained for unesterified cholesterol (grey), GM130 (green), and filamentous actin (red).
Figure 6.15 Dominant-negative ARF1 expression does not inhibit the formation of cholesterol-rich extracellular vesicles. HeLa cells were transiently transfected to express HA-tagged ARF1(T31N), prior to fixation and staining for unesterified cholesterol (grey) and the HA tag (green). The arrow indicates the site of ARF1 enrichment at the vesicle.
Figure 6.16 Microtubule stabilization lowers intracellular cholesterol levels. A) GM03123 NPC1 mutant fibroblasts were treated with DMSO vehicle or 100 nM paclitaxel for 24 hours prior to fixation and staining for unesterified cholesterol. The cells were imaged with the same magnification and exposure settings. B) Average corrected total cell fluorescence was calculated utilizing Image J to obtain the integrated density, area of each cell, and the mean fluorescence of the background.
Figure 6.17 Panobinostat treatment reduces intracellular cholesterol levels. GM03123 NPC1 mutant fibroblasts were treated with DMSO vehicle or panobinostat for 24 hours prior to fixation and staining for unesterified cholesterol (grey), α-tubulin (green), and actin (red). The cholesterol is shown with the same exposure settings.
Figure 6.18 Microtubule stabilization facilitates increased formation of cholesterol-rich extracellular vesicles. GM03123 NPC1 mutant fibroblasts were treated with DMSO vehicle or 100 nM paclitaxel for 24 hours prior to fixation and staining for unesterified cholesterol. The exposure settings have been altered to highlight cholesterol vesicles.
<table>
<thead>
<tr>
<th>Presence at extracellular cholesterol vesicles</th>
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</thead>
<tbody>
<tr>
<td>Actin                                      -</td>
</tr>
<tr>
<td>ApoA1                                      -</td>
</tr>
<tr>
<td>ARF1                                       +</td>
</tr>
<tr>
<td>ARF6                                       -</td>
</tr>
<tr>
<td>Caveolin                                   -</td>
</tr>
<tr>
<td>Clathrin                                   -</td>
</tr>
<tr>
<td>Cytoplasm                                  -</td>
</tr>
<tr>
<td>Doxorubicin                                -</td>
</tr>
<tr>
<td>EB1                                        +</td>
</tr>
<tr>
<td>α-Enolase                                  +</td>
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<tr>
<td>GM1                                         +</td>
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<tr>
<td>GM130                                      +</td>
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<tr>
<td>β1 Integrin                                -</td>
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<td>MT1-MMP                                    -</td>
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<tr>
<td>PIP2                                       -</td>
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<tr>
<td>α-Tubulin                                  +</td>
</tr>
<tr>
<td>Unesterified cholesterol                   +</td>
</tr>
<tr>
<td>uPAR                                       +</td>
</tr>
<tr>
<td>VAMP3                                      -</td>
</tr>
<tr>
<td>Vimentin                                   -</td>
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Figure 6.19 Characterization of the contents of cholesterol-rich extracellular vesicles.
**Figure 6.20** Cholesterol-rich extracellular vesicles are difficult to see via phase-contrast microscopy. LOX-ARF6(Q67L) stained for unesterified cholesterol (green), β₁ integrin (red). Also shown via phase contrast (“phase”).
Figure 6.21 Urokinase plasminogen receptor and α-enolase are localized to cholesterol-rich extracellular vesicles. SW480 cells stained for unesterified cholesterol (grey) and α-enolase/uPAR (green).
Figure 6.22 Cholesterol-rich extracellular vesicles contain ganglioside GM1. LOX-ARF6(Q67L) were grown in a soft layer of FITC-gelatin to better enable free vesicle capture, prior to fixation and staining for unesterified cholesterol, β₁ integrin, and GM1 (via fluorescently tagged cholera toxin B).
Figure 6.23 VEGF inhibition does not block the formation of cholesterol-rich extracellular vesicles. SW480 cells were treated with VEGF inhibitor bevacizumab, prior to fixation and staining for unesterified cholesterol.
Figure 6.24 P-glycoprotein activity is not required for the formation of cholesterol-rich extracellular vesicles. PC-3 cells were treated with P-glycoprotein inhibitor tariquidar prior to fixation and staining for unesterified cholesterol (green) and filamentous actin (red).
Figure 6.25 Doxorubicin does not localize to cholesterol-rich extracellular vesicles in resistant cells. PC-3 cells were conditioned to become resistant to doxorubicin at a 500 nM concentration, and cultured in the presence of doxorubicin prior to fixation and staining for unesterified cholesterol (grey) and filamentous actin (red). Doxorubicin is inherently fluorescent at 488 nm and is shown in green.
6.4 Discussion

Precise regulation of cellular cholesterol is imperative to normal cell function. Aberrations in intra- or extracellular cholesterol regulation can have devastating consequences to normal physiology. Such abnormalities are implicated in a variety of pathologies, from neurodegenerative disorders such as Alzheimer’s disease (AD) and Niemann-Pick Type C disease (NPC) at a cellular level\textsuperscript{143}, to systemic hypercholesterolemia leading to heart disease\textsuperscript{117} and promoting cancer progression\textsuperscript{121; 137}.

