Effect of Myoferlin Depletion on Breast Cancer Cell Motility

THESIS

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By

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Abstract

Cancer metastasis requires tumor cell invasion, migration, and proliferation. Cell migration involves a complex series of processes that extend membrane protrusions to form substrate adhesions that cause cytoskeleton contraction within the cell. Further, cell migration is central to tissue repair, regeneration, development, cancer, and inflammation. The ferlins, an evolutionary conserved protein family, have been implicated as critical to maintaining plasma membranes. Specifically, myoferlin (MYOF), a mammalian ferlin, has been shown as responsible for membrane exocytosis/endocytosis and myoblast fusion. More recent studies have demonstrated that MYOF affects the anti-angiogenic response of endothelial cells and impacts the invasive ability of MDA-MB-231 breast cancer cells. It has also been noted that ablating MYOF using RNAi strategies results in consistent cell proliferation rates and a mesenchymal to epithelial phenotype transformation.

In this study, we present quantified 2D morphologic and migration differences in MDA-MB-231 wild-type (231WT) and RNAi-mediated MYOF-deficient (231MYOFKD) human breast adenocarcinoma (MDA-MB-231) cells. Morphometrics found that MYOF deficiency led to significant differences in lamellipodia number and surface area, filopodia length, and cell surface area. These marked changes could suggest that MYOF plays a role in regulating cytoskeletal arrangement in breast cancer cells.
Prior studies used Boyden Chamber to determine MYOF’s effect on cell migration, which only provided path-independent data without demonstrating expected migration behavior based on MET. These limitations were overcome in this thesis using live-cell imaging platforms to quantify cell migration. The 2D in vitro wound assay showed a marked difference between 231 control (wild-type and lentiviral control) and 231MYOFKD cell size, cell velocity, and cell directionality during wound closure. The more unidirectional movement of 231MYOFKD cells mimicked that of epithelial cell migration, while the 231 control cells exhibited a more mesenchymal-like random migration pattern. Furthermore, 231MYOFKD cells exhibited a greater inclination towards spreading and cobblestone morphology reminiscent of the epithelial phenotype. On the other hand, the 231 control cells with a lack of cell-cell coordination and a more elongated cell shape resembled the mesenchymal phenotype.

It was also demonstrated through immunofluorescence that 231 control cells exhibited a stronger actin and focal adhesion site expression. In addition, atomic force microscopy (AFM) characterized cellular biomechanical properties and demonstrated that 231MYOFKD cells remodeled the actin cytoskeleton with less stress fibers and decreased cell stiffness (Young’s Modulus). Lastly, a cell motility polymerase chain reaction (PCR) kit provided potential genes affected by the MYOF deficiency—Calpain 1, Dipeptidyl Peptidase 4, Epidermal Growth Factor, Integrin Beta-2, and Myosin Heavy Chain 9.

Collectively, these studies demonstrated that MYOF depletion is associated with several notable outcomes: 1) change in cell protrusion characteristics, 2) change in cell...
behavior and phenotype, 3) change from a random to directional migration, 4) a decrease in cell stiffness due to cytoskeletal remodeling, and 5) a possible change in focal adhesion sites. Continued research on MYOF may provide insight into methods that may reduce metastasis and provide a more detailed rationale for the affects described in this study.
Dedication

This thesis is dedicated to my family, my friends, the Kniss Lab, and everyone that helped me reach this point in my life.
Acknowledgments

The research presented in this Master’s thesis would not have been possible without the teamwork and help of all the Kniss laboratory members, Dr. Douglas A. Kniss, Dr. Samir N. Ghadiali, Dr. Cosmin Mihai, and Dr. Heather M. Powell.

Specifically I would like to thank Taryn Summerfield, Dr. Ruth Li, Dr. Douglas Kniss, and Dr. William Ackerman of the OSU Laboratory of Perinatal Research for helping to train me in tissue culture, laboratory techniques, immunofluorescence, polymerase chain reaction, and always taking the time to provide answers to my questions. Dr. Ruth Li, I would also like to thank you for helping to train me on the live-cell imaging system that was such a vital component of this thesis project. I would also like to acknowledge Chris Ahn for his help during the morphometric study. I would like to thank my advisor, Dr. Douglas Kniss, for his guidance, support, and encouragement during the last year and a half. He is a great resource and helped provide me with greater insight into molecular biology and scientific reasoning. He also helped show me my own intense interest in continuing along this path. Additionally, I want to thank Dr. William Ackerman who provided help during the immunofluorescent study, gave critical input during my thesis project, and who helped me with critical and scientific thinking. Dr. Cosmin Mihai was also instrumental in providing cell stiffness data and images using
atomic force microscopy. Without Dr. Mihai, that aspect of the project would not have been possible.

I would also like to thank Drs. Samir Ghadiali and Heather Powell for acting as members of my thesis committee, who gave me significant feedback and critique, and challenged me to think more critically. Dr. Powell, I would also like to thank you for helping me during the scanning electron microscopy sample preparation and answering my own questions on that front.

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Publications


Fields of Study

Major Field: Biomedical Engineering
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<th>Full Form</th>
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<tbody>
<tr>
<td>AC</td>
<td>adenyl cyclase</td>
</tr>
<tr>
<td>Akt/PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CAPN1</td>
<td>calpain 1</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DPP4</td>
<td>dipeptidyl peptidase 4 (CD26)</td>
</tr>
<tr>
<td>DYSF</td>
<td>dysferlin</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EHD2</td>
<td>eps15 homology domain protein 2</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GPRC</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>IF</td>
<td>immunofluorescence</td>
</tr>
<tr>
<td>IGF1</td>
<td>insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF1R</td>
<td>insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>ITGB2</td>
<td>integrin beta 2 (CD18)</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol-triphosphate</td>
</tr>
<tr>
<td>LTVctrl</td>
<td>lentiviral control</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MET</td>
<td>mesenchymal to epithelial transition</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MYH9</td>
<td>non-muscle myosin heavy chain 9</td>
</tr>
<tr>
<td>MYOF</td>
<td>myoferlin</td>
</tr>
<tr>
<td>MYOFKD</td>
<td>myoferlin knockdown</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>SH3PXD2A</td>
<td>SH3 and PX domain-containing protein 2A</td>
</tr>
<tr>
<td>shRNA</td>
<td>small hairpin ribonucleic acid</td>
</tr>
<tr>
<td>TGF β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TIRF</td>
<td>total internal reflection fluorescence</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Chapter 1: Background

Cell motility is central to tissue repair, regeneration, development, cancer, and inflammation (1-3). Cell migration involves a complex series of processes that extend membrane protrusions to form cell-substrate adhesions which cause cytoskeleton contraction within the cell (2-9). Myoferlin (MYOF) is a type II transmembrane protein found on chromosome 10q23.3, adjacent to the phosphatase and tensin homolog (PTEN) gene involved in anti-invasive and anti-proliferative effects (10, 11).

Recent work demonstrated that myoferlin depletion lowers the in vitro invasive ability of highly metastatic breast cancer cells (MDA-MB-231) by lowering the expression of matrix metalloproteinase 1 (MMP1) (12). Moreover, this same dissertation also discovered that ablating MYOF using RNAi strategies resulted in consistent cell proliferation rates and a mesenchymal to epithelial phenotypic transformation (12). It was also noted, using Boyden Chamber, that MYOF depletion demonstrated no change in migration (12). However, this migration result did not correlate with the phenotypic transformation. The likely reason is that Boyden Chamber only provided path independent data (13). To overcome these limitations, this thesis evaluated myoferlin’s affect on 2D cell protrusions, 2D cell migration dynamics (velocity, distance traveled, directionality), and cytoskeletal remodeling.
1.1 Cancer Cell Migration

Cancer metastasis is one of the leading causes of death in the US—responsible for as many as 90% of cancer-related deaths (14, 15). This metastatic process involves three major modes of action—tumor cell migration, invasion, and proliferation (16-20). All three modes work in concert to provide cancer cells the ability to colonize new tissues. Metastasis can be characterized by a series of steps that culminate in the extravasation of tumor cells at a remote site from the primary tumor and involve invasion and migration through the basement membrane and tissue compartments. There are six major steps to metastasis: 1) detachment from primary tumor site, 2) invasion and migration through the surrounding stroma to intravasate into the circulatory and/or lymphatic systems, 3) transit through the circulatory system, 4) extravasation and invasion into the secondary tissue, 5) formation of micrometastases at the secondary tumor site, and finally, 6) the formation of a tumor at the remote site (8, 16-20). During these steps cancer cells have been shown to undergo an epithelial to mesenchymal transition (EMT), which causes epithelial cells to lose their phenotypic markers (E-cadherin, desmoplakin, cytokeratin, occludin) and to become migratory and invasive (14, 16, 21-26). While tumor cell migration alone is insufficient to form distant metastases, this mode of action is integral to many cellular processes (20, 24).

