Influence of matrix and fluid microenvironments on cancer cell migration, survival, and metastasis

James Matthew Barnes

University of Iowa

Copyright 2011 James Matthew Barnes

This dissertation is available at Iowa Research Online: https://ir.uiowa.edu/etd/2818

Recommended Citation
Barnes, James Matthew. "Influence of matrix and fluid microenvironments on cancer cell migration, survival, and metastasis." PhD (Doctor of Philosophy) thesis, University of Iowa, 2011. https://doi.org/10.17077/etd.g77l36sl

Follow this and additional works at: https://ir.uiowa.edu/etd

Part of the Biophysics Commons
INFLUENCE OF MATRIX AND FLUID MICROENVIRONMENTS ON CANCER CELL MIGRATION, SURVIVAL, AND METASTASIS

by

James Matthew Barnes

An Abstract

Of a thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Molecular Physiology and Biophysics in the Graduate College of The University of Iowa

May 2011

Thesis Supervisor: Associate Professor Michael D. Henry
ABSTRACT

Metastasis is the most common cause of lethality in patients with solid tumors. This complex cascade of events begins with invasion of local tissue by cancer cells of the primary tumor and eventually leads to dissemination of cancer cells through the bloodstream. In order to colonize a distant tissue, circulating cancer cells must first survive the physical stresses within the vasculature, and then traverse the endothelium, by a process called extravasation. After extravasation, colonized cancer cells face several additional challenges including proliferation in a nutrient-deprived microenvironment.

Epithelial-mesenchymal transition (EMT) is a process by which cells lose their epithelial characteristics and gain a mesenchymal, often migratory phenotype. There is much evidence that EMT augments cancer cell invasion, however little is known about how mesenchymal cells interact with their microenvironment during metastasis. We investigated the migratory behavior of EMT-like cancer cells on different basement membrane constituents as well as in the presence of other cell types. We showed that ZEB1, a driver of EMT, regulates pro-migratory genes, resulting in cells which must co-opt their matrix and cellular surroundings to elicit invasive migration. Additionally, we show that RNAi-mediated knockdown of ZEB1 results in significantly reduced anchorage-independent growth as well as metastatic colonization in mice. Thus, ZEB1 and EMT-states may facilitate both extravasation and survival of cancer cells in vivo.

In experimental and clinical settings, metastasis is viewed as an inefficient process; of the many cancer cells which enter the bloodstream, very few go on to form secondary tumors. The events which contribute to this inefficiency are debated. A popular theory is that most cancer cells die in circulation, under hemodynamic shear forces. There is evidence, however, which challenges this paradigm. Direct analyses of the response of cancer cells to shear forces are lacking. Therefore, we designed an in vitro model of fluid shear stress, which allows high throughput analysis of various cell
types. In a broad panel of cancer cell lines, derived from various tissues, we found a remarkably conserved inducible shear stress resistance response. This response was absent in normal epithelial cells or non-transformed cell lines. Mechanistically, this response requires extracellular calcium and actin polymerization. These studies revealed a novel mechanism which may be necessary for progressive metastasis, and has practical implications in the study of circulating tumor cells.

To gain insight into the metastatic phenotype, we analyzed a panel of cancer cell lines derived from metastatic passage in mice. We noticed that derivative cells were physically smaller than their respective parental cell lines. Reduced cell size was correlated with attenuated activation of the mTOR pathway, and an increase in autophagic flux. Autophagy allows cells to digest their own proteins and organelles, and thus benefits cells residing in a nutrient-depleted environment. Our data suggest that autophagic cells are selected for in the metastatic microenvironment. Future directions aim to determine the role of autophagy in metastasis. Finally, we show that an aggressive subpopulation of prostate cancer cells exhibit stem cell-like features, which may be regulated by ZEB1.

In sum, these studies provide mechanistic details underlying the interactions of cancer cells with matrix and fluid microenvironments, which in turn affect migration, survival, and metabolism during metastasis.

Abstract Approved: __________________________________________
Thesis Supervisor

________________________________________
Title and Department

________________________________________
Date
INFLUENCE OF MATRIX AND FLUID MICROENVIRONMENTS ON CANCER
CELL MIGRATION, SURVIVAL, AND METASTASIS

by
James Matthew Barnes

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Molecular Physiology and Biophysics
in the Graduate College of
The University of Iowa

May 2011

Thesis Supervisor:  Associate Professor Michael D. Henry
CERTIFICATE OF APPROVAL

____________________________________

PH.D. THESIS

This is to certify that the Ph.D. thesis of

James Matthew Barnes

has been approved by the Examining Committee for the thesis requirement for the Doctor of Philosophy degree in Molecular Physiology and Biophysics at the May 2011 graduation.

Thesis Committee: ____________________________

Michael D. Henry, Thesis Supervisor

____________________________________

Scott Moye Rowley

____________________________________

Christopher Stipp

____________________________________

Mark Stamnes

____________________________________

Wayne Johnson
For my folks
ACKNOWLEDGMENTS

I would like begin by thanking all past and present members of the Henry Lab, whom with I have overlapped, for sharing their time, constructive criticism, and for their friendship. I need to pay special thanks to Dr. Justin Drake and Jones Nauseef for their close collaboration and our co-authorship efforts; Dr. Rob Svensson for daily discussions and many hours of help with animal experiments; Dr. Chris Stipp for helpful guidance, suggestions, and generous supply of materials and reagents; and Dr. Michael Henry for all of his patience, guidance, and enthusiastic mentorship, which I found contagious. Finally, I thank all of my friends and family that made my time at the University of Iowa a productive and fun period of my life.
TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................. vii
LIST OF FIGURES ................................................................................................................ viii
CHAPTER

I. INTRODUCTION ................................................................................................................. 1

Metastasis ........................................................................................................................... 1
Cancer cell extravasation ............................................................................................... 2
Cancer cell adhesion to the endothelium ........................................................................ 6
Epithelial-mesenchmal transition in metastasis .............................................................. 10
Metastatic inefficiency ..................................................................................................... 12
Fluid dynamics of blood flow .......................................................................................... 15
Circulating tumor cells in the clinic ................................................................................ 17
Autophagy and the metastatic microenvironment ............................................................. 19
Relevance and focus of my project ................................................................................ 21

II. ZEB1 COORDINATELY REGULATES LAMININ-332 AND B4 INTEGRIN EXPRESSION ALTERING THE INVASIVE PHENOTYPE OF PROSTATE CANCER CELLS .................................................. 27

Introduction ....................................................................................................................... 27
Materials and Methods .................................................................................................... 28
Antibodies and extracellular matrix proteins ............................................................... 28
Cell lines ............................................................................................................................ 29
Migration assays ............................................................................................................... 29
Quantitative RT-PCR (qRT-PCR) .................................................................................. 31
Flow cytometry ................................................................................................................. 31
Western blot ...................................................................................................................... 32
Chromatin immunoprecipitation (ChIP) ......................................................................... 33
Results ............................................................................................................................... 33
A metastatic subpopulation of PC-3 cells does not express pro- migratory laminin-332 ................................................................................................. 33
Laminin-332 and β4 integrin expression are repressed by ZEB1 .................................... 35
ZEB1 binds directly to LAMC2 and ITGB4 5’ regulatory elements .............................. 37
Laminins and cooperative cancer cell migration .............................................................. 38
Discussion ......................................................................................................................... 40

III. TRANSFORMED CELLS EXHIBIT CALCIUM-INDUCED RESISTANCE TO FLUID SHEAR STRESS ................................................................. 54

Introduction ....................................................................................................................... 54
Materials and Methods .................................................................................................... 56
Cell lines ............................................................................................................................ 56
Human and mouse blood ............................................................................................... 56
Cell size analysis .............................................................................................................. 57
Shear stress equations .................................................................................................... 57
Shear stress models ........................................................................................................ 58
Cell viability assays ........................................................................................................ 59
Flow cytomtery analysis of propidium iodide uptake .................................................60
Statistical analyses ........................................................................................................61
Results ..........................................................................................................................61

In vitro model of fluid shear stress induces cell death in a dose-dependent fashion ................61
Carcinoma cells of various histological origins exhibit unique resistance to fluid shear stress .................................................................63
Shear stress resistance in carcinoma cells is transient and inducible ...............65
Repeated exposure to shear stress results in changes in cellular membrane integrity and induced resistance to shear forces ................65
Induced shear stress resistance requires extracellular calcium .............................67
Induced shear stress resistance requires actin polymerization .............................68
Discussion ....................................................................................................................69

IV. IN VIVO DERIVATIVES OF METASTATIC CANCER CELL LINES EXHIBIT REDUCED mTOR SIGNALING, INCREASED AUTOPHAGY, AND REDUCED CELL SIZE ........................................86

Introduction ................................................................................................................86
Materials and Methods ..............................................................................................88
Cell lines and reagents ...............................................................................................88
Coulter Counter size analysis .....................................................................................88
Flow cytometry analysis of DNA content and cell size ............................................89
Western blot analysis .................................................................................................89
Immunofluorescence microscopy of LC3 puncta .....................................................90
Results .........................................................................................................................90

In vivo derivatives of metastatic cell lines are reduced in size .............................90
Cell cycle distribution does not correlate with cell size .......................................91
Metastatic derivatives exhibit reduced activity of mTOR signaling pathway ........................................................92
Autophagic flux is increased in metastatic derivatives of PC-3 and MDA.MB.231 cells ........................................................93
Epithelial-mesenchymal transition leads to decreased cell size and increased autophagic flux in MDCK cells .........................................................94
Discussion ..................................................................................................................95

V. CHARACTERIZATION OF AN AGGRESSIVE SUBPOPULATION OF PROSTATE CANCER CELLS WITH AN EPITHELIAL-TO-MESENCHYMAL AND STEM CELL-LIKE PHENOTYPE ........................................109

Introduction ..............................................................................................................109
Materials and Methods ............................................................................................111
Cell lines .....................................................................................................................111
Quantitative RT-PCR (qRT-PCR) ............................................................................111
Flow cytometry ..........................................................................................................112
Proliferation and soft agar colony formation assays ..............................................112
Trans-endothelial migration assay ............................................................................113
Metastatic colonization and xenograft implantation .............................................114
Results ......................................................................................................................115

TEM4-18 cells express high levels of the stem cell marker, LGR5 .....................115
Knockdown of LGR5 does not affect aggressive behavior of TEM4-18 cells ..........116
TEM4-18 cells exhibit a CD44high/CD24low profile ............................................116
Epithelial-mesenchymal transition is correlated with stem cell like traits in TEM4-18 cells ................................................................. 117
ZEB1 knockdown reduces colony formation and metastatic colonization in vivo ................................................................. 118
Discussion ........................................................................................................ 119

VI. SUMMARY AND FUTURE DIRECTIONS ........................................ 130

APPENDIX .................................................................................................. 150

REFERENCES ............................................................................................. 157
LIST OF TABLES

Table

3.1 Shear stress values for increasing flow rates. .........................................................74
4.1 Comparative Coulter Counter analysis of cell volume ..............................................100
5.1 TEM4-18 cells form xenografts more potently than PC-3E cells.........................128
LIST OF FIGURES

1. Long-term bioluminescence imaging of tail vein injected cancer cells.................25
2. Short-term serial bioluminescence imaging of tail vein injected cancer cells........26
3. Extracellular matrix deposition by PC-3 cells enhances TEM4-18 cell migration.................................................................44
2. Laminin-332 restores TEM4-18 transwell migration........................................45
3. Antibody inhibition of α3 integrin reduces TEM4-18 migration on laminin-332 coated inserts.................................................................46
4. Western blot analysis of ZEB1 knockdown in TEM4-18 cells .........................47
5. Silencing ZEB1 restores laminin β3 and γ2 chains of laminin-332 and β4 integrin..................................................................................48
6. Silencing ZEB1 restores transwell migration of TEM4-18 cells.......................49
7. Analysis of ZEB1 knockdown in Du145 cells ..................................................50
8. ZEB1 binds specifically to E-boxes within the 5’ regulatory regions of laminin γ2 and β4 integrin.................................................................51
9. ChIP analysis supplemental data ......................................................................52
10. Transwell migration of TEM4-18 cells is enhanced in the presence of PC-3E cells or extracellular matrix deposited by HMVEC cells ........53
3. In vitro model of fluid shear stress induces cell death in a dose-dependent fashion......................................................................................75
4. Clonogenic survival correlates with bioluminescence imaging viability data ......76
5. Viability comparison: needle and syringe vs. rheometer models of shear stress ....77
6. Carcinoma cells of various histological origins exhibit unique resistance to fluid shear stress...........................................................................78
7. Response of PC-3 cells to shear stress is similar over a range of time post- suspension.........................................................................................79
8. Shear stress analysis of a broad panel of cells .....................................................80
9. Shear stress resistance in carcinoma cells is transient and inducible .................81
3.8. The cell-impermeable dye, propidium iodide, accumulates in cells exposed to shear stress in a selective and diminishing manner, reflecting changes in cellular membrane integrity and induced-resistance to shear forces ................................... 82

3.9. Shear stress-induced cancer cell membrane repair requires presence of extracellular calcium ........................................................................................................ 83

3.10. Shear stress resistance response specifically requires calcium .................. 84

3.11. Shear stress resistance requires actin polymerization .............................. 85

4.1. Metastatic derivatives of several cancer cell lines are reduced in size ............ 101

4.2. Size analysis of 22Rv1 and PC-3 liver derivative cells .................................. 102

4.3. Differences in mean cell size are not reflected by differences in cell cycle progression ........................................................................................................ 103

4.4. mTOR activity is reduced in metastatic derivatives ....................................... 104

4.5. Rapamycin treatment reduces size of both parental and derivative cell lines. ... 105

4.6. Autophagic flux is increased in metastatic derivatives .................................... 106

4.7. LC3B puncta in metastatic derivatives of PC-3 cells ..................................... 107

4.8. Epithelial-mesenchymal transition in MDCK cells results in reduced cell size and increased autophagic flux .............................................................. 108

5.1. TEM4-18 cells express the stem cell marker, LGR5 ..................................... 122

5.2. Knockdown of LGR5 does not affect aggressive behavior of TEM4-18 cells ... 123

5.3. TEM4-18 cells exhibit a CD44^{high}/CD24^{low} profile ................................ 124

5.4. TEM4-18 cells express reduced levels of the stem-inhibiting miR-200 family of miRNAs ......................................................................................................... 125

5.5. Knockdown of ZEB1 leads to reduction in levels of LGR5 mRNA in TEM4-18 cells ........................................................................................................ 126

5.6. Knockdown of ZEB1 in TEM4-18 cells reduces soft agar colony formation to level of PC-3E cells .......................................................... 127

5.7. ZEB1 knockdown reduces metastatic colonization in vivo ............................. 129

6.1. A model for ETM and cooperative cell migration ........................................ 145

6.2. Spontaneous metastasis of TEM4-18 cells from orthotopic anterior prostate injections ........................................................................................................ 146

6.3. Histology of PC-3E and TEM4-18 orthotopic tumors ..................................... 147

6.4. Induced shear stress resistance requires Rho-GTPase activity ....................... 148
6.5. Shear stress results in changes in stress-activated protein kinase activity............149
A.1 Proof-of-principle using a fusion-dependent vector ........................................152
A.2. FACS fusion-detection limits ........................................................................153
A.3. A schematic for the use of a fusion-dependent vector.......................................154
A.4. Lack of detectable fusion in pilot studies .........................................................155
Metastasis

Metastasis, or the spread of cancer cells from a primary tumor to a distant tissue, is associated with the lethal consequences of cancer progression. Metastasis is comprised of a series of events, beginning with local tissue invasion, followed by entrance into the bloodstream or lymphatic system, survival within circulation, extravasation (exit from the vasculature), and, finally, colonization of and proliferation at a secondary organ site.

Extravasation remains a relatively poorly understood process in part because tracking the fate of individual cancer cells within circulation and organ vascular beds remains a technically daunting task. Nonetheless, both in vivo and ex vivo analyses have provided insight into the cellular and molecular mechanism of cancer cell extravasation. Although leukocyte diapedesis has provided an instructive paradigm for understanding cancer cell extravasation, the latter exhibits numerous differences and is likely both cell-type and organ-specific. Below, we discuss the mechanisms underlying cancer cell extravasation and focus on another aspect of this process which remains a matter of debate: the extent to which metastatic efficiency is dictated by cancer cell extravasation.

Epithelial-to-mesenchymal transition (EMT) is a process by which a polarized epithelial cell undergoes morphological changes resulting in a mesenchymal phenotype. A growing body of evidence indicates that EMT contributes to metastatic invasion, but the role of EMT in the subsequent steps of metastasis remains unclear. Recent data suggest that a combination of cancer cells with EMT-like features and those with an epithelial phenotype cooperate to lead individual cells from a primary tumor into the bloodstream and eventually to a secondary tissue (Tsuji, Ibaragi et al. 2008); similar
results have been generated using \textit{in vitro} models of cancer cell migration (Drake, Barnes \textit{et al.} 2010). EMT-like traits have also been shown to augment the ability of prostate cancer cells to extravasate \textit{in vitro}, in part through modulating interactions between cancer cells and endothelial cells (Drake, Strohbehn \textit{et al.} 2009). Collectively, these findings indicate that the metastatic behavior of EMT-like cells is contextually dependent on the surrounding microenvironment. A better understanding of the role of EMT in individual steps of metastasis may have an impact on the analysis of circulating tumor cells (CTCs), as well as provide potential therapeutic targets to forestall metastatic disease.

CTCs can be detected in the blood of cancer patients. The isolation and quantification of these cells provides exciting possibilities for prognostic advances. Experimental studies on the survival and fate of CTCs have led to dramatically different conclusions. Early work shows that the majority of cancer cells entering the bloodstream are cleared rapidly under hemodynamic shear stress (Fidler 1970) and mechanical deformation in the microvasculature (Weiss, Nannmark \textit{et al.} 1992). In contrast, there are data from several groups which suggest that most cancer cells are relatively undamaged by the process of circulation and that extravasation occurs efficiently once these cells arrest in microvascular beds (Koop, MacDonald \textit{et al.} 1995; Luzzi, MacDonald \textit{et al.} 1998). A better understanding of the survival and behavior of cancer cells in circulation will have an important impact on the clinical analysis of CTCs and also provide insight into the rate-limiting steps of metastasis which may prove to be vulnerable targets of therapy.

\textbf{Cancer cell extravasation}

In order for CTCs to form metastatic tumors, they must exit the bloodstream and enter the parenchyma of a secondary tissue. Extravasation involves the passage of cells
across the endothelium, termed transendothelial migration (TEM), as well as transit through the subendothelial basal lamina. Several mechanisms of cancer cell extravasation have been reported, and this variation may be due in part to differences in experimental models. Results from many groups support a model by which cancer cells adopt certain leukocyte-like properties, allowing them to adhere to endothelial cells by specific and dynamic cell surface interactions, thereby facilitating passage between or through the endothelial cells and through the underlying basement membrane. Others report static, nonselective mechanisms of cancer cell adhesion followed by either TEM, often involving retraction of endothelial cells, or proliferation within the lumen of the vessel.

Leukocyte extravasation is a highly regulated process which allows immune cells to home to lymphatic and other tissues throughout the body. The cellular and molecular biology of leukocyte extravasation is generally well understood and has been reviewed recently (Vestweber 2007; Friedl and Weigelin 2008; Nourshargh, Hordijk et al. 2010). Each step of leukocyte extravasation is mediated by specific combinatorial interactions between a receptor and a ligand. Temporal and spatial regulation of some of these molecules dictates the selectivity of a leukocyte for a given tissue. Selectins, for example, are surface glycoproteins expressed on the endothelium which initiate rolling of leukocytes by binding to various selectin ligands on the surface of these cells. While rolling, leukocytes encounter a gradient of chemokines presented by endothelial cells. Subsequent activation of chemokine receptor signal transduction facilitates changes in the affinity and avidity of leukocyte integrins for their endothelial ligands, the intercellular adhesion molecules (ICAMs). This results in integrin-mediated firm adhesion of leukocytes which is followed by diapedesis.

Cancer cells of various histological origins have been shown to express cell surface proteins known to be important in leukocyte extravasation, including cell adhesion and chemokine receptors which interact with corresponding ligands at the luminal surface of the endothelium. These protein pairs have been implicated in the organ
tropism of metastasis as recently reviewed (Miles, Pruitt et al. 2008; Strell and Entschladen 2008). As an example, bone marrow endothelial cells facilitate adhesion of circulating leukocytes by constitutive expression of the chemokine SDF-1 and the adhesion molecules VCAM-1 and E-selectin (Kopp, Avecilla et al. 2005). Numerous reports indicate that a variety of cancers express the SDF-1 receptor, CXCR4 (Zlotnik 2006). Antibody neutralization of CXCR4 has been shown to greatly reduce metastatic colonization by breast cancer cells in mice (Muller, Homey et al. 2001). In a panel of prostate cancer cells, CXCR4 expression levels were shown to correlate with metastatic aggressiveness, and targeting this receptor with a small molecule, T140, reduced chemotactic invasion of PC-3 human prostate cancer cells towards SDF-1 (Hart, Brown et al. 2005). Extravasation of PC-3 cells has also been shown to be facilitated by expression of monocyte chemoattractant protein-1 on bone marrow endothelial cells (van Golen, Ying et al. 2008). In vitro-derived bone-tropic clones of the MDA.MB.231 cell line have been shown to overexpress CXCR4 as compared to the heterogeneous parent (Minn, Kang et al. 2005). Recently Jamieson et al. detected overexpression of the monocyte chemokine receptor, CX3CR1, in human prostate cancer tissue samples and showed that its ligand, fractalkine, is expressed on the plasma membrane of osteoblasts, mesenchymal cells, and other stromal cells of the bone marrow (Jamieson, Shimizu et al. 2008). Furthermore, dihydrotestosterone induces cleavage of stromal, but not endothelial plasma membrane-associated fractalkine; consequent establishment of a soluble chemotactic gradient promotes prostate cancer cell homing to bone and ultimately adhesion to the bone marrow endothelium (Jamieson, Shimizu et al. 2008). Forced expression of the E-selectin ligand, sialyl Lewis X, in PC-3 cells has been shown to induce cell rolling and adhesion to IL-1β-stimulated bone marrow endothelial cells in vitro, an interaction blocked by anti-E-selectin antibody (Barthel, Wiese et al. 2009). These and other studies support a role for leukocyte extravasation molecules and activated endothelial cells in the bone tropism of cancers such as breast and prostate.
However, chemokine signaling may play roles relevant to metastasis beyond extravasation (Luboshits, Shina et al. 1999; Burger and Kipps 2006).

Beyond the leukocyte paradigm, N-cadherin is an example of an adhesion molecule implicated in cancer cell extravasation, which is not known to be involved in leukocyte diapedesis. N-cadherin is well-positioned to be involved in the association between cancer cells and endothelial cells as it is present on the luminal surface of endothelia and not enriched at cell junctions (Salomon, Ayalon et al. 1992). Melanoma cells have been shown to adhere to endothelial cells via homophilic N-cadherin binding which leads to retraction of the VE-cadherin and PECAM junctions between neighboring endothelial cells (Voura, Sandig et al. 1998). Engagement of melanoma cell N-cadherin leads to transactivation of Src kinase and nuclear localization of β-catenin, which facilitate the formation of pseudopodia into the disrupted endothelial cell junction and subsequent TEM to the basal side of the endothelium (Qi, Chen et al. 2005; Qi, Wang et al. 2006). Similarly, siRNA-mediated knockdown of N-cadherin in PC-3 cells was shown to reduce TEM (Drake, Strohbehn et al. 2009). Although the signaling consequences of N-cadherin binding were not addressed here, PC-3 TEM has been shown to involve Rac1-mediated invadapodia formation (Sequeira, Dubyk et al. 2008; van Golen, Ying et al. 2008).

Most of the in vitro studies of extravasation discussed above are performed by seeding cancer cells onto a monolayer of endothelial cells growing on a semi-permeable transwell insert or on a layer of extracellular matrix proteins. While this provides a convenient model for extravasation, tumor cells in vivo are circulating in blood. To more accurately mimic physiological conditions, dynamic in vitro models of extravasation have been used in which mixtures of cancer cells and leukocytes are subjected to flow through chambers lined with endothelial monolayers. Using this approach, evidence has been provided for cooperation between leukocytes and cancer cells in extravasation. Strell et al. showed that breast cancer cells, which lack β2-integrin but express its ligand
ICAM-1, bind to β2-expressing neutrophils to form a hetero-cellular complex which then binds to the endothelial cells via ICAM-1 (Strell, Lang et al. 2007). These results are in agreement with other reports of neutrophil-assisted extravasation of breast cancer cells (Wu, Wang et al. 2001) and melanoma cells (Dong, Slattery et al. 2005). Platelets have also been shown to bind to cancer cells of various types, often through selectin-mediated interactions, leading to formation of emboli which exhibit enhanced adhesion to the endothelium (Warren and Vales 1972; Crissman, Hatfield et al. 1988; Borsig, Wong et al. 2002; Camerer, Qazi et al. 2004). It may be the case that certain cancer cells are actually dependent upon leukocyte and/or platelet interactions in order to form metastatic tumors, which is interesting considering the growing understanding of the role of inflammation in cancer progression and metastasis (Grivennikov, Greten et al. 2010).

Although leukocytes provide an attractive mechanistic paradigm for understanding cancer cell extravasation, the selective pressures which would drive epithelial cells to acquire detailed leukocyte gene expression patterns and cellular behavior are not obvious. The process of cancer cell extravasation is not known to follow a strict paradigm and has been reported to occur by a number of mechanisms, which appear to be both cell type- and organ-specific. One feature of cancer cell extravasation, observed primarily in vitro, is that cancer cells, unlike leukocytes, induce the retraction of endothelial cells (Kramer and Nicolson 1979). There are even alternatives to classical extravasation, such as expansive intraluminal proliferation, which have been proposed as a means for metastatic colonization (Al-Mehdi, Tozawa et al. 2000).

**Cancer cell adhesion to the endothelium**

Many studies on extravasation have focused on the mechanisms of tumor cell adhesion to the endothelium, as this is presumed to be the initiating event in this process. Here, intravital videomicroscopy and detailed accounting of cancer cell fate following
systemic injection have provided important insights into the process of extravasation in vivo. Two general mechanisms are thought to be involved: 1) non-selective and passive lodgment of cancer cells in the microvasculature by size restriction and 2) selective adhesion between cancer cells and the vasculature of particular organ sites, including larger diameter vessels. Although these mechanisms are often regarded to be in conflict, both size restriction and selective adhesion could influence the overall process of extravasation. Determining the relative contribution of these mechanisms will be important in potential therapeutic contexts.

In the 1920s, James Ewing proposed that CTCs become entrapped in the first capillary beds they encounter, thus contributing to organ-site preference for metastasis (Ewing 1928). Intuitively, this mechanism derives from the fact that epithelial-derived cancer cells are typically larger than the largest of the white blood cells (human monocytes range from about 12 to 20 micrometers in diameter (van der Meer, van de Gevel et al. 1982)). Although CTCs are deformable and may thus be able to negotiate this restrictive barrier to some degree, they may not be as successful as leukocytes (Vona, Sabile et al. 2000; Zheng, Lin et al. 2007). CTCs would thus seem susceptible to size restriction when encountering the microvasculature (human capillaries are as small as 7 micrometers in diameter (Doerschuk, Beyers et al. 1993)). Indeed, it has long been appreciated in experimental mouse models of metastasis that cancer cells injected into the tail vein accumulate predominantly in the lung (Fidler 1970). Here, this is demonstrated utilizing bioluminescence imaging (Figure 1.1). Early efforts aimed at observing the behavior of CTCs in the microvasculature of the rabbit ear described the association of cancer cells with the endothelium and subsequent extravasation, but did not conclude that size restriction was the sole means of cancer cell arrest at this site (Wood 1958). However, the pioneering work of Ann Chambers, Alan Groom, and colleagues utilizing in vivo video microscopy have provided abundant support for the concept of size restriction in the lung and other organs (Chambers, Schmidt et al. 1992; Morris,
MacDonald et al. 1993; Morris, Koop et al. 1994; Koop, MacDonald et al. 1995; Koop, Schmidt et al. 1996; Luzzi, MacDonald et al. 1998). These studies also strongly challenged the idea that metastatic efficiency is limited by death of CTCs due to hemodynamic shear forces.

Recently, monitoring circulating lung carcinoma and melanoma cells in the brains of living mice via multi-photon microscopy over an extended time course, Kienast et al. have stunningly visualized detailed, individual steps of extravasation (Kienast, von Baumgarten et al. 2010). Minutes after injection, all adherent cells were reported to be arrested by size-restriction in vessels of similar diameter to the cancer cells or in vascular branch points. Some cells were observed dislodging from their original point of arrest only to arrest in another microvascular branch point for periods of up to 48 hours. Although intraluminal proliferation was observed, colonies of cells were never witnessed rupturing through the vascular wall and often would regress (Kienast, von Baumgarten et al. 2010). It is possible that the architecture of the brain vasculature is non-permissive to this type of metastatic colonization. Alternatively, it is possible that by remaining in the lumen of a blood vessel, these cells die due to constant exposure to shear forces and pressures imparted by circulation.