Cells exhibiting NPC mutation provide a striking example of aberrant cholesterol regulation, accumulating high levels of unesterified (“free”) cholesterol in late endosome/lysosome compartments (LE/LY)\textsuperscript{219}. The NPC1 and NPC2 proteins are normally found localized to the LE/LY, however in the case of their mutation, the protein is usually either misfolded and degraded or found retained in the ER and unable to perform its function in cholesterol transport\textsuperscript{55; 56; 197; 238}. In this case, cholesterol accumulation is both a symptom of underlying defects in the cell, as well as being considered a contributor to additional pathology, such as impeding the transport of other cargo within the cell\textsuperscript{219}.

The cholesterol-rich vesicles described here present a very exciting new area of investigation. These structures represent a new class of extracellular vesicles which is not tumor cell specific, and which are distinct from the integrin-positive population which has been studied extensively. These vesicles, which appear to bear some
similarity to the synthetic large liposome vesicles used for drug delivery\textsuperscript{207}, are characterized by their unique membrane which is highly enriched in unesterified cholesterol, a lack of cytoplasm, and the presence of microtubules which appear to support their formation.

The functions of this vesicle population are not yet fully elucidated, however initial data indicates that at least one function may be in cholesterol efflux. The way in which cholesterol is removed from cells is not well-understood, and this may represent one mechanism. While we do not see obvious evidence of endosomal cholesterol being trafficked to these sites, there is a correlation between an increase in cholesterol vesicle formation on the cell surface due to MT stabilization, and a reduction in intracellular cholesterol. Accumulation of unesterified cholesterol is toxic to cells\textsuperscript{202}, and this may be one way to remove it. Other possibilities exist, such as a means to release certain membrane proteins or receptors that selectively bind cholesterol, mechanisms of intercellular communication, or angiogenic stimulation\textsuperscript{47}.

Thus far, efforts to isolate this vesicle population utilizing centrifugal filtration, sucrose gradients, and lipid flotation have not been successful. Future efforts are aimed to isolate these vesicles in order to better characterize their composition, such as with lipidomic and protein mass spectrometry.

The discrepancy between our data and existing data regarding the dependence of invadopodia formation on cholesterol is an interesting one. A study published by Caldieri and colleagues in 2008 demonstrates a reduction in invadopodia seen...
corresponding to small areas of degraded matrix\textsuperscript{20}, following a 40 minute treatment with, and wash-out of, MβCD, followed by a three hour invasion period. Based upon studies conducted in our lab, it appears that the short treatment time utilized by this group and the three hour recovery period allowed afterward may be sufficient for a repopulation of membrane cholesterol, obtained from serum in the culture media. In addition, the alteration of membrane cholesterol obtained in the referenced study does not appear to be a substantial reduction. In addition, in our studies there appeared to be accelerated invadopodia turnover and motility in cells treated with MβCD, suggesting the possibility that these cells may have initiated invadopodia retraction to begin cell movement during the cyclodextrin treatment. The short treatment time and the three hour invasion time point make it difficult to fully evaluate this behavior in comparison with our study. An additional study published in 2009\textsuperscript{232}, also points to a requirement for cholesterol in invadopodia formation. In this manuscript, the authors depleted cells of cholesterol prior to plating on the matrix, and then fixed and stained the cells after a seven hour incubation. The cholera toxin B labeling shows the disruption of lipid rafts, but a persistence of the label at the cell surface. Unfortunately, without labeling for cholesterol, it is not possible to assess the amount of cholesterol depletion in the invading cells to compare the conditions in this manuscript with those used in our studies. Additionally, given the importance of cholesterol in the turnover of focal adhesions and trafficking of adhesion molecules, it appears that it may be beneficial to allow cells to establish these structures prior to cholesterol depletion\textsuperscript{137, 220}. Existing
studies have demonstrated that cholesterol depletion by MβCD affects focal adhesion turnover, but does not induce their disassembly\textsuperscript{137}, and a requirement for cholesterol in the establishment of integrin-mediated matrix attachment\textsuperscript{220, 234}. By allowing the cells to adhere to the matrix prior to cholesterol depletion in our studies, it is possible that we help eliminate effects due to the cells not being able to initiate proper attachment to the substrate. The possibility also exists for different behavior being exhibited due to differences in cell lines used for the experiments. Future experiments using a variety of lines will be helpful in elucidating this.
CONCLUSIONS

The acquisition of invasive ability is a cornerstone in the development of metastatic disease, and it is becoming increasingly evident that tumor cells possess great plasticity in their invasive behavior\textsuperscript{52; 53}. It has previously been demonstrated that tumor cells are able to switch between different modes of motility, amoeboid and mesenchymal, depending on the compliance of their extracellular environment\textsuperscript{46; 101; 176}. This behavior is governed by mutually exclusive signaling through Rac1 and RhoA\textsuperscript{176}. ARF6 has also been shown to be highly influential in the invasive behavior of tumor cells\textsuperscript{133; 203}, and is integral for the formation of invasive microvesicles and invadopodia\textsuperscript{132; 203}. Here, we have helped elucidate the mechanisms which integrate these signaling pathways in the regulation of invadopodia and microvesicle formation in the process of tumor cell invasion.