Cell migration is a well studied mechanism of movement with two modes—single cell and collective migration (2, 3, 6, 9, 27, 28). Single cell migration is the most well understood and studied mechanism of motility in vitro (2, 3, 28). This behavior allows cells to migrate into new tissues and surrounding stroma during morphogenesis,
regeneration, immune response, and cancer. Conversely, collective cell migration involves a group of cohesive cell clusters that migrate in concert. Often times, less invasive and collective cancer cells may behave and migrate as a cell sheet where cells maintain adhesion—a characteristic of epithelial cells. These epithelial cells are also characterized by their cobble-stone like morphology (3, 14, 22, 24, 25, 29).

![Figure 1](image)

Figure 1 Sequence of major steps during metastasis: (1) detachment from primary tumor site, (2) invasion and migration through the surrounding stroma to intravasate into the circulatory and/or lymphatic systems, (3) transit through the circulatory system, (4) extravasation and invasion into the secondary tissue, (5) formation of micrometastases at the secondary tumor site, and finally, (6) the formation of a tumor at the remote site.

Proficient migration entails having distinct leading and trailing edges that allow for the coordination of both single and collective cell motility (2, 3, 28). The cell motility cycle involves steps that occur in many cell types in response to external stimuli, and to
intracellular and intercellular signaling (2-4, 6). These steps include maintaining cell polarity by intracellular signaling to direct leading edge protrusions, integrin-mediated adhesions, focal adhesion development, cytoskeleton remodeling, and directed contraction and detachment of the cell (1, 2, 5, 8, 9, 19, 20, 30-34).

However, less well understood are the mechanisms and kinetics of cancer cells, e.g. action of cell surface and released proteases, structural ECM remodeling, expression of adhesion molecules, and intracellular signaling by external stimuli (1-3, 19). For instance, the forced expression of matrix metalloproteinases (MMPs) to remodel extracellular matrix (ECM) during metastasis provided MDCK (non-cancer kidney cells) cells an *in vivo* invasive ability (8, 9, 35). Such findings show that EMT is not the sole explanation for cancer cell metastasis (14, 24, 35).

The difficulty with EMT remains the identification of cancerous cells at the EMT stage. EMT markers are also either epithelial or mesenchymal, and not all are expressed during an EMT event (24, 35-40). Despite valid studies that show EMT in cancer cells, EMT’s involvement in cancer remains highly debated. One significant factor that gives weight to non-EMT metastasis is the plethora of breast cancer metastases that express E-cadherin (epithelial marker) and maintain the epithelial morphology (15, 35). This suggests that no mesenchymal phenotype is necessary for metastasis. In addition, as previously mention, models have shown that metastasis can be accomplished through TGF-β and MMP induction (15, 35, 36, 41).
1.2 Epithelial to Mesenchymal Transition

In the past thirty years, biologists have defined EMT to be a biological program that is critical for morphogenesis, cell adhesion, fibrosis, wound repair, and cancer metastasis (15, 21, 24, 35). EMT in cancer represents a transdifferentiation program that induces transcription factors in epithelial cells (Snail 1, Snail 2, and Twist) to generate necessary mesenchymal traits for metastasis (14, 21, 24, 26, 35, 39). However, due to difficulties capturing images during this transition, EMT remains a controversial topic (20, 35).

Epithelial and mesenchymal cells are identified by their distinct morphology and formation of multicellular structures (22, 24, 35). Epithelial cells tend to form a polarized sheet with cell-cell contacts and adhesion (2, 3, 6). Mesenchymal cells are typified by random cell migration, less stable cell-cell adhesions, an elongated shape, and possession of front-to-back leading edge polarity (2, 3, 7, 28, 31, 42). The lower cell-cell adhesion is correlated with increased mesenchymal cell motility, which can lead to membrane loss of portions of the trailing edge (31, 32, 43). However, this epithelial and mesenchymal divide is not always present. A metastable cell phenotype has been identified in T24 human bladder carcinoma cells and EGF-treated PMC42 human breast cancer cells (25). This hybrid cell phenotype has been shown to contain a mixture of epithelial and mesenchymal traits, possibly indicative of the carcinoma cells on the tumor cell front prior or during metastasis (25).
EMT changes an epithelial cell by effecting cell morphology, cytoskeletal structure, cell-cell adhesion, cell-matrix adhesion, migration, and invasion (14, 15, 18, 24, 39). Figure 2 depicts the EMT and MET cell transformation and the major differences present during the transition. A number of molecular markers are increased in expression during EMT, e.g. N-cadherin, vimentin, fibronectin, Snail1, Snail2 (Slug), Twist, MMP-2, 3, 9, goosecoid, FOXC2, sox-10, and integrin αvβ6 (15, 24, 26, 29, 38, 39). Proteins that decrease in expression are E-cadherin, desmoplakin, cytokeratin, and occluding (26,
Proteins that accumulate in the nucleus include β-catenin, Smad-2/3, NF-κB, and the transcriptional repressors Snail1, Snail2, and Twist that generate mesenchymal traits (26, 29, 38, 39). Additionally, there are five functional transformations to note: increased migration, increased invasion, increased scattering, increased elongation of cell shape, and resistance to anoikis (cell death associated with loss of cell-matrix adhesion) (15, 24, 29, 35, 39). Of all the markers, the most commonly used for EMT are N-cadherin, vimentin, β-catenin, Snail1, Snail2, Twist, and E-cadherin (24, 29). This panel of EMT markers is required to determine if EMT has occurred because often times not all markers will be affected.

1.3 The Ferlins and Myoferlin

Ferlins are an evolutionary conserved family of proteins that are found in species ranging from non-metazoans to roundworms to mammals (11, 44, 45). This ancient protein family is involved in membrane dynamics, vesicle trafficking, cell motility, and maintenance of plasma membrane integrity (12, 44, 45). Originally discovered in Caenorhabditis elegans (FER-1), ferlins were found necessary for fertilization due to their involvement in the vesicle fusion events during sperm cell migration (11). In fact, FER-1 deficiency correlated with a loss of migration in the C. elegans sperm cells because vesicle fusion is important for amoeboidal migration (11). Phylogenetic studies demonstrated that the ferlin-like proteins have existed through eukaryotic evolution to mammals (45). Such consistent structural motifs include C2 domains, the dysferlin domain, FerA domain, and FerB domain (45).
The major functions of mammalian ferlins. Diagram includes known functions and diseases of the three commonly researched mammalian ferlins: dysferlin, otoferlin, and myoferlin.

Of six mammalian ferlins only otoferlin, myoferlin, and dysferlin are commonly studied. Table 1 describes the known functions and associated human diseases of dysferlin, otoferlin, and myoferlin. Dysferlin (DYSF) expression has been shown to aid in skeletal muscle, heart, and placenta plasma membrane repair due to its role in vesicle fusion and recruitment (46-51). Otoferlin (OTOF) aids in membrane fusion specific to the hair cells of the inner ear that are involved in the cochlear-transmission of signals from the cochlea to postsynaptic auditory neurons by endocytosis/exocytosis (11, 44, 45).

<table>
<thead>
<tr>
<th>Human Diseases</th>
<th>Dysferlin</th>
<th>Otoferlin</th>
<th>Myoferlin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscular Dystrophy Type 2B and Miyoshi Myopathy (47,50)</td>
<td>Reccessive form of deafness (DFNB9) (44)</td>
<td>No human disease (11) Mice: muscle mass effect (53)</td>
<td></td>
</tr>
<tr>
<td>Known Effects</td>
<td>1. Repair mechanism in MC membranes (49)</td>
<td>1. Exocytosis of inner hair cells (44)</td>
<td>1. Stabilizes VEGFR2 by dynamin-2 (52)</td>
</tr>
<tr>
<td></td>
<td>2. Recruitment of intracellular vesicles (47,49,50)</td>
<td>2. Interacts in vesicle fusion with SNARE (44)</td>
<td>2. Endocytosis in ECs (55,56)</td>
</tr>
<tr>
<td></td>
<td>3. Presence during myotube formation (49,50)</td>
<td>3. Role in myoblast fusion (55)</td>
<td>4. IGF response in muscle growth (54)</td>
</tr>
<tr>
<td></td>
<td>5. Responsive to trophoblast fusion in placenta (51)</td>
<td>6. Anti-angiogenic response MYOF also attenuates Tie-2 (57)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Muscle Cells = MCs, Endothelial Cells = ECs, Insulin-like Growth Factor = IGF.
Figure 3  Structural characteristics of the Fer-1 like proteins. Most ferlins have six to seven C2 domains, and a single carboxyl transmembrane domain. FER-1, DYSF, MYOF, and Fer1L5 have FerA and FerB domains, and nested DYSF (inner and outer) domains, which are not present in OTOF many other ferlins. There are two types of ferlins—those with nested DYSF and those without the nested DYSF domain. The black dots on DYSF domains indicate the missense mutations that lead to muscular dystrophy. Reprinted from Current Opinion in Cell Biology, volume 19, Renzhi Han and Kevin P Campbell, Dysferlin and muscle membrane repair, page 411, Copyright (2007), with permission from Elsevier.