Although it can be experimentally demonstrated that the majority of cancer cells injected into the tail vein are retained in the lung following injection, extrapulmonary tumors do develop in some tumor models. PC-3 cells injected intravenously colonize tissues such as the adrenal gland, brain, liver, lung, and kidney (Drake, Strohbehn et al. 2009). Similar results were observed after tail vein injections of DU-145 prostate cancer cells (Glinsky, Glinsky et al. 2001).

β3 integrins have been implicated in cancer cell transendothelial migration, although these are likely to engage ligands in the subendothelial matrix (Wang, Ferreira et al. 2005; Bauer, Mierke et al. 2007). Wang et al. found that blocking α3 and β1 integrin subunits on human fibrosarcoma cells leads to significant reduction of CTC
arrest in the lung vasculature following renal injection in mice (Wang, Fu et al. 2004). Similar results were obtained when infusing blocking antibodies to laminin-332 into mice prior to cancer cell injection. It was concluded that there are small, naturally occurring foci of exposed basement membrane throughout the lung vasculature, exposing laminin-332 to integrins on CTCs, facilitating adhesion (Wang, Fu et al. 2004). However laminin-332 is not normally a component of the subendothelial basement membrane (Hallmann, Horn et al. 2005), so details of this mechanism remain to be explained. The role of laminins and subendothelial matrix proteins in cancer cell migration and extravasation is discussed further below.

To determine if metastatic potential correlates with the ability of cancer cells to adhere to and extravasate through the endothelium, colon cancer cell lines of low, medium, and high aggressiveness were monitored via intravital fluorescence microscopy following systemic injection into Sprague-Dawley rats (Schluter, Gassmann et al. 2006). Regardless of metastatic potential, each cell line bound efficiently to the endothelium of the two most common tissues of colon cancer metastasis, the liver and lung. In less common sites of colon cancer metastasis, including kidney, intestine, skin, muscle, and spleen these cell lines all adhered poorly. Metastatic potential did, however, correlate with the frequency of extravasation and tissue invasion in the lung and liver. These data support the argument that metastatic tumor formation reflects the efficiency of extravasation.

*Ex vivo* fluorescence imaging of isolated perfused lung preparations from mice injected systemically with human and rat fibrosarcoma cells revealed attachment of cancer cells to the endothelial walls of the pre-capillary arterioles, which are much larger in diameter than the cancer cells themselves. Furthermore, these arterioles remained perfusable, showing that the cancer cells had not occluded the lumens of the microvasculature. These data provide support for a model in which specific interactions between cancer and endothelial cells facilitate adhesion (Al-Mehdi, Tozawa et al. 2000).
An unexpected finding of this work was that adherent fibrosarcoma cells rarely extravasated and that most viable cells began to proliferate within the lumen of the blood vessel, leading to colony formation which caused enlargement of the capillaries. Additional support for intravascular proliferation was obtained using this same imaging approach with lungs from mice with subcutaneous breast cancer tumors (Wong, Song et al. 2002). These experimental data support a longstanding hypothesis that intraluminal proliferation and subsequent colony growth through the vessel wall can lead to metastatic colonization in humans (Iwasaki 1915; Takahashi 1915; Crissman, Hatfield et al. 1985).

**Epithelial-mesenchymal transition in metastasis**

EMT is an inducible and reversible process by which a polarized epithelial cell undergoes morphological changes resulting in a mesenchymal phenotype. EMT is characterized by loss of functional epithelial markers, such as E-cadherin and cytokeratin, and gain of mesenchymal markers, such as N-cadherin and vimentin. EMT is often accompanied by increases in protease secretion and large changes in the production of extracellular matrix and basement membrane proteins. These changes result in loss of epithelial polarity and the acquisition of migratory and invasive characteristics. This process is highly regulated both spatially and temporally during embryogenesis (Thiery, Acloque et al. 2009) and also plays a role in the processes of inflammation and wound healing (Thiery 2003). Recent evidence has shown that EMT contributes to the progression of solid tumors by permitting detachment of cells from their primary site and by inducing a migratory phenotype, allowing the cell to invade local tissue and enter the lymphatic system or bloodstream (Thiery 2002). Despite a large number of publications supporting a role for EMT in tumor progression and metastasis, information linking EMT and cancer cell extravasation is lacking. Furthermore, in the field of cancer, definitions of EMT have become ambiguous. It is important to delineate between inducer-driven EMT
(i.e. TGFβ treatment of MDCK cells shown in Chapter IV) versus more stable EMT-like states (TEM4-18 cells, as an example; Drake and Strohbehn et al. 2009).

To identify novel genes involved in cancer cell extravasation, Drake et al. serially passaged PC-3 cells through a monolayer of human lung endothelial cells seeded onto transwell inserts and isolated a subpopulation coined TEM4-18. Compared to the PC-3 input, these cells were enhanced in TEM and were shown to colonize tissues at a significantly higher rate in SCID mice. Furthermore, TEM4-18 cells exhibited a stable EMT-like morphology and near loss of E-cadherin mRNA and protein. Microarray analysis showed upregulation of a zinc-finger homeodomain transcription factor, Zeb1, in TEM4-18 cells (Drake, Strohbehn et al. 2009). Zeb1 had previously been implicated in EMT in developmental and pathological contexts (Spaderna, Schmalhofer et al. 2008; Vandewalle, Van Roy et al. 2008). shRNA-mediated knockdown of Zeb1 in TEM4-18 cells resulted in a partial reversal of EMT, including restoration of E-cadherin and epithelial morphology, and reduction of TEM (Drake, Strohbehn et al. 2009). These data provide evidence that repressors of an epithelial phenotype, such as Zeb1, facilitate extravasation of prostate cancer cells.

However, other studies suggest that cells bearing epithelial characteristics are capable of metastasis. To study the role of EMT in individual steps of metastasis in vivo, Tsuji et al. compared the invasive and metastatic properties of normal epithelial and EMT-like (p12CDK2-AP1-driven) hamster cheek pouch carcinoma cells. Both cell lines formed subcutaneous tumors at a similar rate, but only the EMT cells invaded local tissue and could be detected in the blood, consistent with previous reports of EMT-driven invasion (Tsuji, Ibaragi et al. 2008). When these cell lines were injected intravenously into mice, only the wild-type epithelial cells gave rise to lung tumors, suggesting that the EMT-like cells are incapable of metastatic colonization. When the two cell lines were differentially labeled, mixed, and injected subcutaneously, lung tumors formed which only consisted of wild type cells and, although EMT-like cell DNA was readily
detectable in blood, none was detected in lung tumors. These data suggest that cells with an EMT phenotype are responsible for early events in metastasis, namely invasion and intravasation, and allow neighboring epithelial tumor cells to enter the circulation. During later steps in this model, it appears that only the epithelial cells are able to extravasate and proliferate at a secondary tissue, although extravasation, *per se* was not evaluated. These studies are in agreement with reports that epithelial-like MDA.MB.231 cells are enhanced in metastasis from a subcutaneous site when mixed with mesenchymal stem cells than on their own but that the resulting metastatic tumors do not contain detectable mesenchymal cells (Karnoub, Dash *et al.* 2007). As described below, work by Podsypanina *et al.* provides evidence that epithelial cells are capable of lung colonization (Podsypanina, Du *et al.* 2008), although it is difficult to ascertain whether these cells had, in fact, extravasated in the lung microvasculature. It is possible that epithelial cells have a propensity for intravascular colonization and proliferation, while EMT traits facilitate trans-endothelial migration.

**Metastatic inefficiency**

As mentioned above, early studies on the fate of circulating tumor cells led to the observation that after systemic injection, cells became arrested at the first capillary bed encountered (Fidler 1970; Weiss, Nannmark *et al.* 1992). For example, cells injected via the tail vein would arrest in the lung, and those injected into the hepatic portal vein would arrest in the liver. A sharp drop in cell viability in these experiments occurred immediately after injection, with as many as 99% of cells dying within 24 hours (similar observations are documented here in *Figure 1.2*). It was concluded that the majority of these cells died due to hemodynamic shear stress and by mechanical rupture following size restriction in the microvasculature (Fidler 1970; Weiss 1991). Of the small percentage of surviving cells, fewer yet gave rise to metastatic lesions. Thus, great
emphasis was placed on the ability of CTCs to extravasate and subsequently proliferate in order to successfully colonize a tissue. These observations introduced the concept of metastatic inefficiency, for which there is direct clinical evidence, as discussed below. To date, debate continues over which steps of metastasis contribute most to the inefficiency of this process.

Recently, there has been considerable interest in isolating and quantifying CTCs to develop new prognostic and predictive tools (this is discussed in more detail in a section below). One of the largest challenges here is that the mere presence of CTCs in the blood of patients does not always correlate with poor prognosis or metastasis. As an example, low numbers of circulating cells prior to surgery was shown to be predictive of relapse-free survival in breast cancer patients (Cristofanilli, Budd et al. 2004); and surgeries to remove non-small cell lung carcinoma have been reported to cause increased numbers CTCs in patients, which correlates with relapse (Rolle, Gunzel et al. 2005). Conversely, patients with ovarian or colon cancer who have surgical venous shunts, which introduce many (estimated up to millions) cancer cells into the blood every day, rarely develop metastatic disease (Tarin, Price et al. 1984; Tarin, Price et al. 1984) and disseminated cells are capable of dormancy for periods upwards of decades (Karrison, Ferguson et al. 1999). These data call into question the fate of CTCs which do not complete all steps of metastasis. At the simplest level, it is likely that these cells are either eventually killed in the bloodstream or that once colonizing a secondary tissue that they are unable to proliferate sufficiently to develop into a metastases.

Koop et al. took a quantitative approach to measure the rate-limiting steps of metastasis by adding inert microspheres to suspensions of B16 melanoma cells and injecting these systemically into chick embryos. The cell:sphere ratio was monitored over time to determine the percentage of viable cells at each step of metastasis. Twelve to twenty-four hours post-injection 85% of the melanoma cells survived circulation and had extravasated (Koop, MacDonald et al. 1995). Strikingly similar results were recapitulated
by using the same method of cell accounting in a mouse model following injection into the mesenteric vein (Luzzi, MacDonald et al. 1998). Interestingly, this latter study showed that although the majority of injected cells extravasated, only 0.07% led to micrometastases and 0.02% gave rise to tumors. Because B16F10 cells are a highly aggressive cell line, the question of whether less metastatic cell lines could extravasate was addressed. Using the chick chorioallantoic membrane model, ras-transformed or wild type NIH/3T3 cells, as well as wild type mouse embryonic fibroblasts, were compared for their extravasation potential. No differences were found between the three cell lines, as all were able to successfully extravasate, but only the transformed cells were capable of tumor formation (Koop, Schmidt et al. 1996). These are among the seminal reports arguing that although extravasation is necessary for metastatic tumor formation, it is an efficient step and not related to the overall aggressiveness of the cancer cell. These findings supported the idea that the ability to survive and proliferate in the parenchyma of a secondary tissue ultimately dictates the efficiency of metastasis.

Although non-transfomed cells were analyzed in the studies above, no effort was made to look at the survival and extravasation of normal epithelial cells, which would make a good comparator for the highly malignant melanoma cell lines. Results discussed in Chapter III argue that non-tumorigenic epithelial cells would be incapable of survival after systemic injection; however work from Podsypanina et al. may challenge these expectations. Mice were injected via tail vein with primary mouse mammary cells containing a doxycycline-dependent promoter driving the expression of the oncogenes MYC and Kras\textsuperscript{D12}. At 1.5, 8, and 17 weeks post-injection, mice were placed on either a control or doxycycline diet and in all cases, pulmonary tumor growth was observed in the doxycycline-fed animals using bioluminescence imaging. Intriguingly, although no tumors formed in the control-diet mice, histological analysis of lung tissue showed that in the absence of oncogene expression, these control cells had colonized the lung tissue, were viable, and mitotically active. These findings suggest that non-tumorigenic cells are
capable of surviving circulation, extravasation, and colonization but secondary tumor
growth requires oncogenic transformation (Podsypanina, Du et al. 2008). If these cells
had experienced higher magnitudes of shear forces, such as those in the arterial system,
perhaps selective clearance of non-tumorigenic cells would have been observed.

In contrast to these data showing extravasation to be a common, efficient trait of
cancer cells, recent studies demonstrate that extravasation of a particular breast cancer
cell type occurs at different rates in lung versus liver. Six hours after injection, two-
photon microscopy revealed that most cells were arrested in the lumen of the
microvasculature in both organs. However, at 24 hours post-injection, over half of the
cancer cells had extravasated into liver parenchyma, whereas less than one quarter had in
the lung (Martin, Kremers et al. 2010). Thus, experimental determination of the extent to
which extravasation contributes to metastatic inefficiency may depend on: 1) the cancer
cell type involved, as melanoma cells appear to be an overall more aggressive lung-
metastatic cell than many carcinomas; 2) the organs in which extravasation is being
studied, as structural differences at the vascular level likely have a large impact on the
permissiveness of tumor cell adhesion and extravasation; and 3) the imaging technique
being used to qualify extravasation, as even with high resolution fluorescent imaging,
delineation of intravascular versus extravascular location of cancer cells can be very
challenging, especially in capillary-dense tissues such as the lung. For these same
reasons, the challenge of continuous monitoring of viability and membrane integrity of
cancer cells in the circulation in vivo makes the study of CTC survival difficult.

Fluid dynamics of blood flow

The topic of circulating tumor cells and metastatic inefficiency can be more
completely appreciated when considering the involvement of fluid dynamics. The
constant movement of blood through vessels imparts many dynamic forces, most notably,
pressure differentials and shear force. Shear force is a parallel frictional force exerted on the surface area of an object. Shear stress in the circulation is usually discussed in terms of wall shear stress (WSS) which is constantly applied to the endothelial cells lining the vascular wall. WSS can be calculated using the equation $\tau = 4Q \eta/\pi r^3$ (Davies 2009) where $\tau$ is shear stress in dyn/cm$^2$, $Q$ is flow rate in cm$^3$/s, $\eta$ is the viscosity of the fluid (the viscosity of blood is often reported to be 3.5 dyn/cm$^2$/s), and $r$ is the radius of the vessel (from ~3 cm at the aorta to <5 $\mu$m at the capillary level). As derived from the equation, WSS is inversely correlated with the cube of vessel radius; thus there is an enormous range of shear stresses throughout the vasculature (1-10$^5$ dyn/s corresponding to vessel radii of ~5 $\mu$m-3.5 cm) (Reneman, Arts et al. 2006; Cheng, Helderman et al. 2007; Schneider, Nuschele et al. 2007).

Shear stress is a major attribute of the vascular microenvironment and has important biological implications; for example, endothelial cells are fine-tuned to shear stress, and variations in the magnitude or frequency of shear forces impact the signaling, gene expression, and survival of these cells (Malek and Izumo 1992; Malek, Alper et al. 1999). A well observed phenomenon is that endothelial cells will morphologically align in the direction of fluid flow (Dewey, Bussolari et al. 1981). It is hypothesized that by elongating in line with blood flow, endothelial cells reduce the fluctuations of shear force that they experience. Considering the interplay between WSS and endothelial cell biology, it is not surprising that aberrant changes in WSS levels and in the nature of blood flow (turbulent vs. laminar) are associated with atherosclerosis (Fry 1969; Caro, Fitz-Gerald et al. 1971).

Blood flow and shear stress also have a major influence on the behavior of red blood cells (RBCs). Changes in pressure have a marked effect on the shape and velocity of RBC flow, as well as their location within a blood vessel lumen (flow through small diameter, high shear vessels tends to lead to accumulation of cells along axis of blood flow) (Kamm 2002). RBCs (roughly 8 $\mu$m in diameter) often encounter capillaries of
smaller diameter and thus must significantly deform in order to pass through. Although RBCs are “built” for circulation, at high enough shear forces, they will be torn apart (Abkarian, Faivre et al. 2008). Glandular epithelial cells, from which adenocarcinomas are derived, reside in environments with much lower shear stress than found in the bloodstream (Kamm 2002). It is thus reasonable to believe such cells would be particularly susceptible to destruction by hemodynamic shear forces, as compared to naturally circulating cells.

Although the majority of the research on fluid biomechanics has been focused on RBCs and endothelial cells, shear stress has also been shown to induce changes in the gene expression and adhesive properties of non-RBC circulating cells, including leukocytes and cancer cells (Okuyama, Ohta et al. 1996; Thamilselvan and Basson 2005; Avvisato, Yang et al. 2007; Stroka and Aranda-Espinoza 2010). It is possible that cancer cells have adapted mechanisms typically used by leukocytes and/or endothelial cells to respond to and survive in the hemodynamic shear stress environment of circulation.

Circulating tumor cells in the clinic

A more thorough understanding of cancer cell extravasation and survival in circulation is likely to impact efforts to better predict the likelihood of occult metastasis and perhaps to inhibit metastasis. By definition, CTCs represent the precursor to extravasating cancer cells, and as the forgoing discussion illuminates, their biology is linked. The isolation and quantification of CTCs provides promise as a prognostic and predictive tool in the clinic. Early CTC studies successfully showed correlation of CTC number with poor prognosis in breast cancer patients (Cristofanilli, Budd et al. 2004; Hayes, Cristofanilli et al. 2006). Longitudinal monitoring of CTCs during therapy of metastatic breast cancer patients was also shown to be prognostic of progression-free and overall survival (Hayes, Cristofanilli et al. 2006). These findings encourage clinicians
that CTC quantification may be able to replace testing of disseminated tumor cells (DTCs). DTCs have been identified in the bone marrow of patients with breast, colon, and prostate cancer and have been shown to correlate with poor prognosis and cancer progression (Fehm, Sagalowsky et al. 2002; Morgan, Lange et al. 2009), however isolation of bone marrow is painful and complicated compared to drawing a blood sample. Most current methods of CTC isolation rely on the physical selection of epithelial cells by exploiting their surface expression of epithelial cellular adhesion molecule (EpCAM) (Fehm, Muller et al. 2008). However, with growing support for EMT in tissue invasion and intravasation, it is possible that some CTCs have undergone at least a partial EMT. Such cells will have reduced expression or possible loss of EpCAM, thus potentially important CTCs will be missed entirely by standard isolation approaches. Isolation of CTCs by targeting mesenchymal markers, such as N-cadherin, alongside EpCAM may provide a more powerful prognostic outcome than standard approaches. Furthermore, molecular analysis of CTCs may provide information on the ability of these cells to extravasate and form metastatic tumors; such information would help tailor patient-specific therapies.

One may question whether there is any therapeutic utility in blocking cancer cell extravasation. From the reviewed material it is apparent that a number of cell adhesion and intracellular signaling molecules may be viable targets for inhibiting cancer cell extravasation. To the extent that these targets overlap with those involved in leukocyte trafficking, one might anticipate side effects due to leukocyte dysfunction. In this regard, one particularly interesting target is N-cadherin which may not be involved in leukocyte trafficking and for which antibody and inhibitory peptides already exist (Li, Price et al. 2007; Tanaka, Kono et al. 2010). However, since metastatic dissemination may be an early event, possibly occurring prior to initial diagnosis, whether an opportunity exists to intervene in this step of metastasis is unclear. If secondary metastasis (metastasis not from the primary tumor) plays an important role clinically, blockade of extravasation
may benefit patients who already have disseminated disease. Alternatively, there may be situations such as in the peri-operative state in which large numbers of cancer cells are liberated into the bloodstream for which temporary inhibition of cancer cell extravasation may be useful.

Finally, recent unpublished studies from our laboratory, described in Chapter III, show that in an in vitro model of CTC flow that transformed epithelial cells of various tissue origins are uniquely resistant to shear stress. This observation was independent of the metastatic potential of cancer cells. Primary or non-transformed cells in this model are susceptible to shear-induced death at much lower magnitudes than those required to damage cancer cells. An optimistic application of these findings is that tumorigenic cells could be purified from standard CTC preparations by subjecting them to our shear stress model. By eliminating normal, non-cancerous epithelial cells, the prognostic value of CTC samples may be improved. As discussed in the metastatic inefficiency section, these findings underscore the importance of performing post-test analyses of isolated CTCs to more accurately determine patient prognosis.

**Autophagy and the metastatic microenvironment**

When, and if, a CTC colonizes a secondary tissue, it faces additional barriers to survival and growth. Compared to the site of the primary tumor (i.e. at the prostate, breast, or colon), the microenvironment of a newly colonized tissue is often nutrient-depleted, hypoxic, and contains basement membrane and extracellular matrix proteins foreign to the tumor cell (Chambers, Groom et al. 2002). Collectively, these factors put a great deal of selective pressure on the newly colonized cell. One mechanism which may help cancer cells cope with their metastatic microenvironment is autophagy.

Autophagy is a catabolic process in which a cell utilizes its own cytoplasmic proteins and organelles as an energy source. This mechanism has recently been reviewed
excellently (Rabinowitz and White 2010). Autophagy is finely regulated by the serine/threonine kinase, mTOR, which acts as a molecular sensor of nutrient availability; when extracellular nutrients (amino acids, glucose, etc) are in abundance, mTOR is activated by the PI3K/AKT signaling pathway, which may be engaged by stimulus such as insulin binding to its receptor at the cell surface. Once activated, mTOR phosphorylates S6 kinase and 4E-BP, as well as other targets, resulting in increased ribosomal biogenesis, translation, and, ultimately, cell size and proliferation (Wullschleger, Loewith et al. 2006). When extracellular nutrients become limiting, PI3K/AKT signaling is reduced, allowing induction of autophagy. Recently, induction of autophagy has been shown to occur in a HIF-1α-dependent pathway in response to hypoxia in fibroblasts (Zhang, Bosch-Marce et al. 2008; Chiavarina, Whitaker-Menezes et al. 2010). It is intuitive that such a mechanism would benefit cancer cells which have colonized a nutrient-deprived and avascular tissue site.

An early and necessary event during metastasis is cell-detachment from the basement membrane (Weiss and Ward 1983). Mechanisms such as EMT facilitate this process (see above), allowing cells to migrate away from their primary tumor and eventually enter circulation. In normal epithelial cells, such detachment leads to a specialized type of apoptosis, called anoikis, preventing the detrimental consequences of unregulated cell migration (Frisch and Francis 1994). Experimental inhibition of anoikis in non-transformed epithelial cells has been shown to facilitate metastasis (Douma, Van Laar et al. 2004), and the ability of cells to resist anoikis correlates with poor prognosis in breast cancer (Mori, Chang et al. 2009). These findings underscore the role of anoikis as an anti-metastatic mechanism. Recent evidence has shown that induction of autophagy protects cells from anoikis (Debnath, Mills et al. 2002; Fung, Lock et al. 2008). We discussed above that certain cancer cells appear to colonize specific tissues based on matching of ligand-receptor interactions. Integrin-engagement is also known to be necessary for proper growth factor-induced signal transduction. It appears that autophagy
may allow continued cell growth in the absence of proper growth factor signaling (Miranti and Brugge 2002; Reginato, Mills et al. 2003).

In contrast to the pro-malignant roles of autophagy in cancer, this process is also thought to act as a tumor-suppressor. Evidence for this comes from the observation that autophagy inhibits necrosis, leading to reduced infiltration of pro-tumor macrophage (Bingle, Brown et al. 2002; Degenhardt, Mathew et al. 2006). Furthermore, heterozygous deletion of Beclin1, a protein necessary for autophagy induction, results in tumorigenesis in mice (Qu, Yu et al. 2003). Finally, increased mTOR activity, which reduces autophagy, is correlated with tumor cell growth and survival (Guertin and Sabatini 2007). Thus, the role of autophagy in metastasis is topic of continued debate. It is likely that the balance between mTOR activation and autophagy is dynamically shifted throughout the process of metastasis to meet the demands of the metastatic microenvironment. A better understanding of this balance may enhance the efficacy of therapeutic treatments such as rapamycin and temsirolimus, which antagonize mTOR signaling (Easton and Houghton 2006; Yu, Shi et al. 2010). Furthermore, EMT, which leads to extracellular matrix detachment and enhanced migration, has become a target of clinical interest. Determining if EMT and autophagy are related processes will have important therapeutic implications.

Relevance and focus of my project

Metastasis is the most common cause of lethality in patients with solid tumors. Despite progress in the field, many questions remain about the mechanisms driving metastasis and a consensus has not been reached concerning the rate-limiting steps of metastasis. The answers to these questions will lead to improvements in patient diagnostics and metastatic therapy. Thus, the goal of my graduate work was to contribute to the understanding of the molecular and cellular biology of cancer metastasis and to help clarify the debate over metastatic inefficiency.
I begin this thesis by providing a molecular explanation for a paradoxical finding that TEM4-18 cells, despite enhanced tumor formation in mice, exhibit reduced migration and invasion *in vitro* (derivation and initial characterization of these cells described in Drake, Strohbehn *et al*. 2009). We find that ZEB1, which is partially responsible for an EMT-like phenotype in these cells, directly targets integrin-β4 and two components of its ligand, laminin-332. The latter is a basement membrane protein often associated with pro-migratory characteristics of cancer cells. We show that by supplementing exogenous laminin-332 to TEM4-18 cells, their migratory phenotype is rescued. Importantly, this rescue could also occur by means of paracrine laminin-332 production by cells mixed with TEM4-18 cells. These studies provide a mechanistic basis for context-dependent, or “cooperative”, migration of cancer cells in invasion and metastasis. Future examination of these interactions *in vivo* could provide identification of novel therapeutic targets.

Following invasion and intravasation, cancer cells find themselves in the bloodstream, which is thought to be one of the harshest environments encountered during metastasis. To address the response of cancer cells to shear stress experienced in the bloodstream, I designed an *in vitro* model of fluid shear stress. We show that this model induces cell death in a shear stress dose-dependent fashion. Strikingly, we found that many cancer cell types exhibit an inducible resistance to shear stress. This mechanism requires extracellular calcium and dynamic actin reorganization; furthermore this response is unique to transformed epithelial cells, as primary or non-transformed cells of similar tissue-origin are susceptible to much lower magnitudes of shear force. Future studies will utilize our model to gain further understanding of the mechanism behind this survival response and analyze in depth the physical changes which occur in cells experiencing shear stress. It is also anticipated that our model may have practical clinical applications.
Our studies above show that cancer cells survive fluid shear stress quite well, supporting the hypothesis that late (post-colonization) steps of metastasis largely determine the efficiency of this process. We next sought to examine the physical characteristics of successful metastatic cells. In a comparison of “parental” cancer cell lines and derivatives obtained from metastatic passage in mice, we found that metastatic cancer cells are often reduced in size. This correlated with reduced activity of mTOR signaling, which regulates cell size. Cells with this phenotype often exhibit increased autophagic flux, indicative of an increased ability to survive and proliferate in a nutrient-deprived microenvironment. Ongoing studies aim to determine the effect of autophagy inhibition on metastatic colonization. EMT and autophagy are both thought to contribute to metastasis. Although no connection between the two has been described, these processes may complement one another; for example, EMT results in detachment from the native basement membrane and egress from the nutrient-supplied primary tumor, thus activation of autophagy could protect the cell from detachment-induced death and nutrient dependence. Excitingly, upon induction of an EMT in normal MDCK epithelial cells, we detected a decrease in cell size and increase in autophagic flux. Collectively, these data suggest an important role of autophagy during invasion and upon metastatic colonization.

I conclude my thesis with further characterization of an aggressive subpopulation of the PC-3 human prostate cancer cell line, TEM4-18. These cells exhibit a mesenchymal phenotype reminiscent of EMT, robustly express a marker of adult stem cells (LGR5), and exhibit a CD24lo/44hi profile indicative of a tumor-initiating or “cancer stem cell” population. By subcutaneously injecting titrations of these cells into mice, we find enhanced tumor forming potency versus the epithelial counterpart of TEM4-18 cells. To examine a link between the EMT-like phenotype and stem cell-like profile (which has been shown for breast cancer; et al.) of TEM4-18 cells, I show that RNAi-targeting of the EMT-driver, ZEB1, results in decreased LGR5 expression. Finally, we show that
silencing ZEB1 in \textit{in vivo} derivatives of PC-3, which exhibit a similar phenotype to TEM4-18, leads to a partial reversal of EMT and significant reduction of tumor colonization in experimental metastasis. Future studies will investigate the presence of this phenotype in other cancer cell lines as well as in prostate tissue.