We have presented data which help elucidate signaling downstream of ARF6 that allows tumor cells to switch between the use of two invasive structures, invadopodia and microvesicles, to proteolyze and condition the microenvironment which they are moving through. We have demonstrated how matrix conditions can guide the formation of invadopodia or microvesicles. A soft, compliant matrix favors TMV formation, with a firm matrix favoring invadopodia. Signaling through Rac1 and
RhoA is central for the formation of these structures. The use of invadopodia and microvesicles appears to be mutually exclusive; while individual cells may be able to form both microvesicles and invadopodia, antagonistic signaling through these pathways favors the formation of one type of structure at any given time. An increase in Rac1 activity is important for cells which are encountering a firm barrier which they must use invadopodia to breach, versus a soft matrix which promotes microvesicle formation through Rho activity. We believe that this may represent mechanisms exploited by cells during metastasis, as they move through varied environments to reach a secondary site. Similar to the regulation of amoeboid motility, we have demonstrated that the downregulation of Rac1 activity facilitates microvesicle formation. We have also shown that the Rho signaling which governs microvesicle formation lies downstream of ARF6, as inhibition of Rho kinase in the face of active ARF6 abrogates TMV formation, and expression of active Rho is sufficient to override the blockade of TMV biogenesis seen with the expression of dominant-negative ARF6.

In addition, we have further elucidated the regulation of myosin contractility which facilitates microvesicle release. By suppressing Rac1 activity or expressing constitutively-active RhoA as a means to amplify TMV formation, we found an increase in MLC activation. However, using Rac1 suppression as a model for increased microvesicle formation, we noted that MLCK activity was dispensable for TMV release. We noted a concomitant increase in inhibitory phosphorylation of myosin light chain phosphatase, demonstrating an additional mechanism that may be used to facilitate
myosin activity in microvesicle formation, alongside MLC activation via MLCK\textsuperscript{132}. This inhibitory phosphorylation of MLCP correlates with increased ARF6 activation and microvesicle release. The formation of microvesicles downstream of Rac1 suppression or RhoA activation is dependent on ERK activity, and this may be due to the role for ERK in the inhibitory phosphorylation of MLCP, bridging the pathway between ROCK and myosin light chain phosphatase\textsuperscript{229}.

Next, we described how GTPase activating proteins and exchange factors can be utilized to increase or decrease ARF6 activation in the regulation of migration and tumor cell invasion. The actin scaffolding molecule CD2AP has been demonstrated to play a critical role in the formation of certain membrane protrusions\textsuperscript{94; 239}, and to be involved in regulating cell migration\textsuperscript{135}. Here we have shown that the expression of CD2AP in mammalian epithelium slows migration, with a corresponding decrease in ARF6 activation. A similar phenotype is observed in a Drosophila model, and this decrease in ARF6 activity is dependent on dArfGAP3 and dASAP\textsuperscript{82}. It appears that Cindr forms a stable complex with these GAPs to stabilize them at the cell surface where they can inhibit ARF6 activation\textsuperscript{82}. We have also presented data which help outline a regulation of ARF6 activation downstream of Wnt5a in cell invasion. This is mediated by the ARF6 GEF GEP100 in a Wnt/β-catenin canonical signaling pathway downstream of Wnt5a, which stimulates invasive ability\textsuperscript{59}. In opposition, signaling through SLIT2-ROBO1 opposes ARF6 activation via the cytohesin family of ArfGAPs, which downregulates cell invasion.
Finally, we outlined the development of new tools with which to study ARF6 in tumor cell invasion. Due to the resistance of many invasive lines to traditional transfection means, we have made a lentiviral vector for silencing ARF6, and have described both the promising features of the virus, being its very high transduction efficiency and effectiveness at knocking down ARF6 protein levels, alongside some non-specific effects mediated by the virus infection itself which will require further troubleshooting prior to its use in invasion studies. We also detailed the production of multiple mutants of the ARF6 GAP ACAP2, to assess its role in regulating invadopodia formation. Due to the importance of Src in invadopodia formation\textsuperscript{29, 88, 182, 199}, investigating the effects of altering the Src phosphorylation site of this protein could yield key insight into how ARF6 regulates invadopodia. In addition, we have demonstrated the use of a three-dimensional model for the study of tumor cell invasion, and how this has been useful in illustrating the negative impact that ARF6 activation has on the ability of tumor cells to form a solid mass, helping to explain data previously obtained which showed that \textit{in vivo}, melanoma cells over-expressing active ARF6 form small tumors when implanted subcutaneously, and are unable to establish metastatic lesions when introduced directly into the blood circulation\textsuperscript{133}.

Together, these findings help further our understanding of tumor cell invasion, both in regard to interactions with the microenvironment which prompt the cell to modify its invasive strategy, and the intracellular signaling which regulates this behavior. Continued work aimed at further unraveling the mechanisms of tumor cell invasion will
prove beneficial toward the prevention and management of tumor metastasis, imperative in the fight against cancer.
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