Structurally, the ferlins are a unique protein family due to their multiple C2 domains, as shown in Figure 3. This carries over in MYOF, where there are six C2 domains present in the long cytoplasmic portion of the protein (11). Figure 4 shows the major domains of MYOF. After the C2 domains there is the key carboxyl-terminal transmembrane domain that allows MYOF to anchor itself in the phospholipid bilayer (11). The SH3 domain allows for binding to proline-rich residues and is present in proteins that regulate cytoskeleton, Ras protein, Src kinases, and adaptor proteins (11, 19). Other major domains present in MYOF are the nuclear membrane localization domains, Fer A and Fer B domains, inner and outer DYSF domains (11, 47). On the amino acid level, myoferlin is 40% homologous to the Fer-1 protein found in C. elegans.
and 68% homologous to dysferlin in mammals, which is a significant structural similarity that may signify a profound importance in the maintenance of the plasma membrane of cells across species that express ferlins (11, 45).

**MYOFERLIN FUNCTIONAL DOMAINS**

![Myoferlin functional domains. Illustration compiled from all known sources of the most prominent functional domains in the MYOF protein sequence.]

Myoferlin (~234kDa), one of six mammalian ferlins, is a protein important in myogenesis, including myotube formation by myoblast fusion, endothelial cell membrane repair, stabilization of Vascular Endothelial Growth Factor Receptor-2 (VEGFR-2) in the plasma membrane, and endocytosis (11, 52-57). Studies also show that the interaction of MYOF and eps15 homology domain protein 2 (EHD2) aids in membrane fusion (55, 56). Since both MYOF and EHD2 are expressed in many cell types, this may signify that EHD2 and MYOF interactions may be important for endocytotic recycling of receptors and ligands, e.g., integrins, in cell types beyond endothelial and muscle cells (55, 56). MYOF knockdown has more recently been shown to aid in anti-angiogenic effects by means of anchoring tyrosine kinase receptors at the plasma membrane (12). Recent work also provided evidence that MYOF has a substantial effect on the invasive ability of highly metastatic breast cancer cells (12).
1.4 Purpose

Cancer metastasis is a process that up-regulates cell invasion, cell migration, and cell proliferation (6, 16, 17, 20). MYOF overexpression has also been noted in microarray screening of breast cancer tissue samples (12). Cancer studies have also implicated endocytosis and exocytosis as necessary processes for breast cancer cell invasion through the extracellular matrix during their mesenchymal cell state (12). Recent work demonstrated that MYOF deficiency lowered the invasive capability of MDA-MB-231 cells, did not affect cell proliferation, and demonstrated an MET-like phenotypic change (12). Boyden Chamber migration also demonstrated that MYOF had no affect on migration (12). However, the Boyden Chamber migration assay only determines the path-independent migration and did not provide the expected MET-like differences, e.g. decreased migration velocity and increased collective migration.

To overcome this limitation, this thesis quantified the 2D in vitro cell morphologic and migratory differences of MYOF deficient cells through high-magnification imaging with scanning electron microscopy (SEM) and live-cell imaging platforms. The SEM images allowed for cell protrusion (lamellipodia and filopodia) quantification. The wound assay permitted the quantification of cell velocity, path-dependent distance, path-independent distance, and cell directionality. Measurements performed by atomic force microscopy (AFM) also demonstrated cell stiffness variation. Additionally, a polymerase chain reaction (PCR) array was performed to understand which cell motility-related genes are affected by the MYOF deficiency. The major goal was to understand what consequences MYOF deficient breast cancer cells have on 2D
cell protrusions, 2D cell migration, and cytoskeleton remodeling. This study demonstrated that MYOF deficient breast cancer cells exhibit a marked change in cell morphology, cell protrusions, cell velocity, cell directionality, cell migration distance, and cytoskeleton organization.
Chapter 2: Materials and Methods

2.1 Cell Culture

MDA-MB-231 cells (ATCC, HTB-26) were used in this study. Stable, myoferlin knock-down MDA-MB-231 cells were generated using lentiviral-based delivery of myoferlin shRNA (Sigma, St. Louis, MO). Lentiviral control MDA-MB-231s were generated using non-human target shRNA (Sigma, St. Louis, MO). All MDA-MB-231 cell lines were maintained in DMEM with 4.5 g/L D-glucose supplemented with 10% FBS (Invitrogen, Carlsbad, CA).

2.2 Scanning Electron Microscopy

The samples at sub-confluent seeding, 2 hour post-wound, and 10 hour post-wound were fixed with 3% glutaraldehyde (cat#G-5882, Sigma Chemical Co., St. Louis, MO), post-fixed with 1% OsO₄ and dehydrated with an ethanol dilution series and hexamethyldisilazane (cat#16700, Electron Microscope Sciences, Hatfield, PA) dilution series. The ethanol dilution series was 50% and 70% at 5min., 80% and 95% at 10min., and 100% twice for 10min. The hexamethyldisilazane dilutions series was 3:1 EtOH/HMDS for 15min., 1:1 EtOH/HMDS for 15min., 1:3 EtOH/HMDS for 15min., and 100% HMDS thrice for 15min. After mounting the samples on SEM studs using carbon sticky tap, the samples were prepared for scanning electron microscopy using a Pelco Model 3 sputter coater with gold-palladium (0.07 mbar, 17 mA, 110 sec). The samples
were imaged in an FEI Nova NanoSEM microscope equipped with secondary electron mode at 5.0 kV. All SEM samples were stored in a desiccator after imaging to maintain sample integrity.

2.3 Cell Morphometrics

Cells were washed with PBS, trypsinized, and passaged from 70-80% confluence at a 1:20 ratio in 10% serum containing media. One hundred microliters of cells at a density of 2.5x10^5 cells/mL were seeded on Thermanox plastic coverslips (NUNEC, Rochester, NY). Cells were incubated (37°C, 5% CO2) for 12 hours for attachment to Thermanox plastic coverslips (NUNC, Rochester, NY) in 24-well tissue culture plates. This was repeated for each cell line allowing overnight (12 hours) attachment: MDA-MB-231 wild-type (231WT), lentiviral control (231LTVctrl), and myoferlin knockdown (231MYOFKD) cells. The SEM protocol described above was performed and samples were imaged on FEI Nova NanoSEM. Cell morphology was determined by comparing describing distinct visual characteristics.

ImageJ (NIH) was used for quantifying the cell metrics. Morphometrics analysis was performed on: cell surface area, lamellipodia, and filopodia. Filopodia quantification was performed by measuring the number of filopodia per lamellipodia and the filopodia length. Lamellipodia quantification was performed by estimating the lamellipodia surface area and the number of lamellipodia per cell. All values were converted to relevant units based on scale bar. One coverslip sample was used for each cell line and 30 cells were
quantified from each sample. Figure 5 demonstrates an example of how the measurements were performed using ImageJ.

Figure 5  Sample cell protrusion measurements. This is an example of how the measurements were performed using ImageJ. Yellow denotes the lamellipodia surface area. Red denotes the cell surface area. Blue denotes the filopodial lengths. Lamellipodia and filopodia numbers were determined using these values.

2.4 Sub-Confluent Migration using Live-Cell Imaging

Cells were washed with PBS, trypsinized, and passaged at a ratio of 1:20 in 10% serum containing media at low confluence. Cells were seeded at a concentration of 2.5x10^5 cells/mL (100μL) on polystyrene culture-treated dishes. This was repeated for each cell line: 231WT, 231LTVctrl, and 231MYOFKD cells. The dish was placed in the microchamber of a live-cell imaging system (Marianas, Denver, CO) at 37°C, 5% CO₂. Water was included in the chamber to maintain humidity and decrease drying of media. Time-lapse images (Zeiss Axiovert 200M, Germany) were taken every 10 min over 24 hrs. For each cell line, four simultaneous areas were imaged; three areas at low
confluence and one area at high confluence. Cells were analyzed visually for any significant differences.

2.5 Migration Assay using Live-Cell Imaging

Cells were washed with PBS, trypsinized, counted, pelleted, and resuspended in 10% serum containing media at a concentration of $3.0 \times 10^5$ cells/ml, and 80 μl of cells were placed into each of the wells of a wound assay insert (Ibidi, Martinsried, Germany). Cells were incubated (37°C, 5% CO$_2$) for 24hrs until a confluent monolayer was formed. The insert was removed, 1.5 ml of additional 10% serum containing media was added to dish, and the dish was placed in the microchamber of a live-cell imaging system (Marianas, Denver, CO) at 37°C, 5% CO$_2$. Water was included in the chamber to maintain humidity and decrease drying of media. Time-lapse images (Zeiss Axiovert 200M, Germany) were taken every 10 min over 24 hrs. The experiments were repeated three times per cell line (wild-type, lentiviral control, myoferlin knockdown). One-hundred cells were tracked per experiment using the ImageJ Manual Tracking feature, and the data was analyzed (Ibidi Chemotaxis & Migration Plugin for ImageJ) for average Velocity per wound assay (Eqn.1), Path Dependent Distance (Total Distance Traveled), Path Independent Distance ($d_{i,f}$), Directionality per cell (Eqn. 2), and average Forward Migration Index per wound assay (FMI, Eqn. 3).