In sum, the efforts of my thesis work have provided novel mechanistic insight into cancer cell migration and metastasis. My studies of context-dependent cell migration reveal novel targets of ZEB1 and enhance understanding of the mechanism by which EMT contributes to cancer progression. By examining the response of cancer cells to fluid shear stress, I have revealed novel biological mechanisms which may have an impact on the study of CTCs and also challenge longstanding theories about metastatic inefficiency. My cellular and molecular analyses of metastatic cancer cells have identified physical traits which may provide a selective advantage during metastasis. These changes may lead to altered metabolic states which enhance the survival and growth of metastatic cancer cells, and also lead to expression of molecules which may prove to be useful prognostic biomarkers.
Figure 1.1: Long-term bioluminescence imaging of tail vein injected cancer cells. A luciferase expressing metastatic derivative of PC-3 prostate cancer cells was injected systemically into SCID mice via the tail vein route. In an image taken minutes after injection, these cells are observed arresting in the lung (A). At two months post-injection, colonization of various tissues is observed (B, dorsal image on top, ventral image on bottom). Note that not all mice develop tumors in the region of the lungs, despite their original arrest in this organ.
Figure 1.2: Short-term serial bioluminescence imaging of tail vein injected cancer cells. Luciferase expressing B16.F10 melanoma cancer cells were injected systemically into SCID mice via the tail vein route. At the indicated time points after injection, bioluminescence images were obtained. Note that by 48 hours nearly all signal detected at 4 hours is gone, indicating clearance of cells from the lung vasculature. Between 120 and 168 hours, bioluminescence signal steadily increases, indicating outgrowth of lung-colonized cancer cells. Graphical quantification of the bioluminescence images is shown at the bottom of the figure.
CHAPTER II

ZEB1 COORDINATELY REGULATES LAMININ-332 AND β4 INTEGRIN EXPRESSION ALTERING THE INVASIVE PHENOTYPE OF PROSTATE CANCER CELLS

Introduction

Metastasis involves the invasion of cancer cells across natural barriers such as basement membranes, interstitial matrix, and the endothelium. Considerable effort over the past decades has defined a number of mechanisms that contribute to the invasive behavior of metastatic cancer cells such as elevated secretion of matrix degrading enzymes, altered expression of matrix components or their receptors, and increased motility in response to tumor microenvironmental cues. Epithelial-to-mesenchymal transition (EMT) is a mechanism involved in multiple aspects of mammalian development, such as gastrulation and formation of the neural crest, whereby cells lose epithelial identity and gain the ability to move to distant sites in the organism and thus is an attractive paradigm for understanding metastasis (Thiery, Acloque et al. 2009). Although abundant experimental and some clinical evidence for EMT in cancer exists, the extent to which this mechanism contributes to metastasis remains controversial (Christiansen and Rajasekaran 2006).

We found that TEM4-18 cells differentially express a dual zinc-finger homeodomain transcription factor ZEB1 (a.k.a. ZFHX1a, δEF1, or TCF-8), which represses a variety of epithelial genes in TEM-4-18 cells and has been implicated in EMT in both developmental and pathological contexts, including cancer (for review Vandewalle, Van Roy et al. 2009). ZEB1 expression has also been reported in clinical prostate cancer specimens (Graham, Zhau et al. 2008). In TEM4-18 cells,
transendothelial migration depended on ZEB1, showing that EMT may also facilitate extravasation of cancer cells. Contrary to expectations for cells exhibiting EMT, however, TEM4-18 cells were much less invasive in other experimental paradigms for invasion such as migration across a transwell membrane or invasion through Matrigel (Drake, Strohbehn et al. 2009).

Here we describe the molecular basis to this paradoxical finding. We show that the reduction of transwell migration observed in TEM4-18 cells is due to the loss of expression of laminin-332, a molecule well known to promote cancer cell migration (Miyazaki, Kikkawa et al. 1993; Giannelli, Falk-Marzillier et al. 1997). Further, we show that both β3 and γ2 subunits of laminin-332 as well as β4 integrin, a receptor for laminin-332, are repressed by ZEB1 through direct association with the LAMC2 and ITGB4 promoter regions. Loss of laminin-332 expression and integrin β4 in clinical prostate cancer specimens is a longstanding observation (Nagle, Hao et al. 1995; Hao, Yang et al. 1996). Our study presents a new mechanism which may underlie this observation. Moreover, this study shows that EMT may not always be associated with changes that are cell-autonomously advantageous for invasive behavior and points to cooperative interactions among different cancer cell populations, or with the stroma, to fully support the invasive cellular behavior observed in tumors.

Materials and Methods

Antibodies and extracellular matrix proteins

Laminin-332 was purified from SCC-25 squamous cell carcinoma cells as described previously (Winterwood, Varzavand et al. 2006). Laminin-332 antibody, clone 6F12, (which recognizes the β3 chain and also known as K140) (Marinkovich, Lunstrum et al. 1992) was used for immunofluorescence. Laminin-332 antibody, clone E-6, (which
recognizes the γ2 chain) was purchased from Santa Cruz Biotechnology. Laminin from the Engelbreth-Holm-Swarm mouse tumor, consisting primarily of laminin-111, was purchased from BD Biosciences. Collagen IV was purchased from Sigma.

Cell lines

PC-3 prostate adenocarcinoma (ATCC) cells were stably transduced with a luciferase-expressing retroviral vector and were grown in the ATCC recommended medium (GIBCO) supplemented with 10% FBS (HyClone) and 1 mM non-essential amino acids (GIBCO) as described previously (Drake, Gabriel et al. 2005). All cells were grown at 37°C and 5% CO2. TEM4-18 cells were grown in DMEM/F12 medium supplemented with 400 μg/mL G-418. TEM4-18 cells expressing ZEB1 shRNAs were described previously (Drake, Strohbehn et al. 2009).

Migration assays

Preparation of transwell inserts: 24-well transwell inserts were coated with 1 μg/mL, in 0.005% Tween-20, of either laminin-111 or laminin-332 for 1 hour at room temperature. The inserts were then washed with PBS with 0.005% Tween-20 twice before placing in EGM media (Lonza) for the experiment. Collagen IV coating was performed as previously described (Drake, Strohbehn et al. 2009). For PC-3 and HMVEC conditioned membranes, 1 x 10^5 non-luminescent parental PC-3 or 4 x 10^4 HMVEC cells, respectively, were plated onto 24-well transwell inserts for 24 hours. After 24 hours, the cells were washed in PBS and treated with versene to detach the cells without digesting the extracellular matrix deposited on the inserts.

Transwell migration assays: Prior to plating onto the transwell inserts, PC-3 or TEM4-18 cells were detached with 0.48 mM Versene (Gibco) for 10-15 minutes. Cells
were then resuspended in complete DMEM/F12 media, pelleted at 700 x g for 5 minutes, and resuspended in EGM media at a concentration of 5 x 10^5 cells/mL. Prostate cancer cells (1 x 10^5, 200 μL) were added onto the transwell inserts and allowed to incubate for 18 hours prior to analysis of migration. A standard curve was performed by serial dilution of prostate cancer cells (10,000 to 20 cells) in a 96-well dish followed by bioluminescence imaging (BLI) in a Xenogen IVIS100 imaging system (Caliper Life Sciences). To assay migration using BLI, transwell inserts were placed into a new 24-well dish containing trypsin (400 μL, 10 minutes at 37ºC) to remove only the cells that had migrated. After 10 minutes, trypsin was neutralized with 600 μL of serum-containing DMEM/F12 medium and each insert was washed with medium. 100 μL of sample, in duplicate, from each well was then added to a black 96-well dish (Corning) followed by addition of 100 μL of luciferin (0.3 mg/mL). BLI was determined following 5 minute luciferin incubation. Cell quantification was performed by converting the BLI signal from the sample into the standard curve to derive the number and percent of total cells migrated. Experiments were performed in triplicate and the data presented herein represents one of three individual experiments.

Single cell motility assay: Prior to plating tumor cells, laminin-332-coated dishes were prepared as described above. After coating, 2.3 x 10^5 cells were plated in serum-free medium onto the dish and allowed to incubate at 37ºC for 30 minutes. After incubation, the dish was transferred to a microscope stage incubator (20/20 Technology, Inc.) to maintain a humidified, 5% CO2, 37ºC environment. Images were collected and analyzed as previously described (Winterwood, Varzavand et al. 2006).

Cooperative migration analysis: TEM4-18 cells grown to ~75% confluence were stained with 10 μM CMFDA (Cell Tracker Green, Invitrogen) for 30 minutes in serum-free medium followed by a PBS wash and 30 minute incubation in complete medium. Labeled cells were mixed 1:1 with unlabeled TEM4-18 or PC-3E cells and brought to a final concentration of 5 x 10^5 cells/mL. Co-cultures were then subjected to transwell
migration on collagen IV-coated membranes as described above. After 18 hours, transmigrated cells were removed from the lower chamber as described above, pooled from triplicate wells and pelleted at 700 x g for 5 minutes. Pellets were resuspended in 350 μL FACS buffer containing 1 x 10^5 counting beads (Caltag Laboratories). Live, single cells were gated on forward and side scatter on a BectonDickenson LSR. Stop gates were set on CMFDA cells. The volume of each sample analyzed was calculated by dividing the number of counting beads collected by the total number of counting beads per tube. By dividing the number of CMFDA expressing cells by the volume analyzed, we derived the number of CMFDA-positive cells per tube. This experiment was repeated three times and the results presented are averaged values.

Quantitative RT-PCR (qRT-PCR)

Primers to human ZEB1, LAMA3a, LAMA4, LAMA5, LAMB3, and LAMC2 are listed in Table 1. Total RNA was extracted from low passage PC3, TEM4-18, and ZEB1 knockdown cells using an RNeasy mRNA isolation kit (Qiagen). Reverse transcription was carried out using iScript cDNA synthesis kit (BioRad). The resulting cDNAs were used as PCR template using CYBR Green I (Invitrogen) and data was collected on iCycler thermal cycler (BioRad). Experimental values were normalized to GAPDH values. Relative expression values were calculated using a comparative Ct method (Pfaffl 2001).

Flow cytometry

Cells were detached using 2 mL of 0.48 mM Versene/10cm dish and allowed to incubate for 5-10 minutes. The cells were then harvested, resuspended in 10 mL of serum-containing DMEM/F12 medium and pelleted at 700 x g for 5 minutes. 5 x 10^5
cells per tube were placed into 1.5 mL eppendorf tubes and spun down at 700 x g, 4ºC, for 5 minutes. Supernatant was removed and 50 µl FACS buffer (PBS + 0.02% sodium azide + 5% BSA) + either integrin α3 (1:200, A3-X8, ref Weitzman, Pasqualini et al. 1993), α6 (1:200, A6-ELE, ref Lee, Berditchevski et al. 1995), β1 (1:200, TS2/16, ref Hemler, Sanchez-Madrid et al. 1984), or β4 (1:200, clone 450-9D, GeneTex) antibodies were added to the cells. The cells were then incubated for 20 minutes on ice in the dark, washed with 1 mL of FACS buffer, and pelleted for 5 minutes at 700 x g, 4ºC. 50 µl FACS buffer + secondary antibody (1:100, goat anti-mouse FITC, Chemicon) was added to the cells and incubated for 20 minutes on ice in the dark. Cells were washed again with 1 mL of FACS buffer followed by a 5 minute spin at 700 x g, 4ºC, resuspended in 400 µl of FACS buffer, and transferred to a 12 x 75 mm polystyrene FACS tube (BD Biosciences). Samples were analyzed using the Becton Dickinson FACScan at The University of Iowa Flow Cytometry Core Facility using the WinMDI software (Purdue University).

Western blot

To obtain whole cell extracts, 2% SDS protein lysates were collected. Following protein estimation, whole cell lysates (30 µg) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 5% milk with 0.1% Tween 20 in PBS for 1 hour at room temperature followed by incubation of laminin γ2, clone E-6, (1:500) primary antibody overnight at 4ºC in blocking solution. The membrane was washed 3 times for 5 minutes in blocking solution followed by incubation with donkey anti-mouse (1:2,500) horseradish peroxidase secondary antibody for 1 hour at room temperature. The membrane was then rinsed 3 times for 5 minutes in TBS with 0.1% Tween-20 followed by incubation with SuperSignal West Pico Chemiluminescent substrate (Pierce) for 5 minutes at room
temperature. The membrane was then exposed on Classic Blue autoradiography film BX (Midwest Scientific).

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was carried out using an antibody against ZEB1 (Clone E-20, Santa Cruz). ChIP analyses were performed according to the manufacturer’s instructions in the EZ-ChIP Assay Kit (Upstate Biotechnology, Lake Placid, NY). 5-10 x 10^6 cells were sonicated for 20 seconds at 50% amplitude 10 times, with a 1 minute rest between sonications using a Vibra Cell 150 µL- 5 mL stepped tip (Sonics, Newtown, CT) in 500 µL of lysis buffer to generate fragments of DNA between 200-1000 bp. Purified DNA was used as template to amplify human E-cadherin, laminin γ2, and β4 integrin promoters with the following primers listed in Table 2. 2M Betaine (Sigma) was added to the β4 integrin specific primer set due to its high GC content. All samples were analyzed using qPCR as mentioned above with the fold-change measured as \(2^{(\text{Ct of IgG-Ct of ZEB1})}\).

Results

A metastatic subpopulation of PC-3 cells does not express pro-migratory laminin-332

Previously we found that while TEM4-18 cells more efficiently crossed an endothelial monolayer and colonized tissues in mice following intravenous injection than the bulk PC-3 population from which they were isolated, they were less invasive than PC-3 cells in other in vitro invasion assays (Drake, Strohbehn et al. 2009). To explore these seemingly inconsistent findings, we hypothesized that PC-3 cells might secrete a
matrix-associated component that facilitates cell invasion in transwell assays. To test this, we plated non-luminescent PC-3 cells onto uncoated transwell membranes allowing them to “condition” the membrane before non-enzymatically removing the cells and plating luciferase-expressing PC-3 or TEM4-18 cells onto the conditioned transwell membranes (Figure 2.1A). Consistent with our previous observations, TEM4-18 cells migrated considerably less well compared to PC-3 on unconditioned membranes. In contrast, when plated on PC-3 conditioned membranes; TEM4-18 cells exhibited dramatically enhanced migration, greater than PC-3 cells which also showed a modest increase when plated onto conditioned membranes. This suggests that the bulk PC-3 population secretes an insoluble matrix or matrix-associated component that enhances the migratory activity of TEM4-18 cells, a constituent subpopulation of PC-3 cells.

To gain possible insight into the identity of this factor, we reviewed our previously published microarray data (Drake, Strohbehn et al. 2009). We noted much less expression of LAMB3 and LAMC2 subunits of laminin-332, a matrix molecule well known to be involved in cancer cell motility, in TEM4-18 cells relative to PC-3 cells (data not shown). Since we had shown that TEM4-18 cells are present as an E-cadherin-negative subpopulation of PC-3 cells, we evaluated the expression of laminin-332 subunits in PC-3 parental cells, E-cadherin-positive PC-3 cells (PC-3E) and TEM-4-18 cells (Figure 2.1B, C). qRT-PCR analysis showed that the steady-state mRNA levels of both LAMB3 and, to a greater extent, LAMC2 (Figure 2.1B) were reduced in TEM4-18 cells whereas the levels of LAMA3a (Figure 2.1B), a subunit of several laminin heterotrimers including laminin-332, was not significantly different between the cell lines. Loss of the laminin γ2 protein was also confirmed by western blot (Figure 2.1C), as this subunit was expressed at a much higher level in both the PC-3 and PC-3E cells compared to TEM4-18 cells (Figure 2.1C).

To test the hypothesis that lack of laminin-332 expression was responsible for the reduced migration of TEM4-18 cells, we coated the transwell inserts with various matrix
proteins and measured transwell migration. TEM4-18 cells showed significantly increased transwell migration on laminin-332-coated transwell inserts when compared to other matrix components such as collagen IV and laminin-111 (Figure 2.2A). Laminin-332 restored transwell migration of TEM4-18 cells to levels comparable to PC-3 cells. To determine whether integrins known to support cell motility on laminin-332 are involved in transwell migration of TEM 4-18 cells we performed antibody inhibition experiments. This showed that a3 integrin, but not a6 integrin was involved in transwell migration of TEM 4-18 cells (Figure 2.3). We also evaluated the effect of laminin-332 on migration of isolated PC-3 and TEM 4-18 cells. When plated onto laminin-332, the average velocity (Figure 2.2B), persistence (Figure 2.2C) and distribution of individual cellular velocities (Figure 2.2D) were indistinguishable. Taken together these studies indicate that the TEM4-18 cells do not have an intrinsic deficit in either transwell migration or planar cell motility, but rather they lack the ability to produce laminin-332, which acts as a potent, pro-migratory molecule for these cells.

Laminin-332 and β4 integrin expression are repressed by ZEB1

We previously showed that TEM4-18 cells express the transcription factor ZEB1 at a high level relative to the bulk PC-3 population and that ZEB1 both repressed the epithelial phenotype and promoted transendothelial migration of TEM4-18 cells (Drake, Strohbehn et al. 2009). Another study implicated ZEB1 in the regulation of laminin-332 expression in colorectal cancer cells, however in this context ZEB1 repressed LAMA3 expression and activated LAMC2 expression (Spaderna, Schmalhofer et al. 2006). Although these details were at odds with our observations in TEM-18 cells, as LAMA3a mRNA is unchanged and LAMC2 mRNA is reduced relative to PC-3 cells; we wished to determine whether ZEB1 might repress laminin-332 in TEM4-18 prostate cancer cells.

We previously silenced ZEB1 in TEM4-18 cells and were able to achieve ~60-
70% reduction in ZEB1 protein levels (Figure 2.4) (Drake, Strohbehn et al. 2009). Using these lines we measured the mRNA expression levels of all three laminin-332 chains, LAMA3a, LAMB3, and LAMC2 using qRT-PCR. We detected a significant increase in mRNA of all three subunits of laminin-332, LAMA3a, LAMB3, and LAMC2 after stable ZEB1 silencing (Figure 2.5A). The effect on LAMA3a mRNA, however, was of lower magnitude than LAMB3 or LAMC2 and did not reach significance in one of the ZEB1 shRNA constructs (Figure 2.5A). Furthermore, laminin γ2 protein expression was increased in the ZEB1 knockdown cells when compared to the TEM4-18 or shControl cell lines; though it did not reach the levels detected in PC-3 cells (Figure 2.5B). The latter result is concordant with our findings on the effect of ZEB1 silencing in TEM4-18 cells on E-cadherin expression, which was only partially restored, suggesting that we either have not achieved sufficient inhibition of ZEB1 for a complete rescue of laminin γ2 expression or other factors contribute to the full repression of these genes (Drake, Strohbehn et al. 2009).

We have previously measured the cell surface expression of the three integrin receptors of laminin-332; α3β1, α6β1, and α6β4 integrins in the PC-3 and TEM4-18 cell lines (Drake, Strohbehn et al. 2009). Since we found that β4 integrin was down-regulated in TEM4-18 cells compared to PC-3 cells, this suggested that ZEB1 might coordinately regulate both laminin-332 and β4 integrin. We investigated this possibility using the ZEB1 knockdown TEM4-18 cells. Cell surface β4 integrin expression increased ~2-fold in ZEB1 knockdown TEM4-18 cells; whereas α3 and β1 integrin expression were each reduced ~50% and integrin α6 was unchanged (Figure 2.5C-F). This suggests that ZEB1 represses β4 integrin expression, and confers a shift in laminin-332 receptor expression on the surface of TEM4-18 cells. Thus, ZEB1 coordinately regulates both laminin-332 and its receptor α4β6 integrin.

Since we have shown above that ZEB1 repressed endogenous laminin-332 and that exogenous laminin-332 could rescue transwell migration, we tested the effects of
ZEB1 knockdown on transwell migration. ZEB1 knockdown partially restores transwell migration of TEM4-18 cells (Figure 2.7A) consistent with the observed partial restoration of laminin-332 expression (Figure 2.5A, B). These data are consistent with the idea that ZEB1 represses laminin-332 expression in TEM4-18 cells and thereby results in poor transwell migration of these cells. To extend these findings to other prostate cancer cells, we knocked down ZEB1 expression in Du145, another prostate cancer cell line which we had previously shown to express high levels of ZEB1 (Drake, Strohbehn et al. 2009). In accord with the findings in TEM4-18 cells, ZEB1 knockdown in Du145 resulted in ~2-fold increases in laminin γ2 and β4 integrin expression (Figure 2.6A, B). However, in contrast to our findings in TEM4-18 cells ZEB1 knockdown in Du145 results in decreased cell migration in the transwell assay as has been shown in other cell lines (Figure 2.7B) (Aigner, Dampier et al. 2007; Graham, Zhau et al. 2008; Spaderna, Schmalhofer et al. 2008). To reconcile the disparate effects of ZEB1 knockdown on transwell migration in these two prostate cancer cell lines we considered the possibility that ZEB1 may exert both pro- and anti-migratory effects in cells. Since we showed that ZEB-1 represses the expression of laminin-332, a pro-migratory molecule, this is in essence and anti-migratory effect of ZEB-1. To test this idea, we plated ZEB1 knockdown TEM4-18 cells on laminin-332-coated transwells. In this context, ZEB-1 knockdown in TEM4-18 cells was similar to the effects observed in Du145 cells, i.e. ZEB1 knockdown reduced, instead of enhanced, TEM4-18 cell migration (Figure 2.7C). This shows that if a sufficient level of laminin-332 is present, ZEB1 knockdown reduces migration of TEM4-18 cells.

ZEB1 binds directly to LAMC2 and ITGB4 5’ regulatory elements

To assess whether ZEB1 directly binds to the promoters of laminin γ2 or integrin β4, we performed chromatin immunoprecipitation (ChIP) in TEM4-18 cells. ZEB1 has
previously been shown to interact with either E-box elements (most notably 5’-CAGGTG-3’, 5’-CACCTG-3’, or 5’-CATGTG-3’) or Z-box elements (most notably 5’-TACCTG-3’ or 5’-CAGGTA-3’) in the proximal promoters of other target genes, including CDH1 (E-cadherin) and LAMA3a (laminin α3a) (Eger, Aigner et al. 2005; Spaderna, Schmalhofer et al. 2006; Aigner, Dampier et al. 2007). Therefore, we proceeded to investigate the promoter regions of CDH1, LAMC2 (laminin γ2), and ITGB4 (integrin β4) for E/Z-box elements. We identified regions of clustered E-box or Z-box elements located in the proximal promoter regions of all three genes, including the already published ZEB1 target gene, E-cadherin, which we used as a positive control (Figure 2.9A). We determined that ZEB1 binds specifically to tandem E/Z-boxes within the proximal promoter of all three genes investigated, with enrichment values ranging from 2.5-4-fold when compared to the isotype IgG control (Figure 2.9B-D, primer sets #2; Figure 2.8). Further, when we amplified regions significantly upstream from the proximal promoter that lack E-boxes (~3-5kb), we did not see any enrichment of ZEB1 binding (Figure 2.9B-D, primer sets #1; Figure 2.8) nor did we observe an enrichment of ZEB1 binding to tandem E-boxes within LAMC2 located ~1kb downstream of the transcriptional start site (Figure 2.9A, C, primer set #3; Figure 2.8). These findings indicate that ZEB1 may directly repress laminin γ2 (and thereby laminin-332) and β4 integrin expression by binding to the promoter elements of these genes.

Laminins and cooperative cancer cell migration

Since TEM4-18 cells failed to express laminin-332, and yet laminin-332 facilitates the migratory and invasive phenotype of those cells, we asked if laminin-332 expressing cells within the bulk PC-3 population can support transwell migration of TEM4-18 cells. Indeed, PC-3E cells, which express laminin-332 (see Figure 2.1B), exhibit even greater transwell migration than parental PC-3 cells, perhaps due to the lack
of the poorly migrating TEM4-18 cells (Figure 2.10A). To test if PC-3E cells could support the transwell migration of co-cultured TEM4-18 cells, we mixed CMFDA labeled TEM4-18 cells with unlabeled PC-3E or TEM4-18 cells and assayed migration of the labeled cell population. When mixed with PC-3E cells, TEM4-18 cells were capable of efficient transwell migration (Figure 2.10B). These results are consistent with the notion that PC-3E cells supply laminin-332, which is a limiting factor for cell migration in this context, to TEM4-18 cells.

With the finding that laminin-332 effectively promoted transwell migration of TEM4-18 cells, we considered the possibility that a component of the sub-endothelial matrix might be involved in the enhanced transendothelial migration that we previously documented in these cells (Drake, Strohbehn et al. 2009). Figure 2.10C shows that the relative migration of PC-3E cells and TEM 4-18 cells across collagen IV-treated transwell filters and HMVEC monolayers are consistent with our previous findings, with TEM4-18 cells showing considerably enhanced transendothelial migration. To test whether a component of the subendothelial matrix contributes to this enhanced migration, we non-enzymatically removed HMVEC cells from the transwell insert and plated PC-3E and TEM4-18 cells on these “conditioned” membranes. This showed that both PC-3E and TEM4-18 cells migrated equally well on the HMVEC matrix (Figure 2.10C). Thus, interaction with the sub-endothelial matrix does not explain the difference in transendothelial migration between PC-3E and TEM4-18 cells. Nevertheless, the HMVEC matrix did augment migration of TEM4-18 cells compared to collagen IV-treated filters, suggesting that matrix proteins secreted from endothelial cells may facilitate prostate cancer cell migration. Endothelial cells do not express laminin-332, but do express laminin-411 and -511, both of which are ligands for α3β1 integrin (Hallmann, Horn et al. 2005). qRT-PCR analysis confirmed that HMVEC cells express relatively low levels of mRNA’s encoding laminin-332, but much higher levels of LAMA4 and LAMA5 mRNA’s suggesting the possibility that laminin-411 or -511 may be
components from the HMVEC matrix that cooperatively promote prostate cancer cell migration (Figure 2.10D). Extensive efforts to detect laminin-γ2 chain in HMVECs also produced negative results (data not shown).

Discussion

A principal finding of this study is that ZEB1, which is a known regulator of EMT, represses the expression of laminin-332 and its receptor, β4 integrin in prostate cancer cells. Although notable for its pro-migratory properties and its upregulation in invasive regions of a number of tumor types, laminin-332 and β4 integrin expression is typically reduced in prostate cancer (Nagle, Hao et al. 1995; Hao, Yang et al. 1996; Allen, Smith et al. 1998). This has led to the proposition that loss of these hemidesmosomal cell adhesion components may facilitate dispersion of prostate cancer cells from the primary site. In prostate cancer, specific reduction of the β3 and γ2 polypeptide chains, but not the α3 chain, has been observed (Hao, Yang et al. 1996). However, the mechanism by which this occurs remains unclear. Hao et al. reported that LAMB3 and LAMC2 mRNA could be detected in prostate cancer tissue, suggesting that the mechanism is post-transcriptional (Hao, Jackson et al. 2001). Cleavage of β3 and γ2 proteins by matrix metalloproteases and hepsin has also been described (Giannelli, Falk-Marzillier et al. 1997; Udayakumar, Chen et al. 2003; Tripathi, Nandana et al. 2008). These results may appear at odds with those reported here, however, more than one mechanism may account for the reduction of laminin-332 immunoreactivity in prostate cancer. The continued expression of LAMB3 and LAMC2 mRNA in most tumor tissue does not exclude the existence of a less abundant population of cells that may exhibit ZEB1-dependent repression of these mRNAs (Graham, Zhau et al. 2008). Moreover, proteolysis of laminin-332 might contribute primarily to the generation of pro-migratory
forms of this molecule, rather than its decreased abundance from the extracellular matrix (Giannelli, Falk-Marzillier et al. 1997).

Prior studies have implicated ZEB1 in the regulation of laminin-332 subunits. Spaderna et al. found in colorectal carcinoma that ZEB1 repressed LAMA3a and either directly or indirectly activated LAMC2 (Spaderna, Schmalhofer et al. 2006). The latter finding is consistent with upregulation of laminin γ2 protein at the invasive front of colorectal cancers (Pyke, Salo et al. 1995; Sordat, Bosman et al. 1998; Hlubek, Jung et al. 2001). Loss of the laminin-332 heterotrimer is consistent with the loss of basement membrane structure at the invasive front of colorectal tumors (Spaderna, Schmalhofer et al. 2006). Our findings in prostate cancer are different in that although ZEB1 has some effect on LAMA3a expression, it is clearly associated with repression, not activation, of LAMC2. Together these results indicate that ZEB1 affects transcription of LAMC2 in a manner that depends on tissue context. Since colorectal cancer is generally regarded as a more rapidly progressive disease than prostate cancer; and laminin γ2 may possess intrinsic motility promoting properties independent of the laminin-332 heterotrimer, it is interesting to speculate that the differential function of ZEB1 in these two tissues may contribute to the pace of disease progression. ZEB1 is a dual zinc-finger homeodomain transcription factor that has been shown to recruit both co-repressors including CtBP and histone modifying enzymes and co-activators such as p300 (Postigo and Dean 1999; Shi, Sawada et al. 2003; Pena, Garcia et al. 2006). The differential levels or recruitment of these components, possibly involving post-translational modifications to ZEB1 itself, may underlie the apparent ability of ZEB1 to act as a transcriptional repressor of LAMC2 in one context and an activator in another consistent with other targets of ZEB1 (Costantino, Stearman et al. 2002; Pena, Garcia et al. 2006). Finally, transfection of other cell types with SNAIL1 resulted in decreased expression of laminin-332 and/or β4 integrin suggesting that other master regulators of EMT may control these genes similarly, perhaps explaining why ZEB1 knockdown only partially restores their
expression in our studies (Takkunen, Grenman et al. 2006; Haraguchi, Okubo et al. 2008).