The average cell velocity per wound assay is:

$$Velocity = \frac{1}{n} \sum_{n=1}^{n} \frac{1}{145} \sum_{i=1}^{m} \frac{d_{i+1} - d_{i}}{\Delta t}$$  

Equation 1,
where n=total number of cells per wound (100), m=total time intervals for each cell (145), d=distance traveled for timepoint \( I \), and \( \Delta t \)=time-lapse period (10 minutes).

The individual directionality per cell is:

\[
\text{Directionality} = \frac{d_{\text{path independent}}}{d_{\text{path dependent}}}
\]

Equation 2,

where \( d_{\text{path-independent}} \)=the distance between the initial and final timepoints for each cell, and \( d_{\text{path-dependent}} \)=the summed distance between each successive timepoint during the 24 hours for each cell. Each single cell’s directionality is then averaged to gain an average directionality per cell line.

The average FMI in the x-direction per wound assay is:

\[
X_{\text{FMI}} = \frac{1}{n} \sum_{i=1}^{n} \frac{X_{i, \text{end}}}{d_{i, \text{total}}}
\]

Equation 3,

where n=total number of cells in each wound assay, \( X_{i, \text{end}} \)=the x-distance between the initial and final cell locations, and \( d_{i, \text{total}} \)=the path dependent distance for each successive FMI measured.

2.6 Immunofluorescence

The cells were seeded at a concentration of \( 4.0 \times 10^5 \) cells/mL and 100μL. Cells were seeded onto Thermanox plastic cover slips (NUNECE, Rochester, NY) and placed in a 24-well tissue culture plate for 24 hours. This was repeated for each cell line: 231WT, 231LTVctrl, and 231MYOFKD cells. Following monolayer formation the coverslips were scratched to create a wound, similar to migration assay, with a 10 μl pipette tip. The
cells were washed with PBS and additional media was placed in the wells. After 10 hours of cell migration and wound closure, the cells were fixed for 1 hour in 4% (w/v) paraformaldehyde/PBS, permeabilized with 0.2% (v/v) Triton X-100/PBS for 15 minutes, and blocked in 5% (v/v) horse serum/ PBS overnight at 4°C before the addition of antibodies. Immunolabeling of cultured cells was performed on focal adhesion complexes and the actin cytoskeleton. For the focal adhesion complexes a primary antibody, paxillin (Invitrogen, cat#05-417), was used, conjugated to Alexa Fluor 594 (Invitrogen, Carlsbad, CA). For the filamentous actin, phalloidin (cat#A12379), conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA) was used. Nuclei were counterstained with 5 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI, Sigma). Cells were mounted using ProLong Antifade kit (Molecular Probes) and were imaged using an epifluorescent microscope (Nikon Instruments, Melville, NY).

2.7 Atomic Force Microscopy (AFM) and Biomechanical Analysis

MDA-MB-231 lentiviral control and myoferlin-deficient cells were seeded at a density of 5 to 8x10^5 cells in 60 mm cell culture dishes and allowed to grow in normal culture media to 80-90% confluence for AFM analysis. Prior to AFM analysis, the culture media was exchanged for CO₂ independent media (Invitrogen, Carlsbad, CA) supplemented with 1% Fetal Bovine Serum, 1% Penicillin/Streptomycin and 4 mM L-Glutamine, for optimal cell survival for the duration of the atomic force measurements.

A Bioscope II instrument (Veeco, Santa Barbara, CA) mounted on the stage of an Axiovert 200 inverted optical microscope (Zeiss, Germany) was used in tapping mode for
imaging. All samples were imaged in the supplemented CO$_2$ independent media using a fluid cell. Silicon nitride (SiN) triangular cantilevers (Veeco) 200 μm in length with a tip angle $\theta = 35^\circ$ and a nominal spring constant k of 0.01 N/m were used to image and indent the cells for the calculation of the cells' Young's modulus using a Hertz model.

The force curves for the AFM analysis were determined using a Hertz model below (58, 59):

$$ z - d = z_c + \left( \frac{4(1 - \nu^2)kd}{3E \tan \theta} \right)^{1/2} $$

Equation 4,

where $z$ and $d$ are the actual data and $k$, $\theta$ and $\nu$ are constants. The constants are: $k$ – the cantilever spring constant; $\theta$ - the AFM tip half angle (35 deg for MLCT tips we used), and $\nu$ - the Poisson ratio of the cell assumed to be 0.5 as the cell is mostly made of water. The unknown parameters are $E$ - the Young’s modulus of the cells and $z_c$ - the contact point. The contact point is the $z$ value where the AFM tip touches the sample, in this case a cell. This analysis becomes a polynomial fit with two parameters $E$ and $z_c$. The only useful value is $E$ - the Young’s Modulus. Figure 6 depicts the parameters from the AFM system and the force-deflection curve needed to determine the Young’s modulus and the contact point to solve the Hertz model equation, above.

Images were recorded in height and amplitude modes. All images were recorded with a scan size area of 70 to 80 μm$^2$ covering about 5 cells. Four to five of these scan areas were completed for each cell type, with 30 points per cell used for a total of 150 to 200 points per scan area used for cell stiffness calculations. The experiments were repeated three times. All recorded images and deflection data were analyzed using
version 7.30 of the NanoScope software (Veeco, Plainview, NY). Images were processed for calibration marks using ImageJ (http://rsbweb.nih.gov/ij/). Statistical analysis was performed using Minitab 15.0 (Minitab Inc, State College, PA).

Figure 6  AFM setup and force-deflection graph. Graphical representation of the AFM tip touching and indenting a cell (left), with a single sample force-deflection curve (right).

2.8 Cell Motility Polymerase Chain Reaction Array

RT² Profiler™ PCR array from SABiosciences (cat # PAHS-128F-12) was performed to determine which cell motility genes are affected by MYOF deficiency following manufacturer's protocol. Total RNA was extracted from lentiviral control and MYOF deficient MDA-MB-231 cells using TRIzol® reagent (Invitrogen), according to manufacturer’s protocol up to the chloroform extraction and centrifugation steps. The resultant aqueous phase was mixed with an equal volume of 70% ethanol and applied to an RNeasy mini column (QIAGEN) and processed according to the manufacturer's protocol with DNase on column digestion. Total RNA was quantified by UV spectrophotometry at 260 nm and 280 nm on a NanoDrop 2000, and 1 μg of total RNA from each cell type was reverse-transcribed to complementary DNA (cDNA) using random hexamers and oligo-dT18 primers as part of the RT² First Strand Kit. Equal
amounts of dilute cDNA was mixed with LightCycler® 480 SYBR Green I Master mix (Roche) and aliquoted to each well of the PCR array plate containing the pre-filled gene-specific primer sets, and PCR was performed according to manufacturer’s instructions for the Roche LightCycler® 480.

The array contained 84 genes known to be involved in cell migration mechanisms. See Appendix C.1 for the full array of profiled genes. The LightCycler® 480 Software (Roche) was used to calculate the threshold cycle (crossing point, C_p) values for all the transcripts in the array. The C_p values were then exported into an Excel-based PCR array data analysis template to calculate fold changes in gene expression for pairwise comparisons using the ΔΔCt method.

2.9 Statistical Analysis

Statistical analyses were performed using JMP 9.0 or Minitab 15.0 (Minitab Inc, State College, PA). Statistically significant and non-significant differences were detected using One-way ANOVA with Tukey post-hoc and Student t tests. Statistical significance was considered at p<0.05.
Chapter 3: Results

3.1 Morphology and Morphometrics

3.1.1 Rationale

Initially cell morphology was assessed for the three cell lines, in order to elucidate the effects of myoferlin on the migration of the MDA-MB-231 cells. Live-cell imaging of the cells revealed distinct morphological phenotypes, which can be correlated to migratory differences (2, 3, 5, 6, 9). SEM provided high resolution images of the cell protrusions and the cell shape. These images were used to assess morphological differences between 231MYOFKD and 231 control cells.

3.1.2 Cell Morphology

Cell morphology is one difference that distinguished 231WT and 231LTVctrl cells from 231MYOFKD cells. Figure 7 shows sub-confluent SEM images of each cell line. The 231WT (Figure 7A) and 231LTVctrl (Figure 7B) cells exhibit more mesenchymal morphology; smaller cell surface areas and elongated spindle-like morphologies. The MYOF-deficient (Figure 7C) cells are more similar to an epithelial cells are more similar to an epithelial morphology based on the greater cell spreading, increased filopodia contacts between cells, and greater cell-cell contact formations.
3.1.3 Cell Morphometrics

The notable distinctions in cell morphology and cell protrusions led to the analysis of each cell line at high magnification (2500X). The high resolution SEM images were used to quantify these physiologic differences. Cell morphology metrics were performed to determine the number of lamellipodia per cell, the lamellipodia surface area per cell, the filopodia number per lamellipodia, the filopodia length, and the cell surface area.

The metrics illustrated quantitatively that there exists a significant difference between 231 controls and 231MYOFKD cells. Lamellipodia number (Fig. 8A) decreased between 231WT and 231MYOFKD cells and lamellipodia surface area (Fig. 8B) increased between 231 control and 231MYOFKD cells. (See Appendix A.1 for sample SEM images used during morphometrics.)
Figure 8  Morphometric data of all cell lines. Data included measurements on: (A) Lamellipodia number per cell, (B) Lamellipodia surface area per cell, (C) Filopodia number per lamellipodia, (D) Filopodia length, and (E) Cell surface area. Statistical significance was determined using ANOVA and Student t-test for all cell pairs. Significance interval was p<0.05. Error bars represent standard error.
In addition, the 231LTVctrl cells may in fact be affected by the overexpression of the MYOF gene. Furthermore, as shown in Figure 8D, filopodia length is significantly different between 231 controls and 231MYO FKD cells, and cell surface area (Fig. 8E) is significantly increased between 231 controls and 231MYO FKD cells. Interestingly, MYOF did not affect the filopodia number, whereas lamellipodia numbers were decreased after MYOF deficiency. Of particular note is that both lamellipodia and cell surface area per cell increase by a factor of two in 231MYOFKD cells.

3.2 Cell Migration Quantification

3.2.1 Rationale

Cancer metastasis is associated with tumor cell invasion and migration to distant parts of the body. [17, 34, 79] Cell morphometrics determined a statistically significant difference in cell protrusions using MYOF deficient cells. This study focused on determining MYOF’s affect on sub-confluent migration and cell migration properties, e.g. cell coordination, cell velocity, cell directionality, path-independent distance, path-dependent distance, and wound closure rate. Live-cell imaging was implemented to image sub-confluent migration and wound assay closure.

3.2.2 Sub-Confluent Migration

Low confluence migration studies were imaged over 24 hours for each cell line. Three low confluence and one high confluence sites were selected per cell line to record observations in cell behavior and interactions. Figure 9 illustrates a sample sequence of
four images for one low confluence study in each cell line. Visual comparison of images provided several general observations during sub-confluent migration: 1) control cells exhibited more single-cell migration, more spindle-like morphology, and a more random migration, whereas 2) MYOF deficient cells displayed more coordinated migration. Interestingly, 231LTVctrl cells exhibited a greater coordinated behavior than the 231WT cells, but still less than 231MYOFKD cells.

Figure 9  Sub-confluent cell migration. Phase contrast time-lapse images (10X) of MDA-MB-231 cell lines, using Live-Cell Imaging. These images display cells migrating at sub-confluent concentrations. Images were taken every 10 minutes for 24 hours.
3.2.3 Migration Assay

During the course of each 24 hour wound assay 145 images were compiled. This was clearly repeated three times for each cell line to provide accurate values for analysis. Figure 10 illustrates a sample sequence of four images of one wound assay for each cell line. An image comparison provided several differences during wound closure: 1) 231WT and 231LTVctrl cells exhibit more single-cell movement, greater stretching, more stepping over each other, and a general trend towards random migration, whereas 2) MYOFKD cells display greater collective migration, greater directed migration, and a more ordered wound closure akin to an epithelial cell phenotype. The single notable parallel established in Figure 10 was the consistent wound closure time (18-19 hours) for all wound assays. (See Appendix B.1 for SEM images of wound assays at 2 hours and 10 hours.)
Figure 10  Wound assay cell migration. Phase contrast time-lapse images (10X) of MDA-MB-231 cell lines, using Live-Cell Imaging. These display wound closures taken every 10 min and allowed for Cell Tracking (shown in Figure 11) and comparison of cell migration properties (shown in Figure 12).

Data analysis of the live-cell imaging shown in Figure 10 was performed by tracking 300 cells from three wound assays for each cell line. Figure 11 demonstrates a sample of 50 cells for each cell line—231WT (Figure 11A), 231LTVctrl (Figure 11B), and 231MYOFKD (Figure 11C). As shown, there is a significant difference in cell migration behavior between the 231 control cells and the 231MYOFKD cells. In fact, the
cell tracks depicted in Figure 11 translate into a more random migration behavior for 231WT and 231LTVctrl cells and directed migration behavior for 231MYOFKD cells.

![Cell tracks](image)

Figure 11 Cell track samples of 50 cells from (A) MDA-MB-231 WT, (B) MDA-MB-231 LTVctrl, and (C) MDA-MB-231 MYOFKD. The 231MYOFKD cells migrate more collectively and less random than 231WT and 231LTVctrl.

The cell tracks were analyzed to determine four selected values that highlight comparisons between 231WT, 231LTVctrl, and 231MYOFKD cells—cell velocity (Figure 12A), cell directionality and forward migration index (FMI, Figure 12B), total distance traveled (Figure 12C), and path independent distance traveled (Figure 12D). The wound assay cell tracking results are provided in Figure 11. Cell velocity exhibited an approximate two-fold statistically significant difference between 231 control and 231MYOFKD cells. Conversely, cell directionality and FMI was approximately two-fold greater for the 231MYOFKD cells. FMI was included to provide an x-axis only directional comparison of the cells, whereas directionality is not axis-dependent. The path dependent distance exhibited the same trend found in cell velocity—two-fold increase in 231WT and 231LTVctrl values. No statistically significant differences were ascertained when comparing the path independent distance between 231WT & 231MYOFKD cells.
and 231WT & 231LTVctrl cells. These inconsistent differences between the 231MYOFKD and 231LTVctrl may be associated with the lentiviral vector. However, the path independent distance still corroborates well with the fact that wound closure rates are similar. Also note that cell velocity and path dependent distance were statistically significant between 231LTVctrl and 231WT cells.

Figure 12 Wound assay migration results of MDA-MB-231 cells: (A) Velocity, (B) Directionality and Forward Migration Index (FMI), (C) Total Distance Traveled (Path Dependent Distance), (D) Path Independent Distance Traveled. Each cell line includes 300 cells tracked over 24 hrs from 3 experiments. Error bars represent standard error for each cell line. Bars with different letters (of same color) are statistically significant (p<0.05. Oneway ANOVA with Tukey post hoc test were performed for each data set.
3.3 Immunofluorescence, Biomechanical Analysis, and Cell Motility PCR Array

3.3.1 Rationale

As shown in Figure 7 and 8, 231MYOFKD cells exhibited several quantifiable morphologic and migratory differences with the 231 control cells (231WT and 231LTVctrl). MYOF deficient cells spread out their membrane, migrated more directionally, and behaved as epithelial cells. Conversely, the 231 control cells were more elongated, migrated more randomly, and maintained a mesenchymal behavior. Preliminary studies performed to determine cell adhesion strength further suggested that 231MYOFKD cells had greater adhesion strength than the 231 controls. Immunofluorescence may demonstrate differences in actin remodeling and focal adhesion sites. Biomechanical studies using AFM will be used to determine the cell stiffness. Real-Time (RT) PCR will provide a screening of potential genes that MYOF depletion may affect. Collectively, these studies may help determine MYOF’s involvement in cell migration, cell cytoskeleton architecture, and cell membrane dynamics.

3.3.2 Immunofluorescence

The immunofluorescent study focused on the differences present between 231 control cells (231WT and 231LTVctrl) and 231 MYOFKD cells by staining for actin, paxillin, and the cell nucleus. Paxillin is a cytoskeletal protein and a phosphorylated focal adhesion kinase (FAK) present in actin-membrane junctions involved in focal adhesion (FA) complexes (34, 60, 61).
Figure 13 Immunofluorescence of MDA-MB-231 cells. DIC, actin, and paxillin images of all cell lines at 10 hours post-wound. Actin is represented in green and paxillin is represented in red. Note distribution changes in paxillin and actin. DIC images are provided for reference frame. DAPI not included. All images taken at 20X magnification using confocal epifluorescent microscope.

All the cell lines were seeded on plastic bottom dishes to maintain continuity of results with wound migration assays. The 231WT cells in Figure 13 revealed blebs and a stronger distribution of paxillin throughout the cytoplasm. The 231LTVctrl and 231WT cells were morphologically identical. Blebs, shown by arrows, were present in phase contrast and immunofluorescent images, as shown in Figure 13. Conversely, SEM images in Figure 7 of the 231 control cells hide the presence of blebbing possibly due to the
dehydration and organic solvent preparation of SEM samples. Figure 13 further depicts the presence of actin in the blebbing regions of 231 control cells with a lack of paxillin antibody.

Figure 14 High magnification immunofluorescent images of 231MYOFKD cells. These images show the DIC, actin, and paxillin stains. The paxillin image depicts sites along the leading front with higher concentrations (arrows).