Here we have shown that in prostate cancer cells, ZEB1 coordinately represses both laminin-332 and its receptor integrin β4. Laminin-332 binds preferentially to three integrin receptors, α3β1, α6β1 and α6β4 (Carter, Ryan et al. 1991; Delwel, de Melker et al. 1994; Nishiuchi, Murayama et al. 2003). While both α6β4 integrin and laminin-322 function in stable adhesion complexes in hemidesmosomes, α6β4 integrin is involved in invasion and motility in some cancers (Chao, Lotz et al. 1996; Shaw, Rabinovitz et al. 1997). However, in mammary epithelial cells, the ITGB4 promoter is methylated in association with TGFβ-induced EMT and β4 integrin expression is reduced in invasive prostatic carcinoma when compared to the normal glands or early prostatic intraepithelial neoplasia lesions (Cress, Rabinovitz et al. 1995; Yang, Pursell et al. 2009). Therefore, α3β1 and α6β1 integrins remain as receptors for laminin-332 in TEM4-18 cells as has been noted in prostate cancer specimens. Indeed, here we show that α3 integrin supports transwell migration of prostate cancer cells on laminin-332. Interestingly, in keratinocytes, whether α6β4 integrin binds to laminin-332 may determine whether these cells migrate as collective sheets or in a more mesenchymal mode in response to epidermal growth factor (Russell, Fincher et al. 2003). Thus, ZEB1, by repressing β4 integrin, as well as E-cadherin, may contribute to a change in the mode of cell migration from collective to single cell.

EMT is generally associated with invasive cellular behavior and is mediated by a host of transcription factors, such as ZEB1, which promote this state. Unlike their role in repressing epithelial identity such as inhibiting E-cadherin and polarity gene expression, the detailed mechanisms by which EMT-promoting transcription factors drive invasive behavior remain largely unclear. In fact, here we show that one consequence of ZEB1 activity is repression of laminin-332, a potent pro-migratory molecule. In TEM4-18 cells, ZEB1 silencing results in overall poor transwell migration because the cells cannot
make this protein themselves. In contrast, in Du145 cells, as with other epithelial cell lines, ZEB1 silencing decreases cell migration (Aigner, Dampier et al. 2007; Graham, Zhau et al. 2008; Spaderna, Schmalhofer et al. 2008). Du145 cells, unlike TEM4-18 cells, detectably express laminin-γ2 (see Figure 2.7A). In this case, there is likely some laminin-332 present to support migration in the transwell assay. To further explore the relationship between ZEB1, laminin-332, and transwell migration, we plated TEM4-18 shControl and shZEB1 onto laminin-332-coated transwell inserts. In the presence of exogenous laminin-332, ZEB1 silencing resulted in decreased TEM4-18 migration, mimicking the results observed in Du145 on untreated inserts (Figure 2.6C). This indicates that ZEB1 positively regulates pathways involved in cell migration; however, the relevant target genes for this in prostate cancer are presently unknown. These results again emphasize the contextual nature of ZEB1 function in relation to invasive behavior.

Our work also reveals that laminins are key components within this context. Because ZEB-1 inhibits laminin-332 expression, and this molecule is necessary for efficient prostate cancer cell invasion across matrix, laminin-332 may be supplied by other cells in the tumor microenvironment, including normal prostate basal epithelial cells, to facilitate this event (Yu, Frank et al. 2004). Indeed, our studies here experimentally demonstrate cooperation between PC-3E and TEM4-18 cells in this regard. Cooperation among distinct tumor cell populations or between cancer and stromal cells during cancer progression has a solid theoretical basis and emerging experimental support, including a recent demonstration of cooperative interactions relative to the role of EMT in an experimental metastasis model (Orimo, Gupta et al. 2005; Axelrod, Axelrod et al. 2006; Tsuji, Ibaragi et al. 2009). However, as mentioned above, loss of laminin-332 from the epithelial basement membrane is consistently observed in primary prostate tumors; suggesting that local invasion of prostate cancer does not depend on laminin-332. Interestingly, we show that a matrix preparation from endothelial cells also promotes prostate cancer cell migration and lacks laminin-332.
Figure 2.1: Extracellular matrix deposition by PC-3 cells enhances TEM4-18 cell migration.

(A) Migration of TEM4-18 cells was measured after allowing non-luminescent PC-3 cells to ‘condition’ the transwell membrane for 24 hours. This treatment enhanced TEM4-18 cell migration significantly over ‘non-conditioned’ transwell inserts. *p<0.05, **p<0.01 (paired t-test) vs. ‘non-conditioned’ transwell insert for each cell type. (B) qRT-PCR analysis reveals reduced expression of the laminin β3 and γ2 chains, but not the α3a chain in the TEM4-18 cells when compared to PC-3 and E-cadherin positive PC-3E cells **p<0.01, ***p<0.001 (one-way ANOVA, Bonferroni post test) vs. PC-3E for each gene. (C) Western blot analysis also confirmed the lack of the laminin γ2 subunit (LAMC2) in TEM4-18 cells when compared to PC-3 and PC-3E cells.
Figure 2.2: Laminin-332 restores TEM4-18 transwell migration. (A) TEM4-18 cells were plated onto various extracellular matrix components and migration across transwell inserts was measured. Laminin-332 coated transwell inserts enhances migration of TEM4-18 to levels similar to parental PC-3 cells on uncoated inserts (PC-3 untreated versus TEM4-18 laminin 332 coated). The values are compared to PC-3 migration on untreated transwell inserts *p<0.05 (one-way ANOVA, Bonferroni post test) vs. untreated treatment for each cell type. (B-D) PC-3 parental cells and TEM4-18 cells were plated on purified laminin-332. After 30 minutes for cell attachment and spreading, cell motility was monitored by time-lapse video microscopy every 2 minutes for 5 hours. (B) The mean velocities of PC-3 cells and TEM4-18 cells were similar (p = 0.072, unpaired t test). (C) Directional persistence (the net hourly distance traveled divided by the total hourly distance traveled) was also indistinguishable for PC-3 cells and TEM4-18 cells (p = 0.239, unpaired t test). (D) The velocity distributions of the two cell types were very similar as well (p = 0.386, Kolmogorov-Smirnov test). For PC-3 and TEM4-18 cells, n = 41 and 56 respectively.
Figure 2.3: Antibody inhibition of α3 integrin reduces TEM4-18 migration on laminin-332 coated inserts. Anti-α3 integrin, anti-α6 integrin or mouse IgG isotype antibody was added to suspensions of TEM4-18 cells at a final concentration of 10μg/mL prior to plating onto laminin-332-coated transwell filters. This shows that blockage of α3 integrin, but not α6, at the surface of TEM4-18 cells reduces laminin-332-dependent migration. **p<0.01 (two-way ANOVA, Bonferroni post test).
Figure 2.4: Western blot analysis of ZEB1 knockdown in TEM4-18 cells. Western blot of ZEB1 and β-actin are displayed comparing TEM4-18 cells to the ZEB1 shControl and shRNA cells. This shows ~60-70% reduction of ZEB1 in shZEB1#1 and shZEB1#2. The image of the blot shown removes two lanes between shControl and shZEB1#1 that is not relevant to this figure.
Figure 2.5: Silencing ZEB1 restores laminin β3 and γ2 chains of laminin-332 and β4 integrin. (A) qRT-PCR analysis reveals increased expression of laminin β3 and laminin γ2 chains after stable ZEB1 silencing, ***p<0.001 (one-way ANOVA, Bonferroni post test) vs shControl for each gene. (B) Western blot analysis confirms upregulation of laminin γ2 protein (both the full length 150 kDa and 100 kDa form) in the ZEB1 knockdown cells, although levels are not restored to the PC3 parental cell line. Flow cytometry analysis reveals increased cell surface protein expression of β4 integrin after stable ZEB1 silencing (F), while other laminin-332 receptors are moderately decreased or unchanged (C-E).
Figure 2.6: Silencing ZEB1 restores transwell migration of TEM4-18 cells. Control (shControl) or ZEB1 knockdown cells (shZEB1#1 and #2) were plated onto uncoated transwell inserts and transwell migration was measured after 18 hours. Knockdown of ZEB1 modestly restored migration of TEM4-18 cells to about half the level of the PC-3 parental population *p<0.05, **p<0.01, ***p<0.001 (one-way ANOVA, Bonferroni post test).
Figure 2.7: Analysis of ZEB1 knockdown in Du145 cells. (A) Protein levels of ZEB1, E-cadherin, LAMC2, and β-actin are shown for Du145 cells stably expressing shControl, shZEB1#1, or shZEB1#2. Fold changes in protein are relative to shControl cells. This western blot shows that in response to ZEB1 knockdown, there is a restoration of E-cadherin as well as LAMC2. (B) Flow cytometry analysis reveals increased cell surface protein expression of β4 integrin after stable ZEB1 silencing.
Figure 2.8: ZEB1 binds specifically to E-boxes within the 5' regulatory regions of laminin γ2 and β4 integrin. (A) Promoters of E-cadherin (CDH1), laminin γ2 (LAMC2), and β4 integrin (ITGB4) are depicted from +1kb to -5.5kb of the transcription start site. Exons are shown as light gray boxes and vertical black bars represent E/Z-box elements. Primers used for ChIP analysis are indicated. Primer set #3 for LAMC2 is an area with E-box elements, but shows no enrichment, revealing the resolution of the sonicated DNA to be under 1kb (C). *p<0.05, ***p<0.001 (paired t-test B and D; one-way ANOVA, C) vs. primer set #1 (regions lacking E/Z-box elements).
Figure 2.9: ChIP analysis supplemental data  (A) After performing ChIP and quantitative PCR (described in Materials and Methods) amplification products were run out on a 1% agarose gel. When amplifying total sonicated DNA (input control) or Zeb1-immunoprecipitated DNA with E-box-specific primers, bands are visible in lanes 1 and 3, respectively. No bands are visible when amplifying IgG isotype-immunoprecipitated DNA with E-box-specific primers or when amplifying Zeb1-immunoprecipitated DNA with non-E-box primers in lanes 2 and 4, respectively. (B) Using E-box specific primers for β4 integrin, an example of raw quantitative data showing that Zeb1-immunoprecipitated DNA (3) has a lower cycle threshold than IgG control DNA (2). Total sonicated DNA was used as an input control (1). (C) The single peak of the DNA melt curve indicates the purity of the amplification products.
Figure 2.10: Transwell migration of TEM4-18 cells is enhanced in the presence of PC-3E cells or extracellular matrix deposited by HMVEC cells. (A) Transwell migration of PC-3 parental, PC-3E, and TEM4-18 cells on collagen IV-treated transwell inserts. PC-3E cells display enhanced migration compared to the TEM4-18 cells. *p<0.05 ***p<0.001 (one-way ANOVA, Bonferroni post test) vs. PC-3. (B) TEM4-18 cells were stained with CMFDA and mixed 1:1 with unlabeled TEM4-18 or PC-3E cells and co-cultures were analyzed for transwell migration. In the presence of PC-3E cells, TEM4-18 transwell migration is enhanced approximately 25-fold. *p<0.05 (paired t-test) vs. TEM4-18:TEM4-18. (C) Migration of TEM4-18 cells was measured after allowing HMVEC cells to ‘condition’ the transwell membrane for 72 hours. This treatment enhanced TEM4-18 cell migration significantly over collagen IV-treated transwell inserts but not to the level of migration on a monolayer of HMVECs. *p<0.05, **p<0.01 (two-way ANOVA, Bonferroni post test) vs. ‘untreated’ transwell insert for each cell type. (D) qRT-PCR analysis reveals lack of detectable expression of LAMB3 and LAMC2 in HMVECs when compared to PC-3E cells (used as reference, set to 1 for each gene) but express readily detectable levels of LAMA4 and LAMA5. *p<0.05, ***p<0.001 (two-way ANOVA, Bonferroni post test) vs. PC-3E for each gene.
CHAPTER III

TRANSFORMED CELLS EXHIBIT CALCIUM-INDUCED RESISTANCE TO FLUID SHEAR STRESS

Introduction

Metastasis is the leading cause of mortality in patients with epithelial cancers. This complex process involves the detachment of cells from the primary tumor, invasion into surrounding tissue, entrance to and survival within the bloodstream, extravasation, and, finally, survival and proliferation at the secondary site (Pantel and Brakenhoff 2004; Gupta and Massague 2006). It is widely believed that metastasis is an inefficient process. This can be demonstrated experimentally, when often less than 0.01% of systemically-injected cancer cells develop into metastatic tumors (Fidler 1970; Luzzi, MacDonald et al. 1998). Clinically, metastatic inefficiency can be appreciated by considering that many tumors continuously shed cancer cells into the bloodstream on a daily basis, giving rise to a population of circulating tumor cells (CTCs), yet only a small number of which go on to colonize distant sites in a process which may take decades (Tarin, Price et al. 1984; Karrison, Ferguson et al. 1999). The nature of this inefficiency remains obscure and the extent to which any individual step of metastasis contributes to this is unknown.

In an effort to study the fate of CTCs, early studies were conducted to monitor the destination and viability of tumor cells injected systemically into mice. These authors concluded that the majority of circulating tumor cells are rapidly destroyed in the bloodstream by shear force (Fidler 1970; Fidler 1975) and/or by deformation following size restriction in the microvasculature (Weiss 1991; Weiss, Nannmark et al. 1992). This led to the longstanding assumption that cell death within the circulation is a major contributor to metastatic inefficiency. Observations of significant cell loss following
injection into mice have been reported recently by others as well (Al-Mehdi, Tozawa et al. 2000; Kienast, von Baumgarten et al. 2010). In contrast to these observations, other groups have reported that survival within the bloodstream and subsequent extravasation are completed efficiently by most tumor cells and that the ability to survive and grow at secondary sites is what determines the aggressiveness of a cell type (Koop, MacDonald et al. 1995; Luzzi, MacDonald et al. 1998; Podsyppanina, Du et al. 2008; Tsuji, Ibaragi et al. 2008). Here, we performed a detailed analysis of the survival of various cancer cell types to fluid shear stress in an effort to help clarify this debate.

Studying the response of cancer cells to hemodynamic shear stress in vivo can be a technically daunting and expensive task, requiring large numbers of animals and complex imaging tools. We thus designed a simple, inexpensive model of fluid shear stress, whereby suspensions of cancer cells are repeatedly passed through a needle at a constant flow rate (covering greater than a 10-fold range in shear stress) and cell viability is measured closely throughout the assay. We find that cancer cells derived from several mouse and human tissues exhibit an increased resistance to shear stress over repeated passages of shear stresses at high and supra-physiologic levels. Resistance to shear stress is not further enhanced in cells derived from experimental metastases and is not a stable, genetic trait, but rather a transient, adaptive response requiring the presence of extracellular calcium. Furthermore, this shear stress-activated survival response requires actin reorganization. We show that primary cells and non-transformed, but immortalized cell lines of the breast and prostate do not exhibit this shear stress response and are susceptible to magnitudes of shear stress much lower than required to kill cancer cells.

These data suggest that hemodynamic shear stress is not as large of a contributor to metastatic inefficiency as often assumed and underscore the ability of survival and proliferation at a secondary tissue site as a determinant of metastatic potential. These findings reveal a biological trait which appears to be inherent to transformation and may be necessary for progressive metastatic dissemination. Because this trait is unique to
transformed cells, perhaps it could be exploited to improve the value of clinical CTC studies.

Materials and Methods

Cell lines

All cancer cell lines were obtained from the ATCC and were transduced with an integrating retrovirus encoding firefly luciferase under control of the CMV promoter (pGEM, Promega). Cells were grown in the ATCC-recommended media supplemented with 10% fetal bovine serum and 1% non-essential amino acids. For maintenance of the retrovirus-gene expression, all cells containing this construct were maintained in 200 μg/mL Genetecin (Invitrogen). Primary prostatic and mammary epithelial cells were obtained from Clontech and were cultured in their commercial defined media. For all experiments, cells were grown to ~75% confluence and harvested by trypsinization following neutralization and suspension in complete medium.

Human and mouse blood

To obtain whole human blood, we collected fresh leuko-reduction cones from the University of Iowa Hospitals and Clinics DeGowan Blood Center. Cones were flushed in direction of filtration with normal saline (0.99%NaCl) to reduce red blood cell (RBC) content. Diluted RBCs were collected and brought to a concentration of 5x10^5 cells/mL and used in shear stress experiments. To isolate leukocytes, cones were then eluted in the direction opposite of filtration using 50mL ACK buffer (150mM NH₄Cl, 10mM KHCl₃, 0.10mM ETDA, pH 7.4), which osmotically lyses remaining RBCs. After 15 minutes of incubation at room temperature, cells were centrifuged at 100RCF for 5 minutes,
resuspended in 1mL PBS+xM calcein AM viability dye (Invitrogen), and incubated for an 15 minutes at room temperature. 9mL of ACK buffer was added to this cell suspension, centrifuged once more as above, and brought to a final concentration of 5x10^5 cells/mL in DMEM (Gibco) without supplements.

For whole mouse blood, we performed sub-mandibular bleeds on adult Bl/6 mice. Blood was collected in EDTA-treated tubes (BD biosciences) and diluted using normal saline to a concentration of 5x10^5 cells/mL prior to shear stress treatment.

Cell size analysis

Cells were suspended to a concentration of 5x10^5 cell/mL and analyzed on a Coulter Counter (Beckman Coulter) at a 1:100 dilution in Isoton II (Beckman Coulter). Size analysis was performed using Z2 Acucomp software (Beckman Coulter). Gates were set to exclude cellular debris and aggregates following the manufacturer’s instructions. At least three separate cultures for each cell line were counted and sized in triplicate. Data was plotted using Prism Graph Pad software and cell lines were compared based on mean cell volume. Paired, two-tailed t-tests were used for analysis of statistical significance.

Shear stress equations

Wall shear stress was calculated using Poiseuille’s equation, \( \tau = \frac{4Q\eta}{\pi r^3} \) (Davies 2009), where \( \tau \) is shear stress in dyn/cm²; \( Q \) is flow rate in cm³/s; \( \eta \) is the viscosity of the medium (0.01 dyn/cm/s for culture media); and \( r \) is the radius of the needle (30GA average internal radius = 7.94x10^{-3}cm). For calculating the minimum shear stress experienced by a given cell, we use the equation \( \tau = \frac{\Delta P r}{2L} \) where \( \Delta P \) is the change in pressure, \( r \) is the radius of the cell, and \( L \) is the length of the needle (1.27cm). Here, we make the assumption that cells are flowing along the axis of the needle in single file; thus
keeping everything constant, the cell radius will determine the magnitude of shear force encountered.

Reynolds number was calculated to assume laminar flow using the equation \( R_e = \frac{\rho v D}{\eta} \) where \( \rho \) is the density of the culture media (0.998g/cm\(^3\)), \( v \) is the velocity of flow, \( D \) is the diameter of the needle, and \( \eta \) is the viscosity of the medium. For the 20\( \mu \text{L/sec} \) (low) flow rate, \( R_e \) is 159.58; for the 250\( \mu \text{L/sec} \) (high) flow rate, \( R_e \) is 1998. This indicates that all flow rates in our shear stress model are laminar.

Shear stress models

5mL of a 5x10\(^5\) cell/mL suspension was placed into a small beaker and loaded into a 5mL syringe (BD Biosciences #309603) by slowly drawing the cells into the syringe. A 30 gauge needle (BD Biosciences #305106) was then attached to the syringe and cells were pushed through at a constant flow rate by one of two methods:

Syringe Pump: A Harvard Apparatus PHD-2000 Infuse/Withdraw syringe pump was calibrated for the syringes being used and set to the appropriate flow rate (ranging from 20-350\( \mu \text{L/sec} \)). After securing the syringe to the pump housing, the pump was turned on and the cell suspension was collected into a 15 mL conical test tube (BD Biosciences) angled below the needle. Care was taken not to produce foam or bubbles. The suspension was gently inverted a few times, the syringe was removed from the pump housing, the needle removed, and the cell suspension was re-loaded into the syringe as described above. Cell suspensions were pumped through the needle for 10 passages; at every other passage, duplicate 100 \( \mu \text{L} \) aliquots were taken for viability measurements (described below). As a non-shear stress treated control, cells which had been in suspension for the duration of the shear treatment were used as 100% viability controls.

250\( \mu \text{L/sec} \) flow rate: To facilitate throughput, some experiments at 250\( \mu \text{L/sec} \) were done manually. Here, cell suspensions, prepared as above, were pushed through the
needle by hand and collected into a small beaker. To control the flow rate using this approach, a timer was zeroed before each needle passage and the time taken to push a given volume through the needle was measured. At each passage, the volume in milliliters was divided by the time in seconds to give flow rate in mL/sec. Data for figures was only obtained from shear stress assays in which the average flow rate over 10 passages was \( \pm 5 \mu \text{L/sec} \) of the targeted 250\( \mu \text{L/sec} \).

**Cell viability assays**

**Bioluminescence Imaging (BLI):** This technique was used for all cancer cell lines in the main figures within the text. 100\( \mu \text{L} \) aliquots of shear stress-treated cells or control cells (those sitting in suspension through the duration of shear treatment) were loaded into a black 96 well plate (Costar) in duplicate. Each well was then diluted to 200\( \mu \text{L} \) at final concentration of 150\( \mu \text{g/mL} \) D-luciferin (Promega) using a multichannel pipette. Plates were incubated for 5 minutes at room temperature and then imaged for 5 minutes in an IVIS-100 (Xenogen). Bioluminescence measurements were collected using Living Image 2.50.1 software (Igor Pro). The photon flux of shear treated cells was divided by that of control cells to give % viability. All figures with BLI-derived viability data represent the average of at least 3 experiments.

**WST-1 Viability assay:** For primary cells and cell lines lacking luciferase-expression, we measured cell viability with (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate (WST-1, Roche Applied Science) as directed. As indicated in Figure 1, WST-1 and BLI results were compared in parallel, showing agreement between the two methods.

**Clonogenic plating for quantification of cell viability:** To support cell viability data acquired acutely using BLI and WST-1, clonal survival plating was performed. Here, 5\( \mu \text{L} \) of a 1:10 dilution of control or shear stress-treated cells was plated into 10cm
plate in 10mL of the appropriate complete culture media plus pen/strep. This volume contains an estimated 250 live cells prior to shear stress treatment (5x10^5 cell/mL x 0.5x10^-4 mL = 250 cells). Plates were incubated until visible colonies formed from single cells (depending on the cell line, this takes 1-2 weeks). Colonies were stained overnight with PBS containing 0.01% crystal violet and 0.02% citric acid, washed with distilled water, and counted on a light box. The number colonies from shear treated suspensions are divided by that of control plates to give percent survival. Graphs derived using this approach represent the average of three experiments or more.

Flow cytometry for quantitative analysis of cell viability: This technique was used for leukocytes obtained from whole blood. Leukocytes, prepared as described above, were subjected to shear stress for ten passages. Before shearing and after each passage, 300μL aliquots were placed in 5mL FACS tubes (BD Biosciences) in duplicate. 100μL of DMEM containing 1x10^6 counting beads/mL (Caltag Laboratories) was added to each tube, as well as propidium iodide to a final concentration of 0.5μg/mL. Single cells were gated on forward and side scatter, on a BectonDickenson LSR. Stop gates were set to 5,000 counting beads to assure consistency across samples. The ratio of live (propidium iodide-negative, calcein-AM-positive) cells to counting beads at each passage was compared to non-shear stress-treated controls to provide percent viability.

Flow cytometry analysis of propidium iodide uptake

200μL of cell solution was taken for each sample and added to 200μL of complete culture medium in 5mL polystyrene FACS tubes (BD Biosciences). Propidium iodide (final concentration of 0.5μg/mL) was added either before the first, sixth, eighth, or tenth shear passages. Cells were analyzed on a BectonDickenson LSR with Violet. Single cells were gated by forward and side scatter, consistent with viability, and evaluated for PI and/or Calcein AM positivity.
Statistical analyses

For statistical analysis of cell size and endpoint shear stress survival, paired 2-tailed t-tests were used when comparing two cell lines or flow rates. When comparing the endpoint survival of three or more cell lines or flow rates, one-way ANOVA followed by Bonferroni post tests were performed. When comparing shear stress survival of multiple cell lines over repeated passages, repeated measures ANOVA followed by Bonferroni post tests were used.

Results

In vitro model of fluid shear stress induces cell death in a dose-dependent fashion

To directly test the effect of fluid shear stress on cancer cells, we designed an experimental protocol involving the repeated passage of cell suspensions through a 30 gauge needle (150μm internal diameter). By holding a constant flow rate, we are able to control the magnitude shear stress experienced at the wall of the needle. Changes in cell viability resulting from exposure to shear stress are closely monitored throughout the protocol using bioluminescence imaging (BLI). The range of wall shear stress targeted in our protocol (Table 3.1) encompass high physiological values estimated in the human vasculature, and beyond (Schneider, Nuschele et al. 2007; Reneman and Hoeks 2008).

Using a Coulter Counter, we determined the mean cell radius of PC-3 to be 9.3μm. Applying the shear stress equation shown in the materials and methods section, we are able to use this radius to estimate the minimal (axial) shear forces PC-3 cells encounter in this model. These values are shown for each flow rate in Table 3.1.
Using this protocol, we analyzed the survival of the human prostatic carcinoma cell line, PC-3 after 10 passages of fluid shear stress (Figure 3.1A) and revealed a dose-dependent sensitivity of these cells to shear stress. When analyzing the survival curves of PC-3 cells over repeated shear stress passages (Figure 3.1B) we find little loss of viability at the lowest flow rate and a biphasic loss of viability at the highest flow rate. We confirmed the cell viability measurements acquired with BLI by using other techniques to assess cell viability following the shear stress protocol. We performed a WST-1 assay of mitochondrial function as well as clonogenic survival of control and shear stress-treated PC-3 cells. Results from these other methods mirrored acute changes in viability measurements obtained using BLI (Figure 3.1C). Figure 3.2 shows a comparison of PC-3 clonogenic plating with cells used in later figures.

Since shear force experienced by individual cells in this experiment could be influenced by neighboring cells in suspension, we evaluated PC-3 survival over a 10-fold range of cell concentration. We show that shear stress induced cell death is not affected by cell concentration between $5 \times 10^4$ and $5 \times 10^5$ cells/mL (Figure 3.1D). We also asked whether cell viability in the shear stress protocol is influenced by the confluence (growth phase) of cells prior to suspension. We trypsinized PC-3 cells at low and medium confluence, as well as those which had just reached full confluence (but not over-grown) and found similar shear stress survival (Figure 3.1D). To show that cell viability is not affected by material released by damaged or dead cells, fresh cells were suspended in cell-cleared medium from shear stress-treated cells. Cell viability of these suspensions was not different than cells in fresh media after 10 passages at 250μL/sec (Figure 3.1D). Finally, we show that viability of cells in our protocol is similar between those collected by trypsin and versene (non-enzymatic) treatment (Figure 3.1D). Collectively, these data show that loss of cell viability increases proportionally with the magnitude of shear stress in a manner independent of cell culture and suspension preparation.
To help validate our model, we compared the viability of cells in Figure 1A to PC-3 cells subjected to short pulses of shear force using a rheometer. Over a range of shear stress magnitudes, this approach caused a similar amount of cell death as our needle and syringe system (Figure 3.3).

Carcinoma cells of various histological origins exhibit unique resistance to fluid shear stress.

Next, we employed our shear stress protocol to test for differences in survival between carcinoma cell lines derived from metastatic prostate (PC-3 and 22Rv1), breast (MDA.MB.231), and melanoma (B16f0). Surprisingly, we found only small, insignificant differences in the survival of these cancer cell lines at the 250µL/sec flow rate (black bars in Figure 3.4A and B). We also included in this analysis two immortalized, but non-transformed cell lines of the human prostate (PWR-1E and RWPE-1) as well as primary cells of the human breast and prostate (HMEC and PrEC, respectively). We measured much greater cell death in these epithelial cell types versus all cancer cell lines (Figure 3.4A and B). Freshly isolated mouse red blood cells (RBCs), on the other hand, are robustly resistant to this level of shear stress (Figure 3.4A and B). Similarly, freshly isolated human leukocytes also display great resistance to shear forces (Figure 3.4A and B). This finding underscores the unexpected ability of these transformed epithelial cells to survive these magnitudes of shear stress.

As mentioned above, PC-3 cells exhibited a biphasic survival curve at flow rates above 20µL/sec. Impressively, we found a very similar shape in the survival curves of the other cancer cell lines tested. Meanwhile, cell death in the immortalized, but non-transformed cell lines was much more linear. Although the survival curves of primary cells also appear biphasic, nearly 90% of all cells in suspension had died by the second passage of shear stress, compared to an average of only 30% of cancer cells (Figure
3.4B). The percentage of cell loss per passage of shear stress is shown graphically in Figure 3.4C.