MYOF deficient cells revealed actin arcs (high concentrations of cortical actin), prominent on the leading edges and the migrating front of the cells. These actin arcs were not present in the 231 control cells. Further, the 231MYOFKD cell’s paxillin localized near the nucleus, while 231 controls exhibited more extensive cytoplasmic expression. Paxillin, a biomarker for FA complexes, was also present in several sites along the leading front edges of 231MYOFKD cells, as shown in Figure 14.
3.3.3 Biomechanical Analysis

AFM analysis determined whether MYOF affected the integrity of the cancer cell cytoskeleton (61), since MYOF was revealed to directly affect cell morphology, cell velocity, directionality, and path dependent distance traveled. The cell stiffness was calculated by using an AFM. Figure 15 depicts the 231LTVctrl and 231MYOFKD cells. The AFM image samples demonstrated a distinction between the actin cytoskeleton architecture of 231LTVctrl and 231MYOFKD cells. Further, Figure 15 demonstrates remodeling of actin cytoskeleton. The 231MYOFKD cells exhibit a more rounded cell with less pronounced actin stress fibers, whereas the 231LTVctrl cells exhibit more pronounced stress fibers. Additionally, there was a two-fold decrease in the cell stiffness of MYOF deficient cells.

Figure 15 Cell stiffness measurements. The 231LTVctrl cells (A) have a reorganized (arrows) actin cytoskeleton when compared to the 231MYOFKD cells. Comparison of (B) cell stiffness shows that MYOFKD is associated with decreased Young’s modulus. Error bars represent standard error. Statistically significant differences detected using Oneway ANOVA with Tukey post hoc test.
3.3.4 Cell Motility PCR Array

A wide variety of genes exist that help regulate cell migration. The cell motility reverse transcriptase assay profiles expression levels of 84 genes involved in migration mechanisms. The array included genes that are correlated to growth factors, receptors involved in chemotaxis, Rho family proteins, and additional genes involved in cell protrusion mechanisms. (See Appendix C.1 for a full gene profile of the RT PCR array.)
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<th>T-Test</th>
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<td>ASV, SRC1, e-SRC, pS6-Src</td>
<td>7.18 8.52 6.0E-03 1.1E-02</td>
<td>0.63 N/A -1.58</td>
<td>OKAY</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2: List of genes affected by MYOF deficiency. All genes included that are near the two-fold change in expression when MYOF is removed from MDA-MB-231 cells. All genes highlighted in yellow represent acceptable increases and decreases in expression. Genes highlighted in red were found inconclusive due to expression levels appearing late in PCR cycles. MMP9 was selected in red due to the high fold-change, but was marked inconclusive during gene selection.
Results shown in Table 2 indicated that MYOF loss affected expression levels of calpain 1 (CAPN1), dipeptidyl peptidase 4 or CD26 (DPP4), epidermal growth factor (EGF), insulin-like growth factor 1 receptor (IGF1R), integrin beta 2 or CD18 (ITGB2), matrix metalloprotease 14 (MMP14), non-muscle myosin heavy chain 9 (MYH9), and SH3PXD2A with a 1.7 or greater fold change. CAPN1, with a 2.19 fold expression decrease, is a calcium activated muscle and stomach specific cysteine protease involved in positive regulation of cell proliferation (62). DPP4, with a 2.84 fold expression increase, is involved in metabolism, immune response, cancer adhesion, and cancer proliferation (63). EGF, with a 1.95 fold increase, plays a regulatory role in cell proliferation and differentiation when bound to EGF receptor (64-67). IGF1R, with a 1.85 fold expression decrease, has been implicated in several cancers and its ligand plays an important role in anabolic growth (54, 68). ITGB2, with a 2.01 fold expression increase is involved in a number of partner chains that form specific integrins for cell adhesion (4, 31, 69). MMP14, with a 1.81 fold expression decrease, appears to activate progelatinase A on tumor cell surfaces and may be involved in cytoskeletal reorganization (41, 70). MYH9, with a 2.42 fold expression decrease, plays a role in cytokinesis, cell morphology, cell secretion, and actin capping (71-73). SH3PXD2A, with a 1.76 fold expression decrease, is an “adaptor protein involved in invadopodia and podosome formation, extracellular matrix degradation and cancer invasiveness”, organizer protein for reactive oxygen species generation, and affects the neurotoxicity of β-amyloid. The resultant genes with highest fold changes were CAPN1, DPP4, EGF, ITGB2, and MYH9.
Chapter 4: Discussion of Results

4.1 Morphology and Morphometrics

Cell morphology plays an important role in understanding cell membrane dynamics, cell behavior, cell polarity, cytoskeleton architecture, and cell protrusions (2, 3, 6, 69). The morphometrics study focused on quantifying the cell protrusions and cell size. For a 2D surface, the relevant cell protrusions are lamellipodia and filopodia, in contrast to pseudopodia and invadopodia for a 3D environment (43). Cell morphometrics in Figure 8 showed that MYOF deficient cancer cells have decreased lamellipodia per cell, increased lamellipodia surface area per cell, increased filopodia length per cell, and increased cell surface area per cell. It can also be noted that lamellipodia number correlate with the cell migratory activity (7, 28, 43, 60).

The lamellipodium is a flattened cytoskeletal projection on the leading front of the cell that emerges transiently during cell migration. These lamellipodia are composed of a filamentous actin meshwork, composed of F and G actin monomers (7, 19, 28, 31, 43). Filopodia are thin finger-like actin bundle filament spikes that spread out from the lamellipodia (7, 43, 74). Filopodia also function as environmental sensors of chemokines, surface structures, and adjacent cells. Both lamellipodia and filopodia undergo exocytosis at their protruding end, which represents a region of the clathrin endocytotic cycle (7, 43). Together, focal adhesion development and actin polymerization and depolymerization act in concert to control cell morphology and motility (4, 5, 7, 69).
Figures 7 and 8 provide evidence that 231MYOFKD cells decrease lamellipodia protrusion numbers, increase lamellipodia area, cell area, and filopodia length. This may be corroborated by the partial loss of capping proteins or capping protein expression, which is necessary to control actin polymerization on both lamellipodia and filopodia. (5, 7-9, 33) Mejillano et al studied the loss of capping protein, and determined that filopodial protrusions increased around the cell and lamellipodia became slow and unpredictable (75). While this study imaged static images, such a similar but less potent effect may signify MYOF’s involvement in leading edge exocytosis and actin cytoskeleton dynamics, especially since there is no change in filopodia number for MYOF deficient cells. MYOF’s effect is not pervasive, in any extreme loss of functional pathways that control the polymerization at protrusion leading fronts. Therefore, perhaps MYOF may have a widespread effect on the molecular mechanism shown in Figure 15 by interacting and co-localizing to cortactin, Arp 2/3, cofilin, or capping proteins near the cell membrane. No data currently demonstrates such a behavior, however, this represents a possible explanation.

Figure 8 illustrates the change in cell surface area and one culprit is the two-fold increase in lamellipodia surface area shown in Figure 7 by an effect MYOF may have on capping protein expression. The actin meshwork within the lamellipodia is constructed in part by the Arp 2/3 complexes that, when bound to actin filaments, create branching and a site for microfilament extension (8, 9, 33). The actin microfilaments include a barbed and pointed end (8, 9, 33). The barbed end faces the leading edge to continue actin polymerization, membrane extension, increase tension, and cause membrane treadmilling.
Capping proteins bind to barb ends to halt the polymerization cycle (5, 8-10, 33). Conversely, cofilin severs the actin filaments and causes reactivation of the polymerization process by creating new barbed ends (5, 8-10, 33). Cortactin, implicated in processes of dynamic actin cytoskeleton, is another compound that links tyrosine kinase signaling to actin cytoskeleton remodeling (5, 8-10, 33). Additionally, cortactin is used as an immunofluorescent marker to distinguish the cell lamellipodium from the lamella (5, 8-10, 33).

Figure 16 Effectors involved in protrusion development. Diagram demonstrates how the most notable proteins affect actin polymerization and cell protrusion development (8, 9).

Rho-family GTPases, Rac 1 and Cdc42, are also involved in the cell membrane and structural organization of lamellipodia (5, 7-10, 31, 33). Such structural changes are
present in Figure 7 and quantified by Figure 8 protrusion measurements. Rac1 causes cortactin to localize on cell membrane and bind to actin and Arp 2/3 complexes, and ensure cell migration by promoting lamellipodia formation (5, 7-10, 31, 33, 76). Cdc42 promotes filopodia, when activated, by localizing on WASP receptors to activate Arp 2/3 and increase actin branching and cell migration (5, 7-10, 31, 33, 76). Ena/VASP proteins on the leading edge also promote actin polymerization by preventing capping of barbed ends (77). Figure 16 illustrates additional molecules involved in protrusion extension mechanism.