Resistance to detachment-induced cell death, or anoikis, is a hallmark of cancer (Hanahan and Weinberg 2000). We therefore compared the viability of non-shear stress-treated PC-3 cells to the non-transformed cell lines and primary cells over a one hour period in suspension. The viability of all cells tested was unaffected by detachment within 10 minutes in suspension and there was only a small amount of cell death during a 30 minute period, which is the time that it takes to perform the shear stress protocol at the lowest flow rate, which represents the longest assay flow rate (Figure 3.4D). Therefore, differences in shear stress survival between these cell types are not likely due to exacerbations in detachment-induced cell death.

We questioned whether the biphasic survival response of cancer cells was due to changes which occur in freshly suspended cells (Ren, Kiosses et al. 1999). We made a suspension of PC-3 cells and divided it into three aliquots; shear treatment was started as usual using the first aliquot. The next was sheared following the initial round, which took roughly 10 minutes. The third aliquot was held for one hour before shearing. Viability data for each aliquot was similar (Figure 3.5).

Finally, it is known that serial passage of human or mouse cancer cell lines through mice via systemic injection often selects for cells that exhibit enhanced metastatic potential (Fidler 1975; Fidler and Kripke 1977). We therefore addressed the possibility that experimental metastasis selects for cells of increased shear stress resistance by comparing the survival of PC-3, MDA.MB.231, and B16.f0 cells with their in vivo derivatives in our shear stress protocol. We detected no appreciable differences in shear stress resistance between parent cancer cells versus metastatic derivatives (showed alongside many other cell lines in Figure 3.6). This finding suggests that metastasis does not select for shear stress resistance, however we note that PC-3 and MDA.231 are both
derived from metastatic tumors, thus these cells have previously experienced circulation prior to passage in mice.

Shear stress resistance in carcinoma cells is transient and inducible

The observation that cancer cell death is precipitous over the first two passages of our shear stress protocol, but diminishes with subsequent passage led us to hypothesize that passage selects for shear stress-resistant subpopulations within the cancer cell lines. If this resistance phenotype has a genetic basis, then cells collected following the shear protocol should exhibit enhanced resistance to shear stress. However, we found this not to be the case. In several cell lines, exposing shear stress-surviving cells to a second round of shear protocol after recovery in culture demonstrated that survival was similar to the initial round of the shear protocol (Figure 3.7). This data suggests the interesting possibility that the dramatic reduction in the rate of cell death observed in cancer cell lines results from a transient, physiologic protective response to shear stress.

Repeated exposure to shear stress results in changes in cellular membrane integrity and induced resistance to shear forces.

Our observation of a biphasic viability curve in cells sheared at greater than 20µl/sec led to the hypothesis that cancer cells exposed to shear forces can elaborate changes conferring greater resistance to future shear forces, and thereby resist death by avoiding irreparable membrane damage. Implicit in our hypothesis was the ability of cells to repair a certain degree of membrane damage. To address our hypothesis, the shear protocol was conducted in the presence of propidium iodide (PI). Typically used to mark non-viable cells by virtue of its membrane-impermeability, we evaluated the accumulation of PI within viable cells to represent a cell population that sustained and
repaired membrane damage secondary to shear forces. Before the first, and after each subsequent passage, an aliquot of cell suspension was removed for FACS analysis. When analyzing cell suspensions for PI positive cells within a gate consistent with viability by forward and side scatter parameters, viable non-sheared PC-3 cells demonstrated minimal PI accumulation (mean 0.65% of viable cells, n=9, Figure 3.8Ai). After an initial shear passage, PI accumulated in 7.28% of the viable cell population (Figure 3.8Aii). With continuing passages, more PI accumulated, ultimately maximized after passage 10 with 36.73% of viable cells displaying positive staining (Figure 3.8Aiii). This data indicates that previously undamaged cells sustain membrane damage sufficient to allow PI uptake over repeated passages.

To confirm cell viability as gated by forward and side scatter gates, we employed the vital stain, Calcein AM. Both prior to shearing and after 10 passages, nearly all of the cells in our gate of interest were Calcein AM positive (99.8% and 99.2%, respectively), confirming that all of the cells in gates P1+P2 are, indeed, viable (Figure 3.8B).

When PI was withheld from cell suspension until after passage 9, we observed less uptake of the dye into sheared cells than after passage 1 in constant presence of PI (4.22% versus 7.28%, respectively; Figure 3.8A iv vs. ii). When plotted over repeated passages in constant presence of PI, there is less PI accumulation per passage, on average, in the intervals from passage 3-6 and 7-10 (3.25% and 2.92%, respectively) than in the first passage alone (6.64%). The gradual diminution of additional PI accumulation suggests that fewer cells are being damaged sufficiently to allow PI uptake, reflecting a smaller population eligible for membrane-damaging destruction. If this assumption is true, introduction of PI to cell suspensions at points intermediate in the assay would be expected to reveal less PI accumulation than observed when PI is added prior to passage one. In Figure 3.8C, PI was added either prior to passage 1, 6, 8, or 10, and then sheared to completion (10 passages). Regardless of when PI was added, the first passage in its presence elicited a pronounced increase in PI positivity; however, this increase was
significantly less than that seen at passage 1 in constant presence of PI (7.28% +/- 1.38% versus 3.62% p6, 4.90% p8, and 4.22% p10). Because the FACS analysis is performed on an equal number of viable cells at each passage, cell death is not responsible for the diminution of PI accumulation. Rather, these data suggest that during flow, PC-3 cells in our model experience a range of shear forces; some sufficient to alter the membrane integrity of a cell, but insufficient to cause irreparable membrane damage. The reduced uptake of PI into cells over later passages suggests that PC-3 cells can evoke an induced response that results in raising the threshold for both reparable membrane damage as well as irreversible, lethal damage.

Induced shear stress resistance requires extracellular calcium

Mechanical damage to plasma membranes can be repaired, in certain cell types, by a fusion of vesicles in a mechanism that requires the influx of extracellular calcium (Bement, Yu et al. 2007). This healing process has been reported to require the activity of Rho-GTPases and actin polymerization at the site of the wound (Terasaki, Miyake et al. 1997). We therefore asked whether the shear stress survival response we have observed in cancer cells also requires extracellular calcium. When PC-3 cells are suspended in nominally calcium-free PBS and subjected to our shear stress protocol, we observe a more steady loss of cell viability and an 8-fold increase in total cell death (Figure 3.9A). When suspensions of cells in PBS are supplemented with calcium at the same concentration as complete tissue culture medium we find a survival curve not significantly different from the media control suspensions (Figure 3.9A). These data suggest that shear stress damage to cancer cells triggers a survival response which requires extracellular calcium.

In complete medium, PC-3 cells exhibit little cell death at the 20μL/sec flow rate (as seen in Figure 1). However, when subjected to this flow rate in calcium-free PBS,
these cells exhibit a linear loss of viability with roughly 35% more cell death than in the presence of calcium (Figure 3.9B). This finding indicates that the protective shear stress survival response can be triggered at lower magnitudes of shear force, which may be more commonly encountered physiologically by CTCs.

We found that cells suspended in PBS supplemented with barium were equally susceptible to shear stress as cells in nominally calcium-free PBS (Figure 3.10), indicating specificity for the calcium cation in this survival response.

**Induced shear stress resistance requires actin polymerization**

Next, we queried whether rearrangements of the cytoskeleton occur in response to shear stress and thereby imparts a protective advantage to cells. In support of an active biological process, we found that cell suspensions which were held on ice, and thus expected to have reduced signaling kinetics, for 20 minutes prior to the shear protocol exhibited significantly greater loss of cell viability than under usual conditions (data not shown). To directly test the importance of cytoskeletal remodeling in our hypothesized shear stress response, we briefly treated PC-3 and MDA.MB.231 cells with cytochalasin-D prior to the shear stress protocol. During this time, cell viability was not affected by cytochalasin-D treatment. In both cell lines there was over 3-fold more cell death and an attenuated biphasic survival response in cytochalasin-D treated cells versus DMSO treated controls (Figure 3.11). Thus, shear stress-induced actin polymerization appears to play a protective role in cancer cells during conditions of flow.
Discussion

During hematogenous dissemination, CTCs encounter a wide range of shear stresses \((1-10^5 \text{ dyn/s})\) (Reneman, Arts et al. 2006; Schneider, Nuschele et al. 2007). Shear stress is a major component of the vascular microenvironment and has important biological implications; for example, endothelial cells are fine-tuned to shear stress and variations in the magnitude or frequency of shear forces have effects on the signaling, gene expression, and survival of these cells (Malek and Izumo 1992; Malek, Alper et al. 1999). Shear stress has also been shown to induce changes in the gene expression and adhesive properties of both leukocytes and cancer cells (Okuyama, Ohta et al. 1996; Avvisato, Yang et al. 2007; Stroka and Aranda-Espinoza 2010) Epithelial cells, from which carcinomas are derived, reside in environments with much lower shear stress than found in the bloodstream (Althaus, Bogdan et al. 2007). It is thus reasonable to believe such cells would be particularly susceptible to destruction by hemodynamic shear forces, as compared to naturally circulating cells (i.e. red blood cells and leukocytes). Indeed, it has long been assumed that loss of cancer cells during circulation is a large contributor to metastatic inefficiency, yet there are few studies on the response and survival of cancer cells exposed to fluid shear stress. This may be due, in part, to the difficulty of monitoring individual cell fate and survival in circulation \textit{in vivo}. One early study examined death of B16 melanoma cells subjected to shear stress using a viscometer (Brooks 1984). This report showed dose-dependent killing of cells, however, the earliest viability timepoints analyzed were after one hour of shear stress exposure. Studies focusing on immediate responses of cancer cells to these forces will be more physiologically relevant with regard to hematogenous dissemination.

We thus designed an \textit{in vitro} system to test the survival of suspension cells in fluid shear flow. With the use of a syringe pump, we repeatedly passed cell suspensions through a needle at a constant flow rate, allowing us to control the magnitude of shear stress.
force experienced by the cells. We reveal that most cancer cells, regardless of tissue origin and metastatic potential are constitutively resistant to flow conditions with wall shear forces up to \( \sim500\text{dyn/cm}^2 \). At increasing shear forces, up to \( \sim6,500\text{dyn/cm}^2 \), most of these cancer cells exhibit a biphasic loss of viability. This range of shear forces would be considered high to very high/supraphysiologic (Kamm 2002; Schirmer and Malek 2008). Despite the high magnitude of these values, it is important to note that freshly prepared red and white blood cells endured these forces greater than all cell types tested. During the first two passages of shear flow (each passage roughly between 1 to 6 milliseconds), the rate of cell death is six times greater than in the subsequent eight passages. After 10 passages at the highest shear rate, the average cell death is \( \sim60\% \) of the total suspension. The shape of these survival curves suggests either a selective purification of inherently shear stress-resistant cells, or an adaptive resistance to shear stress. To test these possibilities, we subcultured the surviving fraction of cells subjected to 10 passages of shear stress, and re-exposed these cells to a second round of shear protocol. We show in several cancer cell lines that this approach did not enrich for shear stress-resistant cells. This finding led us to hypothesize that the basis for the observed biphasic survival is an inducible and transient response rather than a stable genetic trait.

After shearing cancer cells in the presence of the membrane-impermeable dye, propidium iodide (PI), we show that viable cells allow rapid PI uptake during the first passage of shear stress, indicating altered membrane integrity. The rate of PI-uptake by viable cells diminishes over repeated passages, suggesting that membrane integrity is inducibly repaired and maintained after the initial “priming” round of shear stress. In support of this conclusion, cells which have been sheared several times prior to the addition of PI allow much less of this dye in than those cells in the presence of PI throughout the assay. To gain mechanistic insight into this shear-induced resistance to continued shear stress, we suspended cells in divalent cation-free PBS. These cells were roughly 10-fold more susceptible to shear stress and had lost the biphasic survival
response. When supplementing PBS suspensions with calcium or barium, we show that calcium addition selectively restores biphasic shear stress survival.

We next questioned if actin polymerization in response to fluid shear stress plays a role in the inducible shear stress-resistance response. Cells treated briefly with a non-cytotoxic dose of cytochalasin-D were significantly more susceptible to shear stress-induced death. Mechanical damage to plasma membranes has been shown to induce a repair response in cardiomyocytes, skeletal muscle cells, and oocytes (Terasaki, Miyake et al. 1997). In these cell types, extracellular calcium rapidly enters the membrane wound, triggering fusion of intercellular vesicles with the damaged plasma membrane domain. This membrane “patching” mechanism has been shown to require activation of small GTPases and rearrangements of the actinomysin cytoskeleton (Bement, Yu et al. 2007). It is possible that cancer cells have adapted such a mechanism to overcome fluid shear stress. Whether or not the calcium-dependent component of cancer cell shear stress damage resistance is couple to actin polymerization will need to be determined.

Importantly, we show that inducible shear stress resistance is unique to transformed epithelial cells. Non-transformed cell lines and primary cells of the human breast and prostate are susceptible to magnitudes of shear force much lower than those required to induce cancer cell death. These cell types exhibit dramatically greater cell loss over the first two passages of shear flow (upwards of 90% in primary cells) and do not elaborate resistance to shear stress at later passages as seen in most carcinoma cell lines. Thus, when comparing the behavior and survival of normal epithelial cells to carcinoma cells, we conclude that carcinoma cells 1) have an intrinsically higher resistance to shear stress-induced cellular damage, and 2) are capable of responding to damage from shear stress, resulting in a transient but efficient repair mechanism.

Perhaps the most striking finding of this study is that multiple cancer cell lines, derived from various tissues, and with a wide range of metastatic potential, exhibit a similar phenotype of shear stress resistance. There is not an obvious explanation for the
lack of shear stress resistance in some of the cancer cell lines we analyzed (i.e. PANC-1 and Jurkat cells). Nonetheless, it is likely that the conservation of this phenotype is intimately linked to cellular transformation. Common transforming oncogenes, such as Ras, AKT, etc. result in constitutive upregulation of Rho-GTPases and changes in cytoskeletal dynamics, and thus cell morphology and tensegrity (Tzima 2006; Cain and Ridley 2009). Mutations which drive primary tumor growth and invasion have been shown to co-opt for metastatic behavior, such as extravasation and angiogenesis (Gupta, Nguyen et al. 2007). It is possible that we have revealed a similar phenomenon with regard to survival during circulation. Our data suggest that killing of circulating tumor cells by hemodynamic shear forces is much lower than often estimated. This would argue that survival of such forces is not a large determinant of metastatic inefficiency.

The topic of metastatic inefficiency is clinically relevant to the study of circulating tumor cells (CTCs). Recently, there has been considerable interest in isolating and quantifying CTCs to develop new prognostic and predictive tools. One of the largest challenges here is that the mere presence of CTCs in the blood of patients does not always correlate with poor prognosis or metastasis. As an example, the number of circulating cells prior to surgery was shown to be predictive of relapse-free survival in breast cancer patients (Cristofanilli, Budd et al. 2004); and surgeries to remove non-small cell lung carcinoma have been reported to cause increased numbers CTCs in patients, which correlates with relapse (Rolle, Gunzel et al. 2005). Conversely, patients with ovarian or colon cancer who have surgical venous shunts, which introduce many (estimated up to millions) cancer cells into the blood every day, rarely develop metastatic disease (Tarin, Price et al. 1984; Tarin, Price et al. 1984). These data call into question the fate of CTCs which do not complete all steps of metastasis. At the simplest level, it is likely that these cells are either eventually killed in the bloodstream or that once colonizing a secondary tissue that they are unable to proliferate sufficiently to develop into a metastases.
If our findings can be extending to a clinical context, it is possible that all CTCs endure shear stress quite well, regardless of their metastatic capabilities. Thus when analyzing a patient’s blood for the presence of CTCs it should be considered that the absolute number of cells detected is a much less meaningful prognostic readout than understanding the ability of these circulating cells to extravasate and proliferate at a distant tissue. Furthermore, with the observation that transformed cancer cell lines are considerably more resistant to shear stress than primary cells, an optimistic possibility is that this biology could be exploited to improve the isolation and analysis of CTCs.
<table>
<thead>
<tr>
<th>Flow Rate (µL/sec)</th>
<th>Wall Shear Stress (dyn/cm²)</th>
<th>Minimum Shear Stress (dyn/cm²)</th>
<th>Time of passage (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>509</td>
<td>59.53216374</td>
<td>12.2</td>
</tr>
<tr>
<td>35</td>
<td>890</td>
<td>104.0935673</td>
<td>6.97</td>
</tr>
<tr>
<td>50</td>
<td>1,271</td>
<td>148.6549708</td>
<td>4.88</td>
</tr>
<tr>
<td>100</td>
<td>2,543</td>
<td>297.4269006</td>
<td>2.44</td>
</tr>
<tr>
<td>150</td>
<td>3,815</td>
<td>446.1988304</td>
<td>1.62</td>
</tr>
<tr>
<td>250</td>
<td>6,358</td>
<td>743.625731</td>
<td>0.89</td>
</tr>
</tbody>
</table>

Table 3.1: Shear stress values for increasing flow rates.
Figure 3.1: *In vitro* model of fluid shear stress induces cell death in a dose-dependent fashion. Suspensions of PC-3 cells were subjected to shear stress at a range in shear forces from $509-6.36 \times 10^3$ dyn/cm$^2$ (Table 1), and monitored for changes in viability. Survival is represented as percent viability of non-shear treated cells which are held in suspension for the duration of the assay. A) Endpoint viability after 10 passages at indicated flow rate. B) Survival over repeated passages at 20 and 250μL/sec. **p<0.01, *** p<0.001 vs. 20μL/sec. The time taken to perform 10 passages at each flow rate is indicated. C) After 10 passages at 250μL/sec, cell viability quantified via bioluminescence imaging (A-C) was compared using WST-1 assay and clonogenic plating. No significant differences between the results were detected. D) Cells were suspended in the following conditions before shear stress treatment: $5 \times 10^4$ vs. $5 \times 10^5$ cells/mL concentration from 70% confluence; 20-30%, 50-60%, or 100% confluence prior to suspension at $5 \times 10^5$ cells/mL (low/medium/high); after shearing cells ten times at 250μL/sec, the suspension was centrifuged to remove cells, and the supernatant was used to suspend a fresh plate of cells (sheared); cells were suspended non-enzymatically (versene). None of these treatments resulted in significantly different endpoint viability.
Figure 3.2: Clonogenic survival correlates with bioluminescence imaging viability data. PC-3, TEM4-18, and PrEC cells which had been subjected to 0, 2, or 10 passages at 250 μL/sec (6.36×10^3 dyn/cm²) were plated at clonal density. Colonies resulting from live, single cells were stained and scored. Data shown on graph is the average of three independent shear treatments and subsequent plating assays. This data correlates well with viability data derived via bioluminescence imaging.
Figure 3.3: Viability comparison: needle and syringe vs. rheometer models of shear stress.

PC-3 cell viability data shown in figure 1A (checkered bars) is compared to PC-3 cells treated at escalating doses of shear stress using a rheometer. All data were acquired using BLI and rheometer experiments were done three times.
Figure 3.4: Carcinoma cells of various histological origins exhibit unique resistance to fluid shear stress. A) A panel of transformed and normal epithelial cells and blood cells was compared for survival after 10 passages of fluid shear stress at 250μL/sec. Cancer cell lines exhibited robust resistance over other epithelial cell types. ***, p<0.001 vs. all cancer cell lines; #, p<0.001 vs. RWPE-1; ├, p<0.001 vs. all non-blood cells (one way ANOVA, Bonferroni post tests). B) The viability of all cells over repeated passage at 250μL/sec. C) The rate of cell death per passage of shear stress at 250μL/sec was averaged all epithelial cell types. The rate of cancer cell death significantly reduces and stabilizes after the second passage, whereas non-transformed cells remain constant. Primary cells lose nearly all viability over the first two passages. ***, p<0.001 vs. passages 1 to 2 of cancer cells; # p<0.05 vs. passages 1 to 2 of primary cells (one way ANOVA, Bonferroni post tests). D) To control for detachment-induced cell death, PC-3 and primary cells were held in suspension at room temperature without shear stress treatment for up to one hour. Loss of viability due to detachment over the first 30 minutes is negligible.
Figure 3.5 Response of PC-3 cells to shear stress is similar over a range of time post-suspension. An aliquot of freshly suspended PC-3 cells was analyzed for shear stress survival (1st). Once this first protocol was finished, another aliquot of the PC-3 stock was subjected to the protocol (2nd). One hour after suspension, a final round of shear protocol was performed (3rd). Regardless of time since preparing the cell suspension, the response of PC-3 cells to shear stress was similar. Survival is represented as percent viability of non-shear treated cells which are held in suspension for the duration of the assay.
Figure 3.6: Shear stress analysis of a broad panel of cells. Cancer cells derived from various epithelial tissues, as well as hematogenous origin were analyzed for survival over ten passages of shear stress at 250μL/sec. A) Endpoint viability and B) viability over repeated passages are indicated. For each cell line, survival is represented as percent viability of non-shear treated cells which are held in suspension for the duration of the assay. Cell lines obtained from experimental metastases in mice were included for PC-3 (AD, adrenal gland; LD, liver), MDA.MB.231 (LuD, lung), B16f0 (B16f10, 10-times serially passaged intravenously to lung), and 22Rv1 (BD, long bone). These *in vivo* derivatives do not exhibit increased shear stress resistance.
Figure 3.7: Shear stress resistance in carcinoma cells is transient and inducible. A) After 10 passages of fluid shear stress at 250μL/sec, surviving PC-C, PC-3 adrenal gland-derivative, and B16.f0 cells were allowed to recover in culture for 24-48 hours. These survivors were then compared for shear stress resistance in parallel with the corresponding shear stress-naïve control cells. Subculturing surviving cells did not enrich for shear stress resistance at 250μL/sec.
Figure 3.8: The cell-impermeable dye, propidium iodide, accumulates in cells exposed to shear stress in a selective and diminishing manner, reflecting changes in cellular membrane integrity and induced-resistance to shear forces. PC-3 cells subjected to shear stress in the presence or absence of propidium iodide (PI) were collected after each passage for flow cytometric analysis. PI was added to the cell suspension either prior to shear passage 1 (p1), 6 (p6), 8 (p8), or 10 (p10). Viable cells (P1+P2 gated), defined by cell size, shape, and density (forward scatter and side scatter), were evaluated for PI positivity. When exposed to PI constantly (Ai), p1 resulted in 7.28% of the population of viable cells accumulating PI intracellularly, as compared to non-sheared cells (0.65%, Ai). With sequential shearing, the p10 sample resulted in a final accumulation of PI in 36.73% in the viable cell population (Aii). In contrast, when PI was introduced to the cells prior to p10 (Aiii), only 4.22% of the cell population accumulated PI. B) To confirm that the P1+P2 gate represented only viable cells, and to eliminate the possibility that PI⁺-dead cells were contributing to our PI⁺ gate, the vital stain Calcein AM was used and confirmed that the P1+P2 gate was predominantly constituted by viable cells (p1 99.8%, p10 99.2%). C) When PI was added prior to passage 6 (pre-6), 8 (pre-8), or 10 (pre-10), less of the viable population of cells accumulated the dye with the first passage with PI. *, p<0.05 vs. 1constant (two-tailed, paired t-tests). This reflects a shear force-resistant phenotype that exists after 1-2 shear passages in a subset of remaining viable cells, and explains the biphasic change in cell viability observed over 10 passages (Figure 2b).
Figure 3.9: Shear stress-induced cancer cell membrane repair requires presence of extracellular calcium. A) PC-3 cells suspended in complete medium, calcium-free PBS, or PBS plus calcium (129.4μg/mL final concentration) were subjected to shear stress at 250μL/sec. The absence of calcium leads to significantly increased cell death whereas cells suspended in calcium-supplemented PBS exhibit cell survival similar to that of cells in media. B) The same experiment as described in A was performed at 20μL/sec. In complete medium, this flow rate induces little loss of viability in PC-3 cells; however in calcium-free PBS, cell death is rapid and linear. *, p<0.05; **, p<0.01; ***, p<0.001 vs. complete media (repeated measures ANOVA followed by Boneferonni’s post tests).
Figure 3.10: Shear stress resistance response specifically requires calcium PC-3 cells suspended in complete medium, calcium-free PBS, or PBS plus either calcium, magnesium, or barium (88mM final concentration) were subjected to shear stress at 250μL/sec. In PBS, shear stress induced is greatly enhanced. Only addition of calcium to PBS rescues the shear stress resistance phenotype.
Figure 3.11: Shear stress resistance requires actin polymerization. PC-3 (A) or MDA.MB.231 (B) cells were treated with 20nM cytochalasin-D for one hour before exposure to the shear stress protocol. Cytochalasin-D treated cells are much more susceptible to shear stress than DMSO control cell suspensions. **p<0.01, ***p<0.001 vs. DMSO control (one way ANOVA, Bonferroni post test).
CHAPTER IV

**IN VIVO DERIVATIVES OF METASTATIC CANCER CELL LINES EXHIBIT REDUCED MTOR SIGNALING, INCREASED AUTOPHAGY, AND REDUCED CELL SIZE**

**Introduction**

Metastasis is the leading cause of death in patients with epithelial cancers. The process of metastasis is complex; beginning with local tissue invasion, entrance into and survival within the bloodstream or lymphatic system, extravasation, and colonization and proliferation at secondary tissue (Chambers, Groom *et al.* 2002). Each of these stages is likely to exert pressures which progressively select successful metastatic cells from less aggressive variants in the tumor cell population. These include: an invasive phenotype, the ability to withstand physical deformation during invasion, survival in the hemodynamic environment of the bloodstream, and upon size restriction in capillary beds, and the ability to grow in a foreign tissue microenvironment. Accumulated evidence confirms that metastatic cells indeed possess traits resulting from some of these selective pressures that distinguish them from non-metastasis-competent cells.

We previously reported the isolation of a PC-3 prostate cancer cell subpopulation with enhanced ability to cross an endothelial monolayer *in vitro*. When injected systemically into the tail veins of SCID mice, these cells formed significantly more tumors than the parental cell line (Drake, Strohbehn *et al.* 2009). In the course of further investigations with this cell line, we found that this derivative was smaller (~23% by mean volume) than the parental cell line. This led us to hypothesize that smaller cancer cells may have enhanced metastatic potential. Reduced cell size may, for example,
facilitate invasion through tissues; enhance survival during hemodynamic shear stress; or reflect the proliferative status or metabolic adaptations of metastatic cells.

To address this possibility, we analyzed the sizes of a panel of epithelial cancer cell lines from various tissues and their derivatives obtained from experimental metastasis. Serial passage of human or mouse cancer cell lines through mice via systemic injection has long been known to select for cell lines that exhibit enhanced metastatic potential (Fidler 1975; Fidler and Kripke 1977). We found that metastatic derivatives of PC-3, MDA MB.231, and B16 cancer cell lines were smaller than their parental counterparts by an average of 18.4% by volume (range 6% to 25%). Cell size is a highly regulated physical trait, which varies greatly across species and within an individual multi-cellular organism, and is often coupled to proliferation (Hall, Raff et al. 2004). Here, we show that size differences between parental and derivative cell lines do not correlate with cell cycle progression. We did, however detect reduced activation of the mTOR (mammalian target of rapamycin) pathway in metastatic derivatives. This data is in agreement with the knowledge that mTOR signaling is a major regulator of cell size (Fumarola, La Monica et al. 2005; Lee, Inoki et al. 2007). Because mTOR signaling is commonly associated with tumorigenesis and progression (Guertin and Sabatini 2007), this data is surprising. However, mTOR also negatively regulates autophagy, a catabolic process of cellular self-degradation which can lead to nutrient independence under conditions of starvation as well as programmed cell death. Consistent with this, we detected increased autophagic flux in some of these metastatic derivatives. It is debated whether autophagy primarily plays a tumor-suppressor role or if it contributes to tumor progression. Our findings suggest that reduced mTOR signaling and subsequent upregulation of autophagy are selected for during metastatic colonization. Finally, we provide evidence that epithelial-mesenchymal transition (EMT), which contributes to invasive phenotypes in certain cancers (Thiery, Acloque et al. 2009) enhances autophagy.
Materials and Methods

Cell lines and reagents

All cancer cell lines were obtained from the ATCC and were transduced with an integrating retrovirus encoding firefly luciferase under control of the CMV promoter (pGEM, Promega). Cells were grown in the ATCC-recommended media supplemented with 10% fetal bovine serum and 1% non-essential amino acids. For maintenance of the retrovirus-gene expression, all cells containing this construct were maintained in 200 μg/mL Genetecin (Invitrogen). For all experiments, cells were grown to ~75% confluence and harvested by trypsinization following neutralization and suspension in complete medium. Pepstatin-A and E-64d (Sigma) and human recombinant TGFβ-I (Peprotech) were used as indicated in figure legends.