Exocytosis of epidermal growth factor (EGF) which binds to receptors on protrusions that activate the mechanisms in Figure 16 may also be affected by MYOF deficiency. MYOF has been implicated in exocytosis and endocytosis. Loss of MYOF may suggest a less localized secretion of EGF to help in directing cell protrusion development. Additionally, 231MYOFKD cells, with lower lamellipodia numbers, may suggest MYOF’s greater involvement in branching mechanisms than microfilament nucleation. Another possible effector by MYOF is Cdc42 because it downregulates matrix metalloproteinase 1 expression by inhibiting the Erk 1/2 pathway—a protein that MYOF loss also downregulates 100 fold (78). MYOFs variety of domains may also play a factor in cell protrusion development and other signaling pathways. In addition, while this study quantified MYOF’s affect on cell protrusions, the method is subject to bias from user input and SEM processing. Immunofluorescent labeling with cortactin or AFM may more accurately quantify MYOF’s affect on cell protrusions.
4.2 Cell Migration Quantification

Cell migration is a vital and highly regulated process that participates in immune response, morphogenesis, tissue repair, regeneration, development, and cancer metastasis (2, 3). The key elements of cell migration are membrane protrusion, cell-cell adhesion, and cell-substrate adhesion, and cytoskeletal rearrangement (32, 43, 61, 69). Contractile forces are generated for motility by actin turnover and adhesion dynamics between leading and trailing edges (8, 9). MYOF has a role in endocytosis, myogenesis, and angiogenesis, suggesting a generalized role in membrane dynamics related to cell migration (11, 54-56, 79). This study, using live-cell imaging platforms, demonstrated that 231MYOFKD cells exhibit increased cobblestone morphology, decreased velocity, decreased path-dependent distances, and increased directionality during wound closure. Moreover, the 231MYOFKD cells maintained a more collective migratory behavior characteristic of epithelial phenotype, while the 231 control cells continued to migrate randomly with a spindle-cell shape reminiscent of a more mesenchymal phenotype.

Cancer cell migration can be regulated by two molecular mechanisms: an integrin signaling pathway and a growth factor signaling pathway (80). Both these distinct initiator pathways influence cell migratory activity (e.g. directed or random migration) and dynamics (e.g. velocity and distance traveled) (80). Integrins are a fundamental component of cell adhesion in most forms of migration, excluding leukocyte migration, because they bind to the ECM and substrates through a number of molecules, e.g. fibronectin and collagen (34, 69, 80). In addition, adhesions of αvβ3 integrins support persistent migration, whereas adhesions of α5β1 integrins prolong random migration (8,
This binding creates intracellular signaling events due to their internal interface with a multi-protein complex, focal adhesion contact (8, 9, 28, 33, 81). In the future, it may be pertinent to quantify the levels of αvβ3 and α5β1 integrins.

Focal adhesion contact is composed of regulatory proteins and structural proteins that interact with the force-generating machinery of the cell (34, 61, 72). Regulatory proteins include focal adhesion kinase (FAK) and src family of protein tyrosine kinases (PTK) (34, 61, 72). Structural proteins include paxillin and vinculin, which allows integrin attachment and interaction with actin cytoskeleton (8, 9, 19, 28). Paxillin and FAK antibodies are often used for immunofluorescent studies that label adhesion complexes (34, 72). MYOF may be involved in either the intracellular signaling pathways or it may be possible that MYOF deficient cells are affecting integrin recycling due to the ferlin family’s known interaction with membrane dynamics. This may further be true for receptors that are being expressed by exocytosis, which may affect the soluble factor initiating pathway leading to force generation.

Soluble factor initiated migration is more active and is induced by a large variety of effectors, e.g. hormones, cytokines, chemokines, neurotransmitters, and other proteins in the ECM (8, 19, 28, 80). In addition, the strongest regulators of migration bind to G protein-coupled receptors (GPRCs), with similar signal transduction pathways initiated by integrins. GPRCs are transmembrane proteins that activate signal pathways to change cell and physiological responses, e.g. motility, photoisomerization, inflammation, and sensing cell densities (8, 19, 28, 80). However, only GPRCs require myosin type II activity for actomyosin contraction (19, 80). Activation of G protein-coupled receptors
activates heterotrimeric G proteins and dissociates the alpha subunit from the beta-gamma subunit (19, 80). The beta-gamma subunit activates phospholipase Cγ (PLCγ) and PTKs, while the alpha subunit activates adenylyl cyclase (AC) (19, 80). AC generates cyclic AMP activating protein kinase A, while PLCγ generates diacylglycerol (DAG) and inositol-triphosphate (IP3) (19, 80). DAG further activates protein kinase C (PKC), and IP3 increases intracellular calcium (19, 80). Together these signal transduction pathways link to the force-generating machinery of the cell (8, 9, 19, 28, 80).

Actin polymerization and depolymerization is/are the primary force generating mechanism of the cell (8, 9, 19, 28, 80). It is through this common pathway that integrins and soluble growth factors and chemokines regulate cell forces, migratory activity, and migratory dynamics. It has been shown that integrin clustering autophosphorylates FAK which allows PTKs to bind and mediate with the actin cytoskeleton (34, 61, 72). By way of vinculin recruitment, Arp 2/3 complexes induce actin polymerization through branching of pre-existing filaments (8, 9, 19, 28, 80). Cell migration and focal adhesion dynamics are also highly regulated by Rho GTPases, such as Cdc42, Rho-A, and Rac1 (8, 9, 19, 28, 80). Cdc42, an effector of N-WASP, stimulates Arp 2/3 complexes to induce actin branching and polymerization (7-9, 78). Rho-A, which effects ROCK, directly causes the phosphorylation of LIM-kinases and cofilin (7-9). Cofilin further induces actin cytoskeleton reorganization by activating actin depolymerization. Rac1 initiates cortactin localization to cell membrane and binds to both actin and Arp 2/3 complexes (7-9, 19).

This study demonstrated MYOF’s general effect on both morphology and migratory properties, as shown in Figures 8 and 12. The migratory properties of MYOF
deficient cells were quantified (Figure 12) with a more directional or persistent form of migration. The control cells maintained the random migration behavior reminiscent of the highly invasive mesenchymal phenotype of MDA-MB-231 cells. It is important to note that both cofilin and the Arp 2/3 complex are involved in force generation at the leading edge of cells (7-9, 19). Arp 2/3 and cofilin act in concert to sever actin and induce actin branching during membrane protrusion development, e.g. lamellipodia. Cofilin is also involved in maintaining the cytoskeleton polarity and can therefore regulate persistent cell migration, found in the 231MYOFKD cells (7, 8, 19). It has also been shown that directional migration is linked to high cofilin activity levels and low Rho-A activity levels, which are a product of fibronectin binding to αvβ3 integrins (8, 19, 28, 69). Alternatively, α5β1 integrin binding to fibronectin induces random migration and increases Rho-A mediated phosphorylation of cofilin.

The molecular mechanism of involvement between MYOF and cell migration is still unidentified. However, with these quantified results continued work can focus on localizing the specific effector(s) of MYOF due to its variety of domains, or whether MYOF’s effect on membrane dynamics represents the root cause. One possible mechanism of involvement, albeit only shown in endothelial cells, may consist of MYOF’s stabilizing effect on VEGFR-2, since αvβ3 integrins and VEGFR-2 should crosstalk to transduce VEGF signals to SAPK2/p38 and FAK (81). In addition, cell surface receptors represent an integral component of stimulating the cell migration molecular mechanism pathways. Therefore, perhaps MYOF deficient cells are exhibiting the change in morphology and migratory properties due to a general effect on integrin
expression or other receptors normally present on membrane rafts of the 231 control cells.

Continued studies may focus on 3D migration to quantify a more *in vivo* affect of MYOF. The 2D *in vitro* studies on cell protrusions and migratory properties allow for quantification of lamellipodia, filopodia, cell size, path-dependent distance, path-independent distance, average cell velocity, and cell directionality. However, MYOF’s *in vivo* affects cannot be determined. For instance, the cell protrusions in 3D (pseudopodia and invadopodia) are different than those in 2D (filopodia and lamellipodia) (33, 60). In addition, 3D cell migration will also involve blebbing (20, 43, 80, 82).

4.3 Immunofluorescence

The 231MYOFKD cells demonstrated a lower presence of paxillin in areas between membrane edge and central cell body. The lower expression of paxillin in the lamella and lamellipodia may affect the cell spreading behavior of 231MYOFKD cells by decreasing actin microfilament tension that would normally be crossed with the trailing edge. The stronger expression of paxillin near the 231WT and 231LTVctrl cell bodies may further be a cause in detachment rate differences during preliminary adhesion studies.

In addition, MYOF was shown to affect the actin distribution, focal adhesion site location, and cell-substrate attachment. The actin cytoskeleton was remodeled in the MYOF deficient cancer cells and forms concentrated actin arcs along the membrane and the larger migrating leading fronts. The 231 control cells also have more pronounced
actin cytoskeleton at the cell edge, which may account for the migration behavior demonstrated in Figures 10, 11, and 12. The paxillin stain for focal adhesion sites demonstrated a change in distribution between 231 control and 231MYOFKD cells. MYOF deficient cells marked a more nuclear-localized expression of paxillin. This change in paxillin expression may be affecting the increased cell protrusion sizes present in 231MYOFKD cells (See Figure 7). Paxillin also plays an intermediary role that binds both to integrins on the membrane and FAK (72). Together, integrins and FAKs promote the translocation of proteins to lipid rafts through ganglioside-one (GM1). FAK is also involved with activating effector Diaphanous, a Rho-GTPase, which regulates microtubule polarity (34, 60, 61, 72).