Coulter Counter cell size analysis

Cells were suspended to a concentration of 5x10^5 cell/mL and analyzed on a Coulter Counter (Beckman Coulter) at a 1:100 dilution in Isoton II (Beckman Coulter). Size analysis was performed using Z2 Acucomp software (Beckman Coulter). Gates were set to exclude cellular debris and aggregates following the manufacturer’s instructions. At least three separate cultures for each cell line were counted and sized in triplicate. Data was plotted using Prism Graph Pad software and cell lines were compared based on mean cell volume. Paired, two-tailed t-tests were used for analysis of statistical significance.
Flow cytometry analysis of DNA content and cell size

1mL of 5x10^5 cell/mL suspension was added directly to a 15mL tube containing 5mL of ice cold 70% ethanol. Ethanol fixation was performed at 4C for at least one hour. Cells were then washed in PBS twice and pellets were re-suspended in 300μL DNA buffer (500nM Tris-HCl + 0.75mM NaCl + 0.5mg/mL RNAse A + 50μg/mL propidium iodide) and incubated for 30 minutes on ice in the dark. Propidium iodide fluorescence intensity, corresponding to DNA content, was analyzed by running cells on a LSR flow cytometer (BD Biosciences), gating on live, single cells. To measure cell cycle distribution, propidium iodide histograms were analyzed using ModFit software to estimate the percentage of cells in G1, S, and G2-M phase of mitosis. To compare the size of cells using FACS, cells prepared as above were gated on G1 and forward scatter histograms were compared based on geometric mean. These experiments were done three times or more.

Western blot analysis

Cells were plated at 2x10^5 cells/well in 6-well plates and grown to ~70% confluence. To obtain whole cell extracts, 2% SDS protein lysates were collected. Following protein estimation, whole cell lysates (30 µg) were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in Odyssey blocking buffer (LiCor Biosciences) mixed 1:1 in PBS for 1 hour at room temperature followed by incubation with anti-β-actin antibody (Sigma, 1:5,000), phospho-specific p70S6KThr389 (Cell Signaling Technologies#9205, 1:1000), phospho-specific S6Ser235/236 (Cell Signaling Technologies#2211, 1:1000), or with anti-LC3B (1:200, Cell Signaling Technologies#2775) antibody overnight at 4°C in blocking solution + 0.1% Tween-20. The membrane was washed 3 times for 5 minutes in blocking
solution followed by incubation with Alexafluor-800 donkey anti-mouse (Roche, 1:10,000) or Alexafluor-650 goat anti-rabbit (Invitrogen, 1:1000) secondary antibody for 1 hour at room temperature in blocking solution. The membrane was then rinsed 3 times for 5 minutes in PBS with 0.1% Tween-20 followed by imaging in an Odyssey scanner (LiCor Biosciences). Quantification of signal intensity was done using Odyssey V3.1 software (LiCor Biosciences) per the manufacturer’s instructions.

Immunofluorescence microscopy of LC3 puncta

The indicated cell lines were plated onto 24-well dish coverslips treated with 15μg/mL rat collagen-I (BD Biosciences) at a density of 1.0x10^4 cells/well and allowed to grow for 48 hours. Cells were fixed with ice cold methanol for 10 minutes, washed in PBS, then blocked for nonspecific protein binding (PBS +1.0% BSA) for 30 minutes. Cells were then incubated with LC3B antibody (1:200, Cell Signaling Technologies#2775) overnight at 4 degrees Celsius, washed in PBS twice, and then incubated with Cy3-anti-rabbit antibody (Jackson Immuno Research) plus DAPI for one hour at room temperature. Cells were washed three times in PBS and then mounted to frosted glass slides using Fluorogel (Electron Microscopy Sciences #17985-10) and imaged using the appropriate filter sets.

Results

In vivo derivatives of metastatic cell lines are reduced in size

We have previously reported the isolation of the TEM4-18 cell line through a transendothelial migration assay (Drake, Strohbehn et al. 2009). In addition to demonstrating enhanced trans-endothelial migration, these cells also colonized tissues in
mice at a higher frequency than PC-3 cells following systemic injection. During a comparative analysis of these two cell lines, we found that TEM4-18 cells are ~25% smaller by volume than PC-3 cells (Table 4.1 and Figure 4.1A; $2614.67 \pm 51.49$ vs. $3375 \pm 62.18 \mu m^3$, respectively, $p<0.001$). This led us to question if smaller cancer cells have enhanced metastatic potential.

To determine if cell size is correlated with metastatic potential, we measured the size of a panel of human and mouse cell lines representing several tissues of origin and for each of them, metastatic derivatives obtained from in-vivo passage in mice (Table 4.1, Figure 4.1, Figure 2.1). PC-3 and 22Rv1 represent human prostatic epithelial cancer cell lines, with metastatic derivatives from the adrenal gland (PC-3 AD), liver (PC-3 LD), and bone (22Rv1 BD). MD.MB.231 (231) is a human mammary carcinoma cell line with a derivative of the lung (231 LuD). B16F0 is a mouse melanoma cell line and B16F10 is a ten-time in vivo passaged derivative of the lung (Fidler 1975). Our measurements revealed a 2-fold range of cell volume across these cell lines (1,600 to 3,375 $\mu m^3$). In four of six parent-derivative pairs, we found that derivatives were significantly smaller (Table 4.1, Figure 4.1). Only in the case of 22Rv1 cells, was the metastatic derivative larger than the parent (Figure 4.2A). It is notable that both 22Rv1 and its derivative are the smallest measured in our study. PC LD cells were subtly smaller than PC-3, but this difference did not reach significance (Figure 4.2B). In sum, these findings suggest that independent of tissue origin, experimental metastasis in mice tends to select for reduced cell size.

Cell cycle distribution does not correlate with cell size

Changes in proliferation rate can account for changes in cell size (Hall, Raff et al. 2004). We therefore considered the possibility that the size distribution of our cancer cells reflected differences in proliferation rate. Dysregulated cell proliferation is a
hallmark of cancer (Hanahan and Weinberg 2000) and because tumors are often heterogeneous, it is likely that cells within a tumor will differ in proliferative rate. To test this, we harvested exponentially growing cells (at the same confluence as those for size analysis) and quantified the DNA content of each cell line to estimate the percentages of cells in each phase of mitosis. Although there were significant differences between some parent and derivative cell lines, we did not detect a consistent shift towards a particular cell cycle distribution which correlated with mean cell size in this set of cell lines (Figure 4.3).

Another possible explanation for these observed size differences could be changes in ploidy. For example, if a parental cell line was tetraploid (4N), and its metastatic derivative were diploid (2N), one would expect a significant size difference. Indeed, there are some mammalian cell types which exhibit a linear correlation between ploidy and cell size (Epstein 1967). Organotropic metastatic derivatives of MDA.MB.231 have been reported to spontaneously become 4N after lung colonization, leading to increased cell size (Lu Organ-specific); in contrast to our studies, larger cells were enhanced in metastatic potential. Our DNA profiles did not reveal large shifts in the PI fluorescence intensity of G1 cells between derivative and parental cell lines, indicating maintenance of ploidy.

Metastatic derivatives exhibit reduced activity of mTOR signaling pathway

The AKT-mTOR signaling pathway is a major regulator of cell survival, proliferation, growth, and size (Kim, Sarbassov et al. 2002; Lee, Inoki et al. 2007). To investigate a molecular mechanism behind the reduction in size in metastatic derivatives, we compared the phosphorylation status of a direct target of mTOR, p70S6 kinase, and its target, ribosomal protein S6. The metastatic derivatives of PC-3, MDA.MB.231, and
B16 all had reduced levels of these phospho-proteins (Figure 4.4). This suggests that the mTOR pathway is attenuated in these lines, and thus have lower levels of ribosomal biogenesis and protein synthesis. Indeed, the protein content per cell is lower in several of the derivatives compared to their parental cell lines (data not shown). Furthermore, to show that this signaling pathway is intact, we treated cells with rapamycin (which binds to and inactivates mTOR) and found dramatically reduced phosphorylation of p70S6K and S6 and a slight reduction in cell size (Figure 4.5).

Autophagic flux is increased in metastatic derivatives of PC-3 and MDA.MB.231 cells

The finding that mTOR signaling is attenuated in the in vivo derivatives of PC-3, MDA.MB.231, and B16 is somewhat surprising, considering the association of PI3K/Akt/mTOR activation with tumor growth, survival, and aggressiveness (Inoki, Corradetti et al. 2005; Yuan and Cantley 2008). Since mTOR regulates the induction of autophagy (Arico, Petiot et al. 2001; Wullschleger, Loewith et al. 2006), we asked whether reduction in p70S6KThr389 and S6Ser235/236 levels correlates with increased autophagic flux.

To test this, we cultured cells in either complete or serum-free medium in the absence or presence of the lysosomal inhibitors, pepstatin-A and E64-d (“P/E”). These inhibitors act to prevent autophagosome turnover, thus allowing accumulation of LC3B-II, which is the most reliable biochemical marker of autophagic induction (Klionsky, Abeliovich et al. 2008; Nakatogawa, Suzuki et al. 2009). In the absence of P/E, a slight increase in LC3B-II can be detected in response to serum starvation in PC-3, MDA.MB.231, and their metastatic derivatives (Figure 4.6A and B), suggesting that all of these cell lines utilize autophagy to cope with nutrient deprivation. When including E/P in the growth conditions, it becomes clear that autophagic flux is higher in the
metastatic derivatives compared to parental cell lines (Figure 4.6C shows relative LC3B-II levels in cells grown in complete media). These data suggest that regardless of nutrient availability (serum starvation vs. nutrient-rich environments) metastatic derivatives have constitutively higher levels of autophagy. A supportive, although less quantitative means to analyze autophagy is by fluorescence microscopy imaging of LC3B puncta, which accumulate during autophagy (Kabeya, Mizushima et al. 2000). Staining of PC-3 and MDA.MB.231, as well as their derivatives, reveals increased LC3B puncta in all cells upon serum starvation. Further, in both growth conditions, metastatic derivatives appear to contain more puncta per cell (Figure 4.7). Surprisingly, despite differences in mTOR signaling, we did not detect a significant difference in autophagic flux between B16.f0 and B16.f10 cells, either biochemically or by immunofluorescence (data not shown).

Epithelial-mesenchymal transition leads to decreased cell size and increased autophagic flux in MDCK cells

The PC-3 LD and AD cell lines used in the studies above share many of the EMT-like characteristics of TEM4-18 cells, such as a fibroblastic-like morphology, loss of E-cadherin expression, and high levels of ZEB1 protein (Drake, Strohbehn et al. 2009). A growing body of evidence shows that EMT-like states plays an important role in the invasive phenotype of many epithelial cancers (Thiery, Acloque et al. 2009). Recent data suggest that EMT-like properties also play an important role in later stages of metastasis, such as extravasation and colonization (Drake, Strohbehn et al. 2009; Drake, Barnes et al. 2010). EMT results in loss of epithelial polarity and basement membrane adhesion, resulting in a migratory phenotype in epithelial cells. Disruption of such cell-extracellular matrix contacts is known to lead to detachment-induced cell death, referred to as anoikis (Frisch and Francis 1994). Autophagy has recently been shown to prevent anoikis in
cancer cells (Fung, Lock et al. 2008). We therefore questioned whether a connection exists between EMT and autophagy.

MDCK, which are canine renal epithelial cells, have been used for EMT studies by exogenous addition of TGF-β, a well described inducer of EMT (Wyatt, Wadham et al. 2007). We added either media or recombinant human TGF-β1 to MDCK cells for 72 hours. At this time, TGF-β-treated cells had undergone morphological changes characteristic of EMT, as well as lost expression of E-cadherin, and began to express ZEB1, a regulator of EMT (Figure 4.8A). Control and TGF-β-treated MDCK cells were analyzed on a Coulter Counter, and we found a ~20% reduction in cell size accompanying EMT (Figure 4.8B). Control and TGF-β-treated MDCK cells were then grown in the conditions described above for Western blot analysis of LC3B in PC-3 and MDA.MB.231 cells (Figure 4.8C and D). Lysates of TGF-β-treated MDCK cells contained a greater LC3B-II to β-actin ratio than control cells, strongly indicating that autophagic flux is increased in MDCK cells upon the induction of EMT.

**Discussion**

Here, we compared the size of human and mouse carcinoma cell lines with derivatives obtained from experimental metastasis in mice. In the case of PC-3 human prostate carcinoma, MDA.MB.231 breast carcinoma, and B16 mouse melanoma cells, we found that experimental metastasis tends to select for smaller cells within the parental population. There were exceptions to this observation, as 22Rv1 human prostate in vivo derivatives were slightly larger than the parental cell line (however these were considerably the smallest cell lines measured, perhaps indicative of a threshold). Using flow cytometry to analyze DNA content in these cells, we showed that differences in cell size were not correlated with changes in cell cycle progression.
To obtain molecular insight for the reduced cell size of metastatic derivatives, we interrogated the mTOR signaling pathway. mTOR is considered a master regulator of cell growth by virtue of its ability to increase the rate of translation and ribosome biosynthesis (Wullschleger, Loewith et al. 2006). We performed SDS-PAGE on lysates from several of our parent-derivative pairs and immunoblotted for activated (phosphorylated) mTOR effectors, p70S6 kinase and the ribosomal protein S6. Detection of these phosphoproteins was reproducibly less in metastatic derivatives versus parental cell lines. Quantification of western blot results revealed similar percentages of p70S6K and S6 attenuation as reduction in cell volume.

Cells derived from experimental metastatic tumors are often enhanced in their malignant properties (Fidler 1975; Fidler and Kripke 1977), and provide a platform for studying the cellular, molecular, and genetic traits unique to metastatic cells. We considered many possibilities to explain the observed selection of reduced cell size during metastasis. A smaller cell may have a survival or colonization advantage in hematogenous circulation; as it is likely to experience lower magnitudes of shear stress than a larger cell, or may be able to more easily negotiate barriers such as passage across the endothelium or its underlying basement membrane that are necessary for productive metastatic colonization. To test the possibility that these size differences have an impact on shear stress resistance, we repeatedly passed suspensions of each cell line through a 30 gauge needle at several constant flow rates, generating a range of shear forces from 500-6,350 dyn/cm². No correlation between cell size and the ability to survive shear stress in vitro was detected (data not shown). Furthermore, we have found no evidence that would suggest smaller cells are generally enhanced in trans-endothelial migration in vitro or in transwell migration. As an example, 22Rv1, which is the smallest cell line of this study, exhibit poor trans-endothelial migration when compared to the PC-3 parental cell line, which is nearly twice the size (Drake, Strohbehn et al. 2009).
An alternative explanation for this observation is that reduced cell size is a secondary characteristic of altered metabolism of metastatic cells. As we have shown, metastatic derivatives of three cancer cell lines, each from a different tissue-of-origin, exhibit reduced activity of the mTOR pathway, which regulates amino acid biosynthesis and glucose homeostasis (Wullschleger, Loewith et al. 2006). Although mTOR activity is often associated with cancer cell growth, proliferation, and survival (Guertin and Sabatini 2007) it also regulates the process of autophagy (Maiuri, Tasdemir et al. 2009).

Autophagy is a catabolic mechanism involving the degradation of a cell’s own macromolecules and organelles (Barth, Glick et al. 2010). Basal levels of autophagy are necessary for tissue homeostasis (Eileen White Refs); however, under conditions of nutrient deprivation or other environmental stress, increased autophagy can provide a cell with energy and metabolites necessary for survival and continued proliferation. Additionally, this process has been shown to inhibit apoptosis in matrix-detached epithelial cells (Fung, Lock et al. 2008) and contribute to dormancy of disseminated tumor cells (Lu, Luo et al. 2008); therefore autophagy is often thought of as a contributor to tumorigenesis. Conversely, autophagy has been proposed to have tumor suppressor functions, due to observations that heterozygous deletion of Beclin-1, a required component of autophagy, results in spontaneous tumor formation in mice (Qu, Yu et al. 2003). The precise role that autophagy plays in tumorigenesis remains debated and very little is known about its role in metastasis (Kenific, Thorburn et al. 2010). Since autophagy is regulated by mTOR and has been reported to lead to reduced cell size (Hosokawa, Hara et al. 2006), we hypothesized that the smaller metastatic derivatives would exhibit increased autophagy compared to parental cell lines.

During autophagy, cytosolic macromolecules and organelles are targeted to a specialized membrane, the phagophore, which elongates into a dual-layered lipid membrane, the autophagosome. Through a series of fusion events, autophagosomes continue to mature and ultimately fuse with lysosomes, leading to the digestion of
phagocytic cargo (Eskelinen 2005). This process resembles endosomal maturation following endocytosis, only begins with engulfment of intracellular material. The most widely used approach to study the levels of autophagy in cells involves biochemical and microscopic analysis of LC3 proteins. Upon induction of autophagy, LC3-I, an ubiquitin-like protein, undergoes phosphatidylethanolamine conjugation (becoming LC3-II) and is then inserted into the membranes of mature autophagosomes (Klionsky, Abeliovich et al. 2008). LC3-II, an ubiquitin-like protein is currently the only faithfully marker of mature autophagosomes (Klionsky, Abeliovich et al. 2008). To analyze autophagy in a semi-quantitative fashion, one must block either the fusion of autophagosomes with lysosomes (bafilomycin-A), or inhibit the proteolytic activity of lysosomal enzymes (pepstatin-A, E64d, etc.); this allows accumulation of LC3-II protein for detection. Autophagic flux (constitutive levels of autophagy in a given cell line) or autophagic capacity (maximum potential of autophagic induction during nutrient deprivation, for example) measurements can thus be made by comparing the relative levels of LC3-II across cell lines or treatments (Mizushima and Yoshimori 2007). Using this approach, we found that both autophagic flux and capacity were reproducibly increased in metastatic derivatives of PC-3 and MDA.MB.231 cells. Additionally, LC3 protein was visualized via fluorescence microscopy, revealing puncta (which act as a surrogate marker for autophagosomes) in all cell lines, but increased in derivatives.

An unexpected and curious finding was that B16f0 and f10 cells exhibit similar levels of autophagic flux, despite reproducible detection of reduced mTOR signaling in f10 cells. Because B16 are the only mouse cancer cells in our analysis, this finding may suggest that the observed increase in autophagic flux in PC-3 and MDA.MB.231 cells derived from experimental metastasis in mice is an artifact of xenotransplantation. Alternatively, this result may simply reflect a cell-type specific phenotype.

Our PC-3 liver and adrenal gland metastatic derivatives represent cells with an EMT-like phenotype (Referred to as 689.Li and GS694.LAd, respectively in Drake,
Strohbehn et al. 2009). EMT appears to play an important role in cancer metastasis, particularly during invasion (Thiery, Acloque et al. 2009), and emerging evidence supports a role for autophagy in metastasis (Kenific, Thorburn et al. 2010). To date, no correlation between autophagy and EMT has been reported. We treated MDCK epithelial cells with the potent EMT-inducer TGF-β, which lead to acquisition of a fibroblastic morphology, and loss of E-cadherin and increase in ZEB1 expression. This EMT was accompanied by a ~20% reduction in cell volume and increased autophagic flux as measured by LC3-II Western blotting. These data support a model for EMT-driven autophagy. Our findings in MDCK cells are in contrast to reports that TGF-β-induced EMT resulted in increased cell size in murine mammary epithelial NMuMG cells (Lamouille and Derynck 2007), thus suggesting that EMT-induced autophagy may be cell-type specific.

We conclude that carcinoma cells with increased autophagy have a selective advantage during metastatic colonization. mTOR activity is associated with inhibition of autophagy. It is often assumed that the metastatic microenvironment is one of low nutrient and oxygen availability. Reduction of mTOR signaling in such an environment would be expected to result in upregulation of autophagy. However, it seems unexpected that the reduction in mTOR signaling (roughly 40%) and increase in autophagic flux (roughly 2-fold) would remain stable in cell culture as we see in our metastatic derivatives. In the case of our lung-derived MDA.MB.231 cells, we observed maintenance of this phenotype for greater than 25 passages, suggesting a genetic or epigenetic basis. Indeed, there have been reports showing histone deacetylase regulation of autophagy in HeLa cells (Oh, Choi et al. 2008); perhaps epigenetic changes during tumor evolution could result in stable changes in autophagic flux. Finally, we may have revealed a regulatory connection between EMT, a driver of cancer invasion, and autophagy, a protective metabolic mechanism which benefits cells detached from their basement membrane and residing in nutrient deprived microenvironments.
<table>
<thead>
<tr>
<th>Parent and Derivative Lines</th>
<th>Volume (µm³)</th>
<th>Volume (% of parent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>3375 ± 62.18</td>
<td>100</td>
</tr>
<tr>
<td>PC-3 LD</td>
<td>3173 ± 36.73</td>
<td>94.01</td>
</tr>
<tr>
<td>PC-3 AD</td>
<td>2811 ± 28.17</td>
<td>83.29</td>
</tr>
<tr>
<td>TEM4-18</td>
<td>2615 ± 51.49</td>
<td>77.48</td>
</tr>
<tr>
<td>22Rv1</td>
<td>1610 ± 164.65</td>
<td>100</td>
</tr>
<tr>
<td>22Rv1 BD</td>
<td>1985 ± 92.65</td>
<td>123</td>
</tr>
<tr>
<td>MDA.MB.231</td>
<td>2721 ± 55.87</td>
<td>100</td>
</tr>
<tr>
<td>MDA.MB.231 LuD</td>
<td>2131 ± 48.83</td>
<td>78.32</td>
</tr>
<tr>
<td>B16.F0</td>
<td>3168 ± 114.0</td>
<td>100</td>
</tr>
<tr>
<td>B16.F10</td>
<td>2370 ± 60.31</td>
<td>74.81</td>
</tr>
<tr>
<td>MDCK Control</td>
<td>2232</td>
<td>100</td>
</tr>
<tr>
<td>MDCK TGFβ</td>
<td>2005</td>
<td>89.83</td>
</tr>
</tbody>
</table>

Table 4.1 Comparative Coulter Counter analysis of cell volume
Figure 4.1: Metastatic derivatives of several cancer cell lines are reduced in size. The cell volume of the following cell line pairs was measured using Coulter Counter analysis (left-hand column) and flow cytometry (right-hand column): A. PC-3 versus TEM4-18 (in vitro-derived from trans-endothelial migration assay) and PC-3 AD (an adrenal gland-derived tumor) B. MDA.MB.231 versus MDA.MB.231 LuD (a lung-derived tumor) C. B16f0 versus 10X in vivo-passaged B16f10. For Coulter Counter analysis, histograms represent mean cell size (averaged for 4 or more experiments). Flow cytometry histograms compare the geometric mean of forward scatter of cells in G1 (n=3 experiments/cell line). *p<0.05, **p<.01, ***p<0.001 vs. parental cell line.
Figure 4.2: Size analysis of 22Rv1 and PC-3 liver derivative cells: A) 22Rv1 and its bone-metastatic derivative, 22Rv1 BD. In contrast to the other parental-metastatic derivative pairs, 22Rv1 BD is slightly larger than its parental cell line; these two cell lines, however, are the smallest measured (see Table I) B) An additional PC-3 derivative of the liver, PC-3 LD, trends towards a mean cell volume smaller than PC-3, but insignificantly so.
Figure 4.3: Differences in mean cell size are not reflected by differences in cell cycle progression. A) Cancer cell lines and in vivo derivatives were analyzed for DNA content by flow cytometry as described in Materials and Methods. This data is used to calculate the percentage of cells in G1, S, and G2-M phase of mitosis. Each bar is representative of at least three independent cell preparations. B) Mitotic distribution was plotted against the volume for each cell line; linear regression analysis reveals that the percentage of cells in G1, S, and G2-M phase does not correlate with cell volume.
Figure 4.4: mTOR activity is reduced in metastatic derivatives. A) Western blot analysis of lysates from PC-3, MDA.MB.231, B16, and metastatic derivatives from each reveals reduced phosphorylation of the mTOR target p70S6 kinase, and ribosomal protein S6. p70S6K$^{Thr389}$, S6$^{Ser235/236}$, and β-actin were probed for on strips of the same membrane; the image shown is representative of multiple experiments, the mean results of which are shown in B. B) Signal intensity from western blots was quantified using Odyssey imaging software, and is plotted in terms of fold-change from parental cell line. Each bar represents the mean of at least four independent lysates. *p<0.05, **p<.01 vs. parent (each pair compared using paired, two-tailed t-test).
Figure 4.5: Rapamycin treatment reduces size of both parental and derivative cell lines. (A) Lysates were prepared from PC-3 and PC-3 AD cells treated with 4nM rapamycin for 24 hours. The image shown is from the same membrane, cropped to show relevant lanes and proteins. This is representative of several independent western blot experiments. (B) PC-3 or PC-3AD cells treated with either 4 or 40nM rapamycin for 24 hours reduces cell size by roughly 20% in both cell lines.
Figure 4.6: Autophagic flux is increased in metastatic derivatives. A) PC-3 and its adrenal-gland derivative and MDA.MB.231 and its lung-derivative (B) were grown in complete (+10% FBS) or nutrient deprived (-10% FBS) media for 4 hours in the presence or absence of pepstatin-A and E-64d (“P/E”, 10μg/mL each). Lysates were then prepared and analyzed for accumulation of LC3B-II, which is indicative of increased autophagy. C) Signal intensity of LC3B-II from cells grown in complete media +P/E was normalized to β-actin. This comparison indicates a constitutive increase in the autophagic flux of metastatic derivatives. This data is representative of three independent experiments. *p<0.05 (paired, two-tailed t-tests).
Figure 4.7: LC3B puncta in metastatic derivatives of PC-3 cells. Cells were treated with complete or serum-free medium for a period of four hours prior to methanol-fixation, and then stained with anti-LC3B antibody and the nuclear stain, DAPI. Fluorescent signal from secondary antibody reveals more cells per field of view with LC3B puncta and more puncta per cell in metastatic derivatives than parental cells in starvation conditions. All images were acquired using the same exposure duration and gain settings.
Figure 4.8: Epithelial-mesenchymal transition in MDCK cells results in reduced cell size and increased autophagic flux. MDCK cells were grown to 50% confluence prior to treatment with 10ng/mL recombinant human TGF-βI for 72 hours. A) Morphological changes and loss of E-cadherin expression and upregulation of ZEB1 indicate an EMT. B) Coutler Counter analysis reveals a 17.06% reduction in size by volume in TGF-βI-treated versus control cells (n=2). C) Lysates of control and TGF-βI-treated cells, grown in either complete or serum-free media in the absence or presence of pepstatin-A and E64-d (“P/E”, 10μg/mL each) were analyzed for LC3B-II content. D) LC3B-II signal intensity from membranes was quantified and normalized to β-actin. In response to TGF-βI-treatment, relative levels of LC3B-II increase in all growth conditions, which indicates an increase in autophagic flux subsequent to EMT.
CHAPTER V

CHARACTERIZATION OF AN AGGRESSIVE SUBPOPULATION OF PROSTATE CANCER CELLS WITH AN EPITHELIAL-TO-MESENCHYMAL AND STEM CELL-LIKE PHENOTYPE

Introduction

Although tumors are thought to originate from one or only a few cells, most are composed of cells with heterogeneous phenotypes. There are several prevailing theories on the basis of tumor heterogeneity, including the cancer stem cell hypothesis, and clonal evolution. The former states that rare cancer cell subsets, with stem cell traits, are able to differentiate into all cell types found within the tumor, while maintaining self-renewal (Reya, Morrison et al. 2001; Wicha, Liu et al. 2006). The clonal evolution model, on the other hand, states that through genetic instability, each cell within a tumor can stochastically acquire unique phenotypic differences, some which may be selected for. Although these theories are often debated, it is becoming appreciated that they need not be mutually exclusive, and that the tissue of tumor-origin may dictate the basis for phenotypic diversity (Shackleton, Quintana et al. 2009). Regardless, tumors from many tissues contain subpopulations of cells which are enriched in tumor initiating and aggressive properties compared to the bulk of the neighboring cells. The identification of such cells will lead to improved prognostics and specific therapeutic treatments.

We previously reported the isolation of a subpopulation of human prostate PC-3 cells via a trans-endothelial migration (TEM) assay, an in vitro model of extravasation (Drake, Strohbehn et al. 2009). These cells were enhanced in TEM and also colonized tissues at a higher frequency than parent PC-3 cells when systemically injected into mice. Microarray analysis of RNA from these cell lines revealed many interesting differences
in transcriptional regulation, including loss of E-cadherin expression in TEM4-18 cells (Drake, Strohbehn et al. 2009). Further, when viewed microscopically, TEM4-18 cells appear to have morphological characteristics like that of an epithelial-to-mesenchymal transition (EMT). Further analysis of microarray data revealed that TEM4-18 cells overexpress ZEB1, a known regulator of EMT. Functional analyses showed that RNAi-mediated knockdown of ZEB1 led to a partial reduction of the EMT-like phenotype, and reduced TEM.

Recent studies have revealed a connection between EMT and stem cell-like phenotypes in cancer cells (Mani, Guo et al. 2008). This connection has been shown to be regulated, in part, by the micro-RNA 200 family, whose transcript targets include ZEB1 and ZEB2 (SIP1) as well as Notch signaling components (Brabletz and Brabletz 2010) and polycomb complex genes (Wellner, Schubert et al. 2009; Iliopoulos, Lindahl-Allen et al. 2010) which play an important role in stem cell renewal.

Here, we investigate the expression of known stem cell markers, primarily those correlated with tumor-initiating potential, in TEM4-18 cells. We show that these cells express robust levels of LGR5, a marker of adult stem cells of the intestine, colon, and hair follicle. Compared to the bulk PC-3 population, TEM4-18 cells exhibit a CD44^{high}/CD24^{low} profile, which is indicative of tumor-initiating potency. Consistent with the recently described link between EMT, miR-200 expression, and stem cell behavior, we found that miR-200a, b, c, and miR-429 are reduced in TEM4-18 cells. Finally, we provide evidence that ZEB1 regulates the expression of stem cell markers and aggressive behavior, both \textit{in vitro} and \textit{in vivo}. These studies may reveal novel transcript and protein biomarkers for the identification of prostate cancer cells enriched in tumor initiation and metastatic potential.
Materials and Methods

Cell lines

PC-3 prostate adenocarcinoma, TEM4-18, and GS689.Li cells all express firefly luciferase via stable retroviral transduction, and have been described previously (Drake, Strohbehn et al. 2009). All cells were grown in DMEM/F-12 (GIBCO) supplemented with 10% FBS (HyClone), 1 mM non-essential amino acids (GIBCO), and supplemented with 400 μg/mL G-418 at 37°C and 5% CO₂.

Quantitative RT-PCR (qRT-PCR)

Sequences of primers to human ZEB1 and ECadherin have been reported previously (Drake, Strohbehn et al. 2009). Sequence to human LGR5: forward, 5’-CTCGTGCCCCCTACTTC-3’; reverse, 5’-AGCACAGGGCAAGGACAGG-3’. RNA was extracted from low passage cells using an RNeasy mRNA isolation kit (Qiagen). Reverse transcription was carried out using iScript cDNA synthesis kit (BioRad). The resulting cDNAs were used as PCR template using CYBR Green I (Invitrogen) and data was collected on iCycler thermal cycler (BioRad). Experimental values were normalized to GAPDH values. Relative expression values were calculated using a comparative Ct method (Pfaffl 2001).
Flow cytometry

Cells were detached using 2 mL of 0.48 mM Versene/10cm dish and allowed to incubate for 5-10 minutes. The cells were then harvested, resuspended in 10 mL of serum-containing DMEM/F12 medium and pelleted at 700 x g for 5 minutes. 5 x 105 cells per tube were placed into 1.5 mL eppendorf tubes and spun down at 700 x g, 4°C, for 5 minutes. Supernatant was removed and cells were suspended in 50 µl FACS buffer (PBS + 0.02% sodium azide + 5% BSA) + either integrin α2 (1:200), CD24-FITC, or CD44-PE antibodies (Sigma, 1:200). The cells were then incubated for 20 minutes on ice in the dark, washed with 1 mL of FACS buffer, and pelleted for 5 minutes at 700 x g, 4°C. For non-directly-conjugated primary antibodies, cells were then suspended in 50 µl FACS buffer + secondary antibody (1:100, goat anti-mouse FITC, Chemicon) and incubated for 20 minutes on ice in the dark. Cells were washed again with 1 mL of FACS buffer followed by a 5 minute spin at 700 x g, 4°C, resuspended in 400 µl of FACS buffer, and transferred to a 12 x 75 mm polystyrene FACS tube (BD Biosciences). Samples were analyzed using the Becton Dickinson FACScan at The University of Iowa Flow Cytometry Core Facility using the WinMDI software (Purdue University).

Proliferation and soft agar colony formation assays

For measurement of cell proliferation, cells were plated into 96-well plates at a density of 5,000 cells per well (day 0). Proliferation was assessed every day (days 1-7) with (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate (WST-1, Roche Applied Science) as directed. For measurements of cell growth in soft
agar, cells (5,000 cells/well in 0.3% agar in complete medium) were plated onto 0.5% base agar in six-well dishes. Colonies were stained at 14–21 d post-plating, with 0.005% crystal violet and 0.1% citric acid in ddH2O. Colony formation was visualized, and scored by two blinded counters the following day.

Trans-endothelial migration assay

Primary HMVEC-Ls (3.5 × 10^4 cells) were plated onto collagen IV (Sigma) coated 8 μm 24-well transwell inserts (Costar) and allowed to form a monolayer over 3-5 days. Integrity of the endothelial monolayer was evaluated by measuring electrical resistance as follows: Using an EVOM Voltmeter (World Precision Instruments) and Endohm-6 transwell insert cup (World Precision Instruments) resistance across the endothelial monolayer was measured on each transwell insert; with a target resistance of greater than 10 Ω/cm^2 prior to use in transendothelial migration assays. Prior to plating onto HMVEC-Ls, prostate cancer cells were detached with 0.48 mM Versene (Gibco) for 10-15 minutes. Cells were then resuspended in complete DMEM/F12 media, spun at 200 x g for 5 minutes, and resuspended in EGM media at a concentration of 5 x 10^5 cells/mL. Prostate cancer cells (1 x 10^5, 200 μL) were added onto the HMVEC-L monolayer and allowed to incubate for 18 hours prior to analysis of migration. A standard curve was performed by serial dilution of prostate cancer cells (10,000 to 20 cells) in a 96-well dish followed by bioluminescence imaging (BLI) in a Xenogen IVIS100 imaging system (Caliper Life Sciences). To quantify migration using BLI, transwell inserts were placed into a new 24-well dish containing trypsin (400 μL, 10 minutes at 37°C). After 10 minutes, trypsin was neutralized with 600 μL of serum-containing DMEM/F12 medium and each insert was washed with medium. 100 μL of sample, in duplicate, from each well
was then added to a black 96-well dish followed by addition of 100 μL of luciferin (0.3 mg/mL). BLI was determined following a 5 minute luciferin incubation. Cell quantification was performed by converting the BLI signal from the sample into the standard curve to derive the number and percent of total cells migrated.

Metastatic colonization and xenograft implantation

We performed all procedures involving animals according to The University of Iowa Animal Care and Use Committee policies. Using a 27 gauge needle, we injected 200 μL (1 x 10^6) of cell suspension into the tail vein into 5-8 week old male scid mice (Taconic). We performed BLI in a IVIS100 imaging system (Caliper Life Science) as described previously (Drake et al., 2005). Whole body tumor growth rates were measured as follows: A rectangular region of interest was placed around the dorsal and ventral images of each mouse and total photon flux was quantified using Living Image software v2.50 (Xenogen) with the units of photons/sec. The dorsal and ventral values were then summed and plotted weekly for each animal. Total photon flux was quantified using Living Image software with the units of photons/sec. The values were plotted weekly for each animal. To adjust for the 2-fold difference in BLI intensity between vector control and shZEB1-GS689.Li cells, we multiplied the photon flux in the control group accordingly. For subcutaneous tumor models, PC-3E or TEM4-18 cells (2.5 x 10^5 or 5 x 10^4) were injected into the subcutis of each rear flank of male athymic nude mice (5 per group, NCI).
Results

TEM4-18 cells express high levels of the stem cell marker, LGR5

TEM4-18 cells are an aggressive subpopulation of PC-3 cells isolated via serial passage through a trans-endothelial migration (TEM) assay. These cells exhibit significantly enhanced TEM and tumor formation in vivo, compared to bulk PC-3 cells, and also display morphological and phenotypic characteristics of epithelial-mesenchymal transition (EMT) (Drake, Strohbehn et al. 2009). Since EMT of cancer cells has recently been shown to correlate with stem cell-like traits (Mani, Guo et al. 2008), we asked whether known stem cell markers were differentially expressed in TEM4-18 versus PC-3 cells.

Analyzing microarray data (described in Drake, Strohbehn et al. 2009), we found that one of the most upregulated transcripts was LGR5. LGR5 has elegantly been shown to mark stem cells in the crypts of the small intestine and colon (Barker, van Es et al. 2007), in the hair follicle (Jaks, Barker et al. 2008), and most recently, in the stomach (Barker, Huch et al. 2010). Expression of this gene has also been correlated with colon and ovarian cancer (McClanahan, Koseoglu et al. 2006). To confirm our microarray data, we performed quantitative real-time PCR (qPCR) on cDNA from PC-3 and TEM4-18 cells. We found that LGR5 mRNA is overexpressed roughly 75-fold in TEM4-18 versus PC-3 cells; the latter did contain low, yet detectable levels as indicated by cycle threshold values (Figure 2.1). This magnitude of LGR5 overexpression suggests that TEM4-18 cells are representative of a small percentage of the total PC-3 population. This finding suggests that LGR5 may be a novel marker of prostate cancer cells with an aggressive phenotype.
Knockdown of LGR5 does not affect aggressive behavior of TEM4-18 cells

Although the ligand of LGR5 is not known and a function has not been described, LGR5 null mice exhibit neonatal lethality due to ankyloglossia (which inhibits the ability to breast feed) and gastrointestinal distention (Morita, Mazerbourg et al. 2004). Transcript levels of LGR5 are shown to correlate with aggressiveness in colon cancer (McClanahan, Koseoglu et al. 2006; Uchida, Yamazaki et al. 2010); and in basal cell carcinoma, knockdown of LGR5 was reported to reduce proliferation and tumor xenograft formation in mice (Tanese, Fukuma et al. 2008). We therefore asked whether LGR5 serves a functional role in the aggressive phenotype of TEM4-18 cells.

Using lentiviruses encoding shRNAs which target LGR5, we were able to stably reduce LGR5 expression by more than half in TEM4-18 cells (Figure 2.2A). The proliferation rate of LGR5 knockdown cells was compared to vector controls (Figure 2.2B) and no changes were observed. We then tested the effect of knockdown on soft agar colony formation (Figure 2.2C) and TEM (Figure 2.2D); assays commonly used to assess tumorigenicity and metastatic extravasation, respectively. LGR5 deficient TEM4-18 cells did not behave differently than vector controls in these assays, suggesting that LGR5 does not functionally contribute to the malignant phenotype of TEM4-18 cells.

TEM4-18 cells exhibit a CD44high/CD24low profile

The ability to discriminate tumor-initiating from benign cancer cells within bulk populations will lead to improvements in prognostics and therapy. Potent tumor-forming cancer cells have been prospectively sorted out of bulk tumors of the brain (Singh, Hawkins et al. 2004), colon (Dalerba, Dylla et al. 2007), and breast (Al-Hajj, Wicha et al. 2003) by exploiting hierarchical expression of surface proteins present on these cell
types. The CD44\textsuperscript{high}/CD24\textsuperscript{low} profile has been shown to mark such cells in breast cancer (Al-Hajj, Wicha et al. 2003; Shipitsin, Campbell et al. 2007), and has since become used to identify “cancer stem cells”. We stained TEM4-18 and PC-3 cells with antibodies recognizing CD24 and CD44 and quantified surface expression differences using flow cytometry (Figure 2.3). We found that compared to PC-3, TEM4-18 cells exhibit an enriched CD44\textsuperscript{high}/CD24\textsuperscript{low} phenotype.

Epithelial stem cells of the prostate have been shown to express high levels of α2β1 integrin (Collins, Habib et al. 2001). We were unable to detect differences in the levels of α2 integrin between PC-3 and TEM4-18 cells (Figure 2.3); however, all cells within the PC-3 population appear to uniformly express α2β1 integrin.

Epithelial-mesenchymal transition is correlated with stem cell like traits in TEM4-18 cells

As stated above, emerging evidence suggests that EMT and tumor initiating (stem cell-like) phenotypes are correlated. Mechanistic studies suggest that master regulators of EMT promote the expression of stem cell-related genes. For example, Twist has been shown to lead to increased β-catenin and CD44 expression in breast cancer (Zhou and Li 2011) and ZEB1 leads to increased Notch signaling in pancreatic cancer (Brabletz and Brabletz 2010). miR200 miRNAs, which target ZEB1&2, also regulate several genes required for self-renewal of stem cells; furthermore ZEB1 and miR200 reciprocally inhibit one another, thus creating a complex regulatory network for EMT and stemness (Brabletz and Brabletz 2010; Iliopoulos, Lindahl-Allen et al. 2010; Lo, Yu et al. 2010).

Since TEM4-18 cells display an EMT-like phenotype, we hypothesized that miR200-family micro-RNAs (miRNAs) would be down-regulated compared to FACS-sorted E-cadherin-expressing PC-3 cells (PC-3E). This latter population represents the most epithelial-like subpopulation within PC-3. Using qPCR, we showed that this was
indeed the case (Figure 2.4). We showed that there is an inverse correlation between miR200 family miRNAs and ZEB1 expression. Likewise, we showed that high levels of miR200s correlate with E-cadherin expression (Figure 2.4).

This finding led us to question if ZEB1 and LGR5 expression are related. We have previously shown that shRNA-mediated knockdown of Zeb1 in TEM4-18 cells leads to a partial reversal of EMT, restoration of E-cadherin, and reduced TEM efficiency (Drake, Strohbehn et al. 2009). While LGR5 knockdown has no influence on ZEB1 mRNA levels, ZEB1 knockdown led to a similar reduction in LGR5 mRNA (Figure 2.5). This finding is in support of a regulatory loop between EMT- and stem cell-like states in prostate cancer.

ZEB1 knockdown reduces colony formation and metastatic colonization in vivo

We next tested the effect of ZEB1 knockdown on soft agar colony formation. This assay directly tests the growth of epithelial cells in an anchorage-independent environment; the ability to do so is often correlated with survival and proliferative properties in vivo. Vector control TEM4-18 cells formed colonies robustly, whereas PC-3E cells are about 25% as efficient (Figure 2.6). This data is in agreement with the finding that when low numbers of cells are injected into nude mice, TEM4-18 cells form more subcutaneous tumors than PC-3E cells (Table 2.1). ZEB1 knockdown in TEM4-18 reduced colony formation to levels similar to that of PC-3E cells (Figure 2.6).

Finally, we asked whether ZEB1 enhances metastatic colonization. As shown previously, ZEB1-deficient TEM4-18 cells are inhibited in TEM (Drake, Strohbehn et al. 2009), which leads us to believe that ZEB1 plays an important role in extravasation. We knocked ZEB1 expression down in GS689.Li cells, which are an in vivo derivative of PC-3 obtained from a liver metastases. These cells share many of the same characteristics as
TEM4-18 and were chosen because they form experimental lesions in mice relatively efficient. ZEB1-deficient GS689.Li cells formed fewer tumors and at a reduced rate when compared to vector control cells (Figure 2.7). Collectively, these data suggest that ZEB1 drives an EMT-like state in prostate cancer cells and this contributes to metastatic behavior in vitro and in vivo.

Discussion

This study focuses on the molecular characterization of an aggressive subset of PC-3 cells, TEM4-18. These cells exhibit enhanced TEM in vitro and are significantly more efficient at tumor formation in vivo when compared to PC-3 cells (Drake, Strohbehn et al. 2009). TEM4-18 cells exhibit morphologic and molecular traits reminiscent of EMT. Recent evidence suggests that cancer cell EMT correlates with stem cell-like traits, such as self-renewal and enhanced tumorigenesis (see above). We therefore questioned if TEM4-18 cells exhibit markers used to identify tumor-initiating cells, or “cancer stem cells”.

A comparative genome-wide microarray was performed on RNA from PC-3 and TEM4-18 cells (Drake, Strohbehn et al. 2009). Focusing on differentially expressed stem cell-related genes, we noted a dramatic upregulation of LGR5 in TEM4-18 cells. LGR5, also known as GPR49, is an orphan G protein-coupled receptor with a leucine-rich ectodomain (Hsu, Liang et al. 1998). Originally identified as a Wnt-target gene in colon cancer (McClanahan, Koseoglu et al. 2006; Van der Flier, Sabates-Bellver et al. 2007), LGR5 has been shown to reliably mark stem cells of the adult human intestine, colon, stomach, and hair follicles (Barker, van Es et al. 2007; Barker, van Es et al. 2008). We did not detect differences in proliferation, TEM, or anchorage-independent growth in LGR5-knockdown TEM4-18 cells. This led us to conclude that LGR5 acts as a marker of
aggressive prostate cancer cells, rather than providing an important functional role in tumor growth or metastasis.

Additionally, we showed that TEM4-18 cells exhibit a CD44_{high}/CD24_{low} profile when compared to bulk PC-3 cells. This led us to compare the tumor initiating potential of PC-3E and TEM4-18 cells. At low injection numbers, TEM4-18 cells formed twice as many subcutaneous tumors as PC-3E cells. Although this difference is not large, the titrations were not low enough to reveal the minimum cell number required to form tumors in these cell lines. The CD44_{high}/CD24_{low} phenotype was first shown to mark tumor initiating cells in breast cancer (Al-Hajj, Wicha et al. 2003). More recently, CD44_{high} cancer cells of the prostate (Patrawala, Calhoun et al. 2006; Li, Chen et al. 2008) and colon (Dalerba, Dylla et al. 2007) have been shown to exhibit self-renewal and tumor-initiation. It is becoming appreciated, however, that this marker not always reliably identifies tumor-initiating cells, and that other markers should be looked at alongside CD44 (Levin, Powell et al. 2010).

After establishing that TEM4-18 cells express several markers of tumor initiating cells, we asked whether this phenotype is correlated with EMT. Interestingly, we detected reduced expression of LGR5 upon Zeb1 knockdown. This is the first evidence that we know of that ZEB1 regulates expression of LGR5; however, this finding is consistent with reports that ZEB1 promotes a de-differentiated phenotype by direct regulation of epithelial polarity genes (Aigner, Dampier et al. 2007). EMT is regulated, in part, by the expression of miR-200 miRNAs, which target ZEB1 and ZEB2 (Hurteau, Carlson et al. 2007). Recently, the miR-200s have shown to promote epithelial differentiation through their silencing of multiple stem cell genes (Yi, Poy et al. 2008). This led us to hypothesize that these miRNAs regulate both EMT and the stem cell-like traits in TEM4-18 cells. Indeed, qPCR revealed that miR-200a and b and miR-429 are significantly reduced in TEM4-18 versus PC-3 parental cells; future work will determine the
functional consequences of “antagomiR” silencing of miR200s in these cells. These data further underscore the complex role of micro-RNAs in tumorigenesis and metastasis.

Finally, we tested the role of ZEB1 in soft agar colony formation and metastatic colonization in vivo. ZEB1 knockdown in TEM4-18 cells results in a partial reversal of EMT (Drake, Strohbehn et al. 2009; Drake, Barnes et al. 2010) as well as reduction in LGR5, thus by extension, ZEB1 deficiency should correlate with a partial loss of stem cell-like functional traits. Concomitant with their differences in xenograft formation, PC-3E cells are much less efficient at soft agar colony formation than TEM4-18 cells. RNAi-mediated ZEB1 knockdown in TEM4-18 cells led to colony formation similar to that of PC-3E cells, indicative of reduced tumor formation potential. Future studies will aim to confirm that ZEB1-deficient TEM4-18 cells form fewer subcutaneous tumors than control cells, as has been documented in pancreatic cancer cell lines (Wellner, Schubert et al. 2009). To test for a role of ZEB1 in metastatic colonization, ZEB1-knockdown and control in vivo passaged PC-3 were injected intravenously into SCID mice. ZEB1-knockdown led to significantly reduced tumor size and frequency.

Collectively, our results indicate that EMT regulates stem cell markers and characteristics in prostate cancer cells. We may have revealed a novel combination of markers, LGR5^+CD44^{high}CD24^{low}α2^+, which label aggressive subsets of prostate cancer cells. In order to validate this as a signature of tumor-initiating cells, our studies will need to be extended to other prostate cancer cell lines. Additionally, if cells with this phenotype are important in vivo, we should be able to detect small subsets of them in tissue samples. We have shown that Zeb1 plays a critical role in the aggressive behavior of prostate cancer cells in vivo. ZEB1 augments trans-endothelial migration and survival in vitro and also appears to regulate stem cell features. We argue that cells with EMT- and stem cell-like phenotype have an increased propensity for metastatic extravasation and colonization; this finding has important implications in the study of circulating tumor cells.
Figure 5.1: TEM4-18 cells express the stem cell marker, LGR5. Quantitative real-time PCR was used to confirm microarray data to show upregulation of LGR5 in TEM4-18 cells. ***, p<0.001 vs. PC-3 (paired t-test). Graphs are representative of at least three independent RNA preparations; error bars represent standard error of the mean. The numbers represent cycle threshold ratios of LGR5/GAPDH.
Figure 5.2: Knockdown of LGR5 does not affect aggressive behavior of TEM4-18 cells. A) TEM4-18 cells were stably transduced with either vector control or anti-LGR5 shRNA-encoding lentivirus. Two independent shRNA constructs (sh1 and sh3) led to significant reduction in LGR5 mRNA. *, p<0.01 vs. vector control (one way ANOVA followed by Bonferonni’s post tests). Error bars represent standard error of the mean. Vector control and sh3-expressing TEM4-18 cells were compared for differences in B) proliferation rate, C) colony formation in soft agar, and D) trans-endothelial migration. LGR5-deficiency did not affect any of these assays.
Figure 5.3: TEM4-18 cells exhibit a CD44$^{\text{high}}$/CD24$^{\text{low}}$ profile. PC-3 and TEM4-18 cells were compared for surface expression of CD44, CD24, and α2 integrin via flow cytometry. CD44 is increased TEM4-18 while CD24 is reduced, indicative of a tumor-initiating phenotype. α2 integrin, a marker associated with stem cells of the adult prostate, is expressed similarly in PC-3 and TEM4-18 cells.
Figure 5.4: TEM4-18 cells express reduced levels of the stem-inhibiting miR-200 family of miRNAs. Quantitative real-time PCR was used to measure levels of miR200 a,b,c, miR429, Zeb1, and E-cadherin in PC-3E and TEM4-18 cells. *, p<0.05; **, p<0.01; ***, p<0.001 vs. PC-3E cells (one way ANOVA followed by Bonferroni’s post tests). Error bars represent standard error of the mean.
Figure 5.5: Knockdown of ZEB1 leads to reduction in levels of LGR5 mRNA in TEM4-18 cells. TEM4-18 cells expressing shRNAs targeting either Zeb1 or LGR5 were analyzed for mRNA expression of these two transcripts at early and late passages using quantitative real-time PCR. While LGR5 knockdown has no effect on ZEB1 expression, ZEB1 knockdown significantly reduces LGR5 expression at early passages after transduction of shRNA constructs. In later passages, however, LGR5 expression returns to that of the vector control, despite maintenance of LGR5 mRNA knockdown.
Figure 5.6: Knockdown of ZEB1 in TEM4-18 cells reduces soft agar colony formation to level of PC-3E cells. Cells suspended in soft agar were allowed to form colonies for a period of two weeks. Colonies formation was scored by counting crystal violet stained agar plates. Colony formation was normalized to the number of colonies in shControl plates, set to 100%. This experiment is representative of three independent experiments, each counted in a double-blinded fashion. *** p<0.001 vs. control (shCTL) TEM4-18 cells (one way ANOVA followed by Bonferroni’s post tests). Error bars are representative of standard error of the mean.
Table 5.1: TEM4-18 cells form xenografts more potently than PC-3E cells.
The values in the lower box indicate the number of subcutaneous
tumors formed at a given cellular concentration (n=5 mice/group.)

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Number of cells injected subcutaneously</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5x10^4</td>
</tr>
<tr>
<td>PC-3E</td>
<td>3</td>
</tr>
<tr>
<td>TEM4-18</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 5.7: ZEB1 knockdown reduces metastatic colonization in vivo. ZEB1 was knocked down in GS689.Li, a metastatic in vivo derivative of PC-3. These cells, as well as vector controls, were injected into the tail veins of mice and tumor colonization and growth was monitored via bioluminescence imaging. A) Mice injected with ZEB1 deficient cells exhibited less tumor burden, indicated by total body photon flux (n=8 mice per group). B) This table indicates the location of tumor formation in individual mice within each group. Collectively, these data show that ZEB1 knockdown reduced tumor size and frequency. *, p<0.05; ***, p<0.001 vs. shCTL (Repeated measures ANOVA followed by Bonferroni’s post tests).
Metastasis, which is the leading cause of death in patients with solid tumors, begins with invasion of tissue and blood vessels by cells of the primary tumor, followed by extravasation and colonization of a secondary tissue. Once colonized, cancer cells must survive and proliferate in order to establish a metastatic tumor. My thesis projects focus on the migratory phenotypes of aggressive cancer cells, extravasation, and survival within the circulatory system. I conclude by describing unique molecular and physical characteristics of metastatic cells.

We began by providing a detailed molecular and cellular characterization of the migratory behavior of a subset of PC-3 prostate cancer cells, TEM4-18. These cells were previously derived in our lab by serial passage of PC-3 human prostate cancer cells through a trans-endothelial migration (TEM) assay. This assay involves plating cancer cells onto an established monolayer of human endothelial cells which rests on a porous (8µm) transwell filter. By isolating cells which migrate to the underside of the filter, we were able to study cells with a propensity for extravasation. Despite their increased metastatic potential \textit{in vivo}, a paradoxical finding was that TEM4-18 cells are poor at migration through extracellular matrix proteins and non-treated transwells, which are \textit{in vitro} measurements of metastatic aggressiveness (Drake, Strohbehn \textit{et al.} 2009). We hypothesized that TEM4-18 cells lack a pro-migratory factor, which is present within the heterogeneous PC-3 population. In support of this idea, when PC-3 cells were cultured over time on transwell membranes prior to non-enzymatic removal, we found that TEM4-18 cells migrated through these “conditioned” membranes. We thus investigated transcriptional differences between PC-3 and TEM4-18 which would explain this migratory phenotype. We detected a loss of the pro-migratory basement membrane.
molecule, laminin-γ2 (a subunit of laminin-332), and its cell surface receptor, integrin-β4 in TEM4-18 cells. These genes were shown to be novel targets of the transcription factor ZEB1. To show the specific role of laminin-332 in this context, transwells were treated with exogenous laminin-332 which rescued the migratory deficit of TEM4-18 cells. These results could be recapitulated by mixing TEM4-18 cells with an E-cadherin-expressing subpopulation of PC-3 cells (PC-3E), which were shown to produce high levels of laminin-332. Finally, ZEB1-deficient TEM4-18 cells were shown to be less migratory on exogenous laminin-332, as were ZEB1-knockdown Du145 cells, which maintain low-level expression of laminin-332. These results are in agreement with the predominant view that ZEB1 activity is associated with pro-invasive functions.

Intriguingly, our results argue that context-dependent cooperation between cancer and normal cells as well as extracellular matrix components in the microenvironment may be necessary for full aggressive potential of cells with epithelial-mesenchymal-transition (EMT)-like features. In the specific case of TEM4-18 cells, loss of laminin-332 and integrin-β4 greatly reduces autonomous invasive migration of these cells in vitro. However, these cells maintained expression of integrin-α3, another receptor for laminin-332. Thus, in a physiological context, TEM4-18 cells need only to come into contact with laminin-332 provided in-trans by neighboring cells to invade effectively. Normal epithelial cells of the human prostate, as well as several stromal cell-types indeed deposit laminin-332 (Hao, Yang et al. 1996). Laminin-411 and -511, additional pro-migratory ligands of integrin-α3, are present in the subendothelial matrix and may also aide in intravasation and/or extravasation. It is thus feasible that such cooperative migration may facilitate each step of metastasis. A model of cooperative migration and EMT in the process of metastasis is provided in Figure 6.1.

Another report has been published which supports a model for cooperation between epithelial and mesenchymal cancer cells in the process of metastasis (Tsuji, Ibaragi et al. 2008). Surprisingly, the study by Tsuji et al. showed that EMT-like cells are
only required for invasion, allowing epithelial cells entrance into the bloodstream. Although cells of both type intravasated, only the epithelial cells were able to extravasate and form metastatic tumors (Tsuji, Ibaragi et al. 2008). This is in contrast to our studies which show that EMT-like cells are enhanced at trans-endothelial migration, and are fully capable of metastatic colonization. This discrepancy may argue that the role of EMT in metastasis is cell-type-specific (prostate vs. hamster cheek cells). Unlike the spontaneous metastasis model of Tsuji et al., we injected our cells directly into the tail veins of mice. It is possible that circumventing the process of invasion may eliminate negative selection of certain cell types and lead to an overestimation of metastatic potential. However, preliminary data from our lab shows that TEM4-18 cells injected orthotopically into the anterior prostates of mice are able to spontaneously metastasize to the liver whereas PC-3E cells remain confined to the injection site (Figure 6.2). Interestingly, histology reveals stark differences between PC-3E tumors, which appear to be encapsulated, and TEM4-18 tumors, which contain a diffuse cell distribution (Figure 6.3). Importantly, TEM4-18 cells at the metastatic site were shown be E-cadherin negative, supporting maintenance of the mesenchymal state.

Collectively, these results argue that changes accompanying an EMT-like state are capable of driving all steps of metastasis. Support for this comes from work by Acevedo et al. developed a genetic mouse model of inducible prostate-specific EMT. They found that induction of EMT resulted in step-wise disease progression, beginning with prostatic intra-epithelial neoplasia (mPIN) and ultimately, distant metastasis. Importantly, metastatic cells were shown to maintain their EMT phenotype, characterized by their lack of E-cadherin and spindle-shaped morphology (Acevedo, Gangula et al. 2007). Our own work also shows that knockdown of ZEB1, which results in a partial reversal of EMT, leads to significantly reduced metastatic colonization in an aggressive PC-3 derivative.
Although our studies support a role for EMT in the process of prostate cancer metastasis, this is a topic of some debate, stemming from the fact that a true EMT, characterized by inducible and reversible transdifferentiation, has yet to be observed in prostate cancer progression. Furthermore, histological examination of secondary tumors resulting from the dissemination of prostate cancer cells reveals predominant epithelial features (De Marzo, Knudsen et al. 1999). Nonetheless, EMT-like states are observed in prostate cancer, characterized by loss of E-cadherin, and gain of mesenchymal markers, namely, N-cadherin (Tomita, van Bokhoven et al. 2000) and vimentin (Zhang, Helfand et al. 2009). Our future studies will test for EMT in prostate cancer by transduction of cancer cells with a strong epithelial phenotype with fluorescent reporters driven by E-cadherin, vimentin, and N-cadherin promoters. These cells will then be implanted orthotopically into the mouse anterior prostate and tumor growth will be monitored using BLI as described previously in this thesis. If EMT occurs during tumor growth or invasion, we would expect reduction of the E-cadherin reporter and activation of the vimentin (or another mesenchymal gene-driven) reporter. If metastases result (which would be the case for a cell line such as PC-3), histologic examination of these tumors will reveal whether the EMT phenotype is maintained upon metastatic colonization. Finally, this type of reporter model could be used in vitro to further elucidate the cooperative migration model.

The theme of the next part of my thesis is survival of cancer cells in the circulatory system. The inevitable step between invasion and extravasation is dissemination through the circulation (either through blood or lymphatic vessels). As detailed in the introduction, the fate of CTCs remains controversial. Many reviews on the process of metastasis make mention of CTCs being rapidly cleared by hemodynamic shear forces (Gupta and Massague 2006), thus “survival during circulation” is often considered a critical determinant to metastatic efficiency. However, there are those that challenge this assumption ((Chambers, Groom et al. 2002); for an example of a point-
counterpoint debate on this topic, see (Weiss 1993)). This debate continues, in part, due to the difficulty of studying the survival of individual circulating cells in vivo. We thus designed an in vitro model to focus directly on the response of cancer cells to shear stress. By repeatedly passing cell suspensions through a 30 gauge needle at a controlled flow rate, we were able to compare the sensitivity of various cancer and normal cell types over a 10-fold range of shear forces. A striking observation of these studies was that every cancer cell line initially analyzed was quite resistant to high levels of shear stress (suspensions remained ~40% viability after 10 passages at 6,358 dyn/cm², which is a value measured physiologically, yet likely present in few anatomical regions). Intriguingly, each of these cancer cell lines exhibited a biphasic survival curve, characterized by precipitous cell death over the first two passages, followed by a slow and stable rate of cell death over the remaining passages. Primary and non-transformed cells, however, did not display this survival phenotype and were susceptible to shear-induced cell death at much lower magnitudes. We showed that this observation is due to an inducible response to shear stress rather than the selection of genetically shear-resistant subpopulations. We provided mechanistic insight into this survival response by displaying a requirement for extracellular calcium as well as dynamic actin reorganization.

The primary impact of these findings is that transformed epithelial cells are uniquely resistant to fluid shear stress. Of note, the majority of our cancer cell lines behaved similarly, although there were rare exceptions to this finding, such as the pancreatic carcinoma cell line, PANC-1. The conservation of this mechanism across numerous cancer cell lines, derived from various tissues, leads us to the hypothesis that the process of transformation (which initiates tumorigenesis and countless changes in cell signaling (Hanahan and Weinberg 2000) results in a shear stress-resistance phenotype. We sought to determine if transformation of normal (i.e. PWR-1E or RWPE-1) cells with oncogenic RASV12 will lead to enhanced shear stress resistance. Thus far, these
experiments have not shown survival differences between vector- and RAS$^{V12}$-expressing PWR-1E cells. However, we have not done the appropriate controls to show that RAS$^{V12}$ transduced cells are actually transformed; thus future experiments will test the ability of these cells to form colonies in soft agar and/or subcutaneous tumors in immune-compromised mice. If these cells are unable to perform these tasks, this explains the lack of a result in shear stress experiments. In contrast, if these cells do lead to colony and tumor formation, this would indicate that the shear stress resistant phenotype is not due to transformation per se.

Common oncogenes, such as RAS, play an important role in the physiologic response of endothelial cells to shear stress, in part through regulation of small GTPases of the Rho family, RhoA/B/C, Rac1/2, and Cdc42 (Tzima 2006). PI3K signaling, which is often unregulated in cancer, also activates these GTPases (Cain and Ridley 2009). Changes in the activation state of these molecular switches has a marked effect on gene expression, cytoskeletal dynamics, and membrane permeability of endothelial cells (Tzima 2006). It is therefore possible that these signaling networks, which are commonly augmented in transformed cells, contribute to cancer cell shears stress resistance. In support of our hypothesis, we found that inhibition of actin polymerization dramatically reduces shear stress survival. Furthermore, specific inhibition of the Rho-GTPase effector ROCK reproducibly reduces shear stress resistance in both PC-3 and MDA.MB.231 cells (Figure 6.4A). ROCK-inhibitor-treated cells still exhibit a biphasic survival curve, although percent viability is significantly less than control cells at most passages. This suggests that molecules other than ROCK and its targets regulate this survival response. Similar results were seen in a single experiment by treating cells with toxin-B (an inhibitor of RhoA and C, Figure 6.4B). These results indicate an important role for GTPase activation in the inducible shear stress response of cancer cells. To directly test whether shear stress results in activation of Rho, we prepared lysates of control and sheared PC-3 cells and attempted to immunoprecipitate GTP-bound (active) Rho.
Unfortunately, the Western blot did not show successful Rho-pulldown in any samples. These experiments will be revisited in the future.

We investigated additional signaling pathways which may play a role in the response of cancer cells to shear forces. JNK and ERK, members of the MAPK stress activated protein kinases, are known to become activated in response to mechanical stress in cardiac cells. Additionally, JNK and ERK signaling leads to many transcriptional and post-transcriptional changes, and converges with Rho-GTPase signaling and cytoskeletal regulation (Ruwhof and van der Laarse 2000). We prepared lysates of control and shear stress treated PC-3 and B16.f10 cells and performed Western blot analysis with antibodies specific to the active forms of JNK and ERK. Surprisingly, we found that shear stress led to reduced levels of phosphorylated ERK in B16.f10 cells. Sheared PC-3 cells, on the other hand, exhibited increased phosphorylation of JNK (Figure 6.5) ERK activation appeared unchanged in sheared PC-3 cells as did JNK in sheared B16.f10 cells. These preliminary data suggest that the activity of stress-activated kinases is altered in response to shear stress, and in a cell-type specific fashion. These changes may be important in the survival response of cancer cells, but it is also possible that these kinases are simply being chaotically activated and inactivated due to this mechanical stress. Future experiments will employ specific small molecule inhibitors to elucidate the role of JNK/ERK, and other MAP kinases, in the shear stress responses of cancer cells.

An interesting finding is that cancer cells require the presence of extracellular calcium in order to promote the downstream survival response when exposed to shear stress. Mechanical stress has been shown to induce calcium influx in other cell types (Tatsukawa, Kiyosue et al. 1997; Bement, Yu et al. 2007). In cardiomyocytes, skeletal muscle, and oocytes, rapid calcium influx, following stress-induced membrane damage, triggers the fusion of intracellular vesicles to damaged membrane domains. This “patching” process has been shown to require dynamic reorganization of the actinomysin cytoskeleton (Terasaki, Miyake et al. 1997; Bement, Yu et al. 2007). We hypothesized
that this type of repair process may be upregulated in carcinoma cells, resulting in robust resistance to shear stress. Calcium influx promotes release of intracellular stores through calcium-induced-calcium release processes (Endo 2009). Together, both influx and release of calcium activate signal transduction pathways, including MAPK (Ruwhof and van der Laarse 2000). Therefore, it is possible that calcium-dependent signal transduction is important in shear stress survival instead of or in addition to calcium-activated membrane patching.

Because we showed that membrane integrity is transiently altered in viable cancer cells during shear stress treatment, we can assume extracellular calcium enters these cells in passive fashion. However, stretch-activated ion channels (SACs) have been described in cardiac cells (Sigurdson, Ruknudin et al. 1992) and may allow the specific entry of calcium into cancer cells during shear stress treatment. Some of these channels can be blocked using drugs such as streptomycin (Gannier, White et al. 1994), and future studies could be done to examine the role of SACs in this process. To test that increases in intracellular calcium levels lead to sustained shear stress resistance, cells will be treated with titrating levels of ionomycin (which allows calcium entry via release of intracellular stores (Morgan and Jacob 1994)) and then shear stress treated. In these experiments we may expect a reduction in the amount of cell death at early passages, because intracellular calcium has accumulated without the need for shear stress-induced membrane permeabilization.

The more that we investigate the molecular mechanism responsible for the survival response of cancer cells, the clearer it becomes how utterly complex and cell-type specific this mechanism likely is. Therefore, it may be more fruitful to concentrate future experiments on cellular responses which are consistent amongst cell lines under shear stress, rather than attempting to dissect the entire signaling pathway(s) which result in resistance. Our data show that actin polymerization and Rho-GTPases are necessary for efficient shear stress survival and therefore lead us to consider that cells transiently
alter their physical state during shear stress, thus “shielding” them from mechanical damage. We aim to determine if the viscoelastic properties, or “stiffness”, of cells change after being subjected to shear at the high flow rate (250µL/sec). This will be determined by collecting cells at various passages and probing the membrane of individual cells with micropipettes with an end diameter of roughly 8µm. By applying negative pressure to a cell, a piece of its membrane will be taken into the pipette. Differences in viscoelasticity correlate with the amount of backpressure required to stretch a given amount of membrane. We would expect that after two passages of shear stress, which is the inflection point of the cancer cell survival, most cells will have become stiffer than non-sheared controls. These experiments will be done in collaboration with the labs of Drs. Sarah Vigmostad and Krishnan Chandran in the College of Engineering.

Additionally, to visualize cells during shear flow, we plan to employ a model where cell suspensions are passed through glass micropipettes, which are being imaged using light and fluorescence microscopy. By mixing cancer cells with whole blood, we will be able to study the response of cancer cells to shear stress in a more physiologic context. We may observe interactions between cancer cells and platelets, which is assumed to shield cancer cells and contribute to the resistance to shear stress (Nash, Turner et al. 2002). It is also possible that cancer cells interact with one another during flow, which may contribute to their response to this force. During laminar flow, shear stress is greatest at the wall and minimal at the axis of the channel. At high levels of shear stress, red blood cells orient along the axis of flow whereas platelets accumulate along the vessel wall, this process is referred to as “platelet skimming” (Aarts, van den Broek et al. 1988; Kamm 2002). It is not clear how cancer cells distribute during laminar flow. Using our original model, we were unable to report this distribution due to the fact we cannot image through a steel needle. The glass-pipette approach would allow us to map cancer cell distribution over a range of shear forces, which will provide a function to estimate the shear forces experienced by individual cells.
The remainder of my thesis focuses on physical and molecular traits unique to metastatic cells. Early morphologic comparisons of PC-3 and TEM4-18 cells revealed an EMT-like appearance, as described above. Additionally, TEM4-18 cells appeared to have a reduced surface area compared to PC-3 cells. To determine if this was because these cells are actually smaller or if this was only an effect of differences in cell spreading, we measured the size of these cell lines using a Coulter Counter and flow cytometry. TEM4-18 cells were indeed ~75% the volume of the bulk PC-3 population. This led to our initial hypothesis that smaller cells are selected for during metastasis. We then compared the size of cells derived from experimental metastasis in mice with their respective parental input cell lines. In support of our hypothesis, we found that in most cases the derivative cells were smaller than the parent; this was true for cancer cells of the human prostate (PC-3), human breast (MDA.MB.231), and mouse skin (B16 melanoma), and the exception was another human prostate cancer cell line (22Rv1). To help explain these size differences, Western blots were performed to analyze activity of the mTOR pathway. Our results demonstrated a negative correlation between the level of phosphorylated mTOR targets proteins and the mean size of individual cell lines, consistent with the regulation of cell growth by mTOR. However, these results are somewhat surprising because increased mTOR activity has been associated with enhanced tumor growth and aggressive behavior (Guertin and Sabatini 2007). The selective advantages a smaller cell would have during metastasis seem intuitive. A smaller cell could perhaps negotiate invasion through matrix proteins and cell junctions with less steric hindrance. However, data from our lab would suggest there is no correlation between cell size and matrigel invasion, blank transwell migration, or trans-endothelial migration. In this light, it seems unlikely that cell size per se would alter the metastatic properties of a cancer cell. Therefore, we began to hypothesize that the reduced size of metastatic cells was a trait secondary to a selected metastatic phenotype.
In addition to its regulation of translation and ribosome biogenesis, the mTOR pathway is known to inhibit autophagy, a self-degrading catabolic process (Rabinowitz and White 2010). Using Western blot and immunofluorescence we showed that the smaller derivatives of PC-3 and MDA.MB.231 cells are more autophagic than their parental cell lines. Both in conditions of nutrient-rich growth and during starvation, derivative cells contained a higher content of mature autophagosomes. As detailed in the introduction, autophagy has a debated role in tumor progression. Its activation allows cells to survive in nutrient-depleted conditions, and in anchorage-independent states, but also slows tumor growth and blunts progression-promoting inflammatory responses. Most research on autophagy in cancer has focused on the site of the primary tumor and in the invasive front, while there is very little known about its possible role in metastasis (Kenific, Thorburn et al. 2010). Our data lead to our current hypothesis, that this altered metabolic state facilitates survival and self-sufficiency during the process of colonization.

Curiously, despite reduced mTOR activity and cell size, we did not detect an increase in autophagy in B16.f10 cells compared to the f0 parent. Two possibilities explain this: 1) since autophagy within the B16.f0 parent is already very high, an increase beyond this level may lead to autophagic cell death; or 2) the observed increase in autophagy within PC-3 and MDA.MB.231 derivative cells is an artifact of xenotransplantation. To discriminate between these possibilities, future experiments will be performed to test the autophagic state of additional syngeneic carcinoma cell lines and their metastatic derivatives (this can be done using the mouse prostate carcinoma line, RM1, and the mouse mammary carcinoma line, 4T1). Experiments also need to be conducted to determine if growth of PC-3 and MDA.MB.231 in a subcutaneous site leads to changes in cell size, mTOR activity, and autophagy. If cells derived from xenograft tumors exhibit a phenotype similar to the parental input cells, it suggests that growth in a mouse host does not necessarily lead to increased autophagy. This would solidify the hypothesis that individual CTCs with increased autophagy are selected for upon
metastatic colonization. To directly test this hypothesis, we will inhibit autophagy by transducing PC-3 and MDA.MB.231 cells with shRNAs targeting ATG5, which is absolutely required for autophagosome formation (Hosokawa, Hara et al. 2006). We expect that these ATG5-deficient cells will colonize tissues at a lower frequency than vector controls, and that the resulting tumors will be smaller in size. Other possible outcomes may be that increased autophagy is selected for in certain metastatic sites and not others. It would be interesting if such metastatic sites correlate with the degree of tissue vascularization.

If data from these future experiments support for our current hypothesis, it would be of interest to inject mice systemically with cancer cells, such as PC-3, and begin a rapamycin treatment regimen. Rapamycin is a potent and specific inhibitor of mTOR, and is shown to activate autophagy (Jung, Ro et al. 2010). Because mTOR promotes cell growth, proliferation, and migration, it is not surprising that rapamycin and other drugs which inhibit mTOR signaling (“rapalogs”) are the subject of numerous clinical trials (Easton and Houghton 2006). Some of these trials have showed positive results with regard to inhibiting primary tumor growth and in some cases, resulting in partial tumor regression (Dancey 2002). Our results may reveal that rapamycin treatment leads to increased colonization of CTCs. These findings would have implications on the timing of rapamycin treatment; perhaps targeting mTOR in non-invasive primary tumors is an effective therapeutic approach, but once cancer cells have invaded and are present in circulation, this treatment may cause a selective advantage during the remaining steps of the metastatic cascade.

Our results provide evidence, for the first time, that EMT may regulate autophagy by showing increased autophagic flux in TGFβ-treated MDCK epithelial cells. As discussed in detail in the introduction, EMT is believed to drive invasion in many cancer types. However, this process leads to enhanced cell migration, in part due to forcing detachment of cells from their extracellular matrix substrates. This process, however
could make cells more prone to detachment-induced apoptosis (anoikis), as well as lead cells away from their nutrient-rich primary tissues. Autophagy protects cells from anoikis and from starvation, thus it seems logical that upon EMT, autophagy may be increased as well. Future experiments are needed to solidify our findings in MDCK cells. Something we have yet to reconcile is the report by Lamouille and Derynck, showing that TGFβ-treatment of mouse mammary epithelial cells (NMuMG) led to increased cell size, which was explained by upregulation of mTOR signaling (Lamouille and Derynck 2007).

Although the authors did not assess autophagy in these cells, it would be predicted that an increase in mTOR would lead to decreased autophagic flux. We will recapitulate our experiments using NMuMG cells, as well as other epithelial cell lines, such as PrEC (primary prostate) and BPH-1 (benign prostatic hyperplasia). It is possible that we will reveal cell type-specificity with regard to EMT regulation of autophagy.

Finally, we analyzed TEM4-18 cells for markers characteristic of a tumor-initiating, or cancer “stem cell” phenotype. Rationale for these studies comes, in part, from the fact that TEM4-18 cells represent a subpopulation of the heterogeneous PC-3 cell line; exhibit enhanced aggressive properties; and present EMT-like traits. Recent studies have revealed a connection between EMT and stem cell-like phenotypes in cancer cells (Mani, Guo et al. 2008). Analysis of our microarray data revealed dramatic upregulation of the stem cell marker, LGR5 in TEM4-18 cells. Furthermore, these cells were enriched for a CD44\textsuperscript{high}/CD24\textsuperscript{low} surface expression profile, indicative of tumor initiating potency in breast cancer cells (Al-Hajj, Wicha et al. 2003). Fitting with the EMT-like features, we found that members of the miR-200 family of microRNAs were significantly reduced in TEM4-18 cells compared to the epithelial-like PC-3E cells. miR-200 not only targets ZEB1 and ZEB2, promoting epithelial differentiation, but also targets multiple genes necessary for stem cell maintenance (Wellner, Schubert et al. 2009; Brabletz and Brabletz 2010; Iliopoulos, Lindahl-Allen et al. 2010). Together, these results suggest that ZEB1 drives an EMT-like state in aggressive prostate cancer cells and
this leads to the acquisition of stem cell-like traits. In support of this, ZEB1-knockdown in TEM4-18 cells reduced LGR5 expression, and greatly reduced colony formation in soft agar. In order to determine if this phenotype (ZEB1+/LGR5+/CD44\textsuperscript{high}/CD24\textsuperscript{low}) has potential in the identification of aggressive cells physiologically, additional cell lines and tissue samples must be analyzed.

Much of our research has focused on the migratory effects of ZEB1; however, our results in the colony formation assay suggest pro-survival roles for ZEB1. Survival upon metastatic colonization is thought to contribute greatly to metastatic efficiency (Chambers, Groom \textit{et al.} 2002). To begin examining the role of ZEB1 in this process, we transduced the aggressive PC-3 liver-derivative with shRNAs which target ZEB1. When injected systemically into mice, these cells formed significantly fewer tumors than vector control cells. We have shown that ZEB1 augments TEM \textit{in vitro}, thus it is possible that the reduced tissue colonization of ZEB1-knockdown cells is due to defects in extravasation. However, we were unable to determine if ZEB1 reduction affects extravasation \textit{in vivo} (as discussed in the introduction, examination of this process in living animals is technically daunting). Future studies could elucidate the role of ZEB1 in metastasis by utilizing tetracycline-inducible vectors to control its expression at various timepoints during tumor progression. For example, ZEB1 could be knocked down just after metastatic colonization to specifically study its role in secondary tumor growth.

In conclusion, my thesis projects have covered multiple aspects of cancer metastasis. We have revealed mechanistic insight into the roles of ZEB1 and EMT-like changes in cancer cell migration and metastasis. Our fluid shear stress model has allowed us to characterize novel biological properties of transformed cells. These findings challenge longstanding dogma about metastatic efficiency and may have practical applications in the isolation and study of CTCs. Our analyses of metastatic cell lines provide new information about physical and metabolic changes which may occur during
metastatic colonization. An optimistic outcome of these studies is the identification of novel biomarkers and therapeutic approaches.
Figure 6.1 A model for ETM and cooperative cell migration. Steps 1 and 2: EMT-like cancer cells of a primary tumor (characterized by expression of EMT regulators, such as ZEB1 and lack of E-cadherin expression) are surrounded by other cell types, including epithelial cancer cells, and normal prostatic epithelial and stromal cells. The process of EMT generally leads to increased invasive potential, but may reduce the production of extracellular proteins needed to facilitate migration (in this case, ZEB1 directly regulates laminin-332). Neighboring "bystander" cells in the microenvironment which deposit laminin-332 will allow the invasion and intravasation of EMT-like cells. Additional pro-migratory extracellular proteins such as laminin-411 and -511, present in the subendothelial matrix, may also facilitate intravasation. Steps 3 and 4: There is growing support for a role of EMT in extravasation. In this case, N-cadherin-mediated interactions between endothelial and cancer cells contributes to this process. As in the case of intravasation, subendothelial matrix proteins may facilitate trans-migration of EMT-like cells. Once colonized, it remains debated whether the reversal of EMT, mesenchymal-epithelial-transition (MET), occurs. This is largely due to the fact that epithelial features dominate in prostate metastatic tissue. Nonetheless, the EMT phenotype may play a role in colonization by facilitating enhanced survival and evasion of apoptosis.
Figure 6.2: Spontaneous metastasis of TEM4-18 cells from orthotopic anterior prostate injections. 2.0x10^4 TEM4-18 or PC-3E cells were injected into the anterior prostate on week 0. Tumor growth and metastasis was monitored using BLI over the course of 8 weeks. A) At week one, both cell types remain confined to prostate. B) After 8 weeks, only TEM4-18 cells had disseminated. Regions of Interest (ROI) show that signal is specific and not the result of light-scatter. C) Longitudinal BLI images were quantified and log-transformed. This study only compared one animal per group, thus no statistics were possible.
Figure 6.3: Histology of PC-3E and TEM4-18 orthotopic tumors. Paraffin embedded tumors were sectioned and stained with hematoxylin and eosin and imaged at 20X.
Figure 6.4: Induced shear stress resistance requires Rho-GTPase activity A) PC-3 and MDA.MB.231 cells were cultured in 100nM Y27632 for 20 hours then subjected to 10 passages of shear stress at 250 μL/sec (6.36x10³dyn/cm²). The shear stress survival of these drug-treated cells was compared in parallel with media control cells. *p<0.05, **p<0.01, ***p<0.001 vs. control. B) MDA.MB.231 cells were treated with 400ng/mL toxin-B for three hours prior to shear stress treatment. For each condition, survival is represented as percent viability of non-shear treated cells which are held in suspension for the duration of the assay.
Figure 6.5: Shear stress results in changes in stress-activated protein kinase activity. B16.f10 or PC-3 cells which had been sheared 5 or 10 times at 250 μL/sec (6.36x10³ dyn/cm²). Lysates were prepared and equal amounts of protein from each were analyzed for phosphorylation of ERK and JNK via western blot. The image above each membrane shows the BLI signal from control and sheared cells to highlight reduction in cell viability.
APPENDIX

THE ROLE OF CELL FUSION IN CANCER PROGRESSION AND METASTASIS

Background and rationale

Cell fusion is involved in many normal human physiological processes such as zygote formation, heart development, skeletal muscle development, and stem cell-mediated tissue renewal. Spurious cell fusion, however, may play a role in cancer. Hybrid cells resulting from unregulated cell fusion may exhibit characteristics which favor carcinogenesis and malignancy, such as stem cell-like self renewal, drug resistance, aneuploidy, and invasiveness. A variety of stem cell types are being evaluated as therapy in the treatment of many diseases, including cardiovascular disease. Studies have suggested that stem cells which integrate in the damaged heart and help reconstitute normal cardiac function do so in part by fusing with cells in and around the injured tissue. Transdifferentiation is another mechanism by which this is believed to occur. Despite these encouraging findings, studies in mice and in humans have suggested that bone marrow-derived cells are capable of fusing with normal and transformed host cells and that this may lead to tumorigenesis. Further studies have shown that tumor-host cell hybrids derived in vivo and in vitro exhibit greater metastatic potential than their parental tumor cell counterparts. Therefore it is important to understand the relationship between cell fusion and cancer both in a spontaneous setting and following stem cell treatment. With this understanding, we might be able to avoid this potential side effect of stem cell therapy. The long term goal of our research is to understand the cellular and molecular basis for cell fusion in cancer metastasis. The objective of this proposal is to elucidate the relevance of tumor-host cell fusion in vivo and to characterize the metastatic phenotype of the resulting hybrids.
Experimental Design

To begin studying the process of cell fusion in cancer progression, we designed a fusion-dependent reporter construct, whereby fluorescence reporter activity is conditional on the presence of Cre recombinase. The design of this construct and its functionality is described in Figure A.1 below. This construct was stably transduced into the mouse breast carcinoma cell line, 4T07. These cells were shown to be negative for fluorescence activity using FACS. When these cells were infected with a Cre-expressing adenovirus (leading to excision of the stop cassette as shown in Figure A.1), they became uniformly fluorescence-positive. These fluorescent cells were titrated into non-Cre-expressing cells and the limit of detection was determined using FACS (Figure A.2). These cells were then injected into the mammary fat pads of mice which ubiquitously express Cre recombinase, as described in Figure A.3. Once tumors had grown, we excised and dissociated them, and analyzed for the presence of fluorescence-positive cells (Figure A.4). The results from these experiments are summarized below the figures.
Figure A.1: Proof-of-principle using a fusion-dependent vector. A) Structure of LSL-GFP vector before (top) and after (bottom) Cre-mediated recombination between LoxP sites. This vector is was later modified to contain mCherry in the place of GFP. B) FACS analysis of B16.f0.LSL-GFP and B16.f0.LSL-GFP.Cre showing that GFP expression is dependent upon Cre activity. C) Cells from B were treated with either vehicle control, 1, or 10μM ganciclovir and viability assays were performed showing that after Cre recombination, thymidine kinase is efficiently removed and cells are resistant to ganciclovir. The Y-axis represents viability in terms of percent of control treated cells. CMV = cytomegalovirus promoter; HSV = Herpes Simplex Virus; GFP = green fluorescent protein; IRES = Internal Ribosomal Entry Site
Figure A.2: FACS fusion-detection limits. 4T07 cells expressing the mCherry-modified construct described in Figure 1 were mixed at various ratios with mCherry-positive cells (obtained by Cre-adenovirus infection of the same cells). These cells were then analyzed via flow cytometry for the quantitative analysis of mCherry-positive events. We observed that at a frequency below 1:10,000 unambiguous detection of mCherry cells diminishes. Gates on all plots are equivalent and set using 100% mCherry negative population as control. Percent of mCherry positive cells detected is shown above gate marker in each panel.
Figure A.3: A schematic for the use of a fusion-dependent vector. A) The “Deleter” mouse strain, a Balb/C mouse which ubiquitously expresses Cre recombinase under control of the CMV promoter. B) 4T07 cells expressing a Cre-dependent mCherry fluorescent protein vector. In the absence of Cre recombinase, the stop sequence upstream of mCherry prevents translation of this protein. Thus, only when fusion between a cell of the Deleter mouse and a 4T07 cell occurs, will the floxed stop sequence be excised, resulting in constitutive expression of mCherry (C). These cells were injected orthotopically into mammary fat pads of Deleter mice Picture below C. Tumors were allowed to develop (growth monitored via BLI) and were then excised, dissociated, and cultured. Upon recovery of tumor cells, flow cytometry was performed to detect fusion events.
Figure A.4: Lack of detectable fusion in pilot studies. Cells from tumors which were excised and recovered as described in figure 3 were analyzed for mCherry fluorescence using FACS. The top panel shows a positive sample. The bottom panel is representative of 4 tumors. We did not detect mCherry in these samples. However, as shown in figure 2, our detection limit falls of below 1:10,000. We expect spontaneous fusion in vivo could be well below this threshold; thus there is a possibility we are missing positive events by this analysis.
Summary

- We have established an orthotopic model of breast cancer metastasis and can monitor primary tumor growth and metastasis using BLI.
- We have demonstrated the feasibility of the fusion-dependent reporter system in cell culture experiments.
- We have established a practical limit of detection by FACS of fusion events at 1 in 1000.
- A pilot study using this model did not lead to detectable fusion events.
REFERENCES


Ewing, J. (1928). "Neoplastic Diseases."