These marked differences in F-actin and focal adhesion sites may also be affecting the cell stiffness, cell-substrate adhesion, and cell migration properties (61, 78). Changes in cell morphology, cell coordination, and cell migration are shown in Figures 7-12. Immunofluorescent images corroborated the morphometric data in Figure 6 by demonstrating that 231MYOFKD cells deregulate protrusion formation and disrupt paxillin binding on the cell membrane. This may further affect protrusion formation, membrane treadmilling, membrane tension, and the loss of spindle-like morphology. Additional fluorescent imaging may need to use total internal reflection fluorescence (TIRF) to quantify focal adhesion expression.
4.4 Cell Biomechanics

Cancer cells undergo many genetic and epigenetic changes during EMT or MET. Tissue architecture transformation further leads to changes in metastatic potential (14, 35, 38, 39). In addition, significant advances have been made towards understanding molecular cues which influence breast cancer metastasis (14, 38, 39, 82). However, the past decade has focused on understanding and characterizing mechanical differences between cancer cell phenotypes, e.g. cell structure, force-induced changes, cell-directed remodeling of tissues, matrix stiffness, and micromechanical cues (7, 32). Furthermore, studies to date on cell mechanobiology suggest that there is a positive correlation between intracellular stiffness, cell migration, and affected mechanotransduction mechanisms in breast cancer cells (10, 30, 82).

Metastasis entails cancer cell migration and invasion through ECM and stroma, and reestablishment of secondary tumors within distant organs (16, 17). Studies have shown that increasing matrix stiffness affects breast cancer cells by disrupting cell-cell contacts and altering epithelial morphology to a more spindle-like morphology (22, 40, 83). These effects are similarly found in EMT and MET, which provides an interesting coupling between mechanical properties and metastatic potential. Another study, performed with *C. elegans*, determined that relaxed membrane conditions could result in larger cell sizes and that membrane tension changes can affect actin polymerization, cell-substrate adhesion, and trailing edge retraction (10). In fact, this same study quantified actin polymerization rates by measuring leading and trailing edge extension and retraction, respectively. Tension relaxation was found to lower actin polymerization rates
of actin, while increased tension resulted in increased actin polymerization rates. In addition, this reduction in membrane tension lowered cell velocities, cell displacement, and led to an unorganized rough lamellipodium (10). Increased tension was further shown to reduce lateral membrane protrusions and entailed longer filaments in direction of migration (10).

Based on these observations, MYOF may have affected the cancer cells by remodeling the actin cytoskeleton, interacting with membrane rafts, and interfering with the mechanical coupling between actin and the plasma membrane. Such an interaction would further alter the cell-cell interactions, the cell-substrate interactions, the cell protrusion mechanism, and the actin remodeling mechanisms. In the context of cell contraction and stiffness, Rho GTPases are likely involved by their ability to activate Rho-associated kinases (ROCK) which inhibit myosin light chain phosphatase (19, 43, 76, 84). Rho activation is also known to affect actomyosin contraction, formation of stress fiber bundles, and focal adhesion site maturation (8, 9, 19, 43, 76, 84). Continued work is necessary to understand whether MYOF affects molecular mechanisms during migration or affects the membrane dynamics.

4.5 Cell Motility Real Time PCR Array

CAPN1 is a calcium-regulated protease subunit that makes up the calpain enzymes (62). Calpain has been shown to help regulate cell migration by modifying proteins involved in cell adhesion and cytoskeletal components (62). This in turn can affect cytoskeletal remodeling. Moreover, calpain activity is involved in cellular
signaling, apoptosis, and cell survival. In addition, Perrin et al demonstrated that decreasing calpain activity decreases cancer invasiveness and chronic inflammation since calpain expression is altered during tumorigenesis (62). This suggests that 231MYOFKD cells may influence CAPN1 by reducing the enzyme expression 2.19 fold, which may be one of several reasons behind the reduced invasive capacity of 231MYOFKD cells. Similar reasoning may provide a possible effector of MYOF that may explain the distinct changes in cell morphology and migration shown in Figures 7-12.

Alternatively, DPP4 or CD26 was overexpressed in 231MYOFKD cells. DPP4 is a membrane-bound enzyme that is known to play a role in cancer proliferation and adhesion (63). Studies have also shown that DPP4 acts as a suppressive regulator during cancer development and metastasis, while in other cancers can create the reverse effect (63). In addition, DPP4 interacts with ECM proteins, which may play an influential role in its affect on cancer migration and metastasis. DPP4 is also useful as an efficient biomarker for specific types of cancer, due to its location on cell surface. Generally, DPP4 expression is associated with less aggressive cancers (63), as may be found in the 231MYOFKD cells. Further studies are necessary to better understand such an interaction.

EGF is another factor that was overexpressed in 231MYOFKD cells. EGF plays a regulatory role, when bound to its receptor (EGFR), in cell proliferation, differentiation, and survival (64, 65). In addition, EGF expression is known to correlate with a poor prognosis in cancer patients. Therefore, therapies have attempted to reduce EGFR expression (66, 67). The overexpression of EGF may be producing stronger signals and
activate more aggressive cell behaviors and invasive characteristics (64-67). However, it is interesting to note that the opposite effect has been shown in studies on 231MYOFKD cells, especially since it is known that overexpression of EGFR results in poor prognostic breast cancers (64, 65, 67).

ITGB2 may represent one possible cause for the changes found in cell adhesion, cell protrusions, and cell migration. ITGB2 is an integrin involved in adhesion and cell-surface mediated signaling (4, 31). Since cell migration can be regulated by integrin-mediated signaling events, this ITGB2 overexpression in 231MYOFKD cells may present a possible root cause (31, 69). Conversely, MYH9, involved in force generation and cell migration through regulation of cell protrusions, has a decreased expression in 231MYOFKD cells (71, 72). MYH9 encodes a myosin IIA heavy chain that has been assessed for lamellar spreading in MDA-MB-231 breast cancer cells (71, 72). In fact, studies demonstrate that myosin IIA and IIB are both recruited to MDA-MB-231 lamellar regions (71-73). Another study depleted myosin IIA using siRNA and produced impaired migration and increases lamellar spreading (73). Furthermore, this same phenomenon of cell spreading is shown in 231MYOFKD cells, indicating a possible rationale for the morphology changes in Figure 7. Additionally, since myosin IIA is involved in the actomyosin cytoskeleton (72, 73) this 2.42 fold decrease in expression may be affecting the cell migratory properties depicted in Figure 12.

The cell motility gene profiling array provided some possible rationales for the differences demonstrated in Chapter 3 regarding cell morphology and migration. CAPN1, DPP4, and MYH9 may represent possible effectors of MYOF that alter cell morphology
and migration. In addition, ITGB2 overexpression suggests a possible signaling pathway that may be highly activated in 231MYOFKD cells. However, ITGB2 may primarily be involved in cell adhesion. This single array needs to be repeated several times prior to conclusive results on gene profiling, but this study provides some insights into MYOF’s effect on the cell migration and adhesion mechanisms.
Chapter 5: Summary and Future Work

Cancer metastasis is one of the leading causes of death (17, 18, 20). Therefore, understanding this lethal mechanism can impact treatment practices and improve patient survival. This process accelerates three key elements—cell invasion, cell migration, and cell proliferation—that enable the cancer to invade surrounding tissues, blood vessels, and lymphatic systems (18, 20). Cell migration is essential to tissue repair, regeneration, development, cancer, and immune response (2, 3, 6). Consequently, cell migration is regulated by a complex series of molecular mechanisms that extend protrusions, affect cell polarity, affect cytoskeleton architecture and dynamics, and form cell-substrate adhesions (5, 8, 9, 19, 32, 69).

*Caenorhabditis elegans* sperm migrate in an amoeboid fashion by membrane recycling from back to front (70, 85). FER-1, a *C. elegans* protein, is known to regulate calcium-dependent membrane fusion in sperm. In fact, membrane fusion is such a vital component of *C. elegans* sperm that FER-1 mutations and deficiencies lead to infertility and immotile sperm cells (11). Myoferlin is one of three studied mammalian ferlins with a 40% homologous structure to Fer-1 (11). Myoferlin has been shown to be involved in membrane exocytosis/endocytosis and myoblast fusion during musculogenesis, *in vitro* breast cancer cell invasion, stabilizing tyrosine kinase membrane receptors, angiogenesis, and decreased muscle mass in mouse models (11, 12, 52-57). Given myoferlin’s
functions, the importance of endocytosis in breast cancer metastasis (12, 15), and the importance of cell migration during invasion (2), we hypothesized that myoferlin may also contribute to breast cancer cell migration.

Initially it was observed that myoferlin deficient MDA-MB-231 cells exhibited a mesenchymal to epithelial transition. Control cells maintained a mesenchymal phenotype (spindle-shape), while myoferlin deficient cells reverted to an epithelial phenotype (cuboidal cells). Interestingly, another study determined that the EMT markers (Figure 2) did not show expected changes in expression during this morphologic transformation. Myoferlin deficient cells exhibited increased E-cadherin, lower fibronectin, and no change in vimentin expression (12). Cell morphometrics were performed to establish myoferlin’s affect on 2D cell protrusions. Figure 8 showed that myoferlin deficient MDA-MB-231 cells affected lamellipodia number, lamellipodia surface area, filopodial length, and cell surface area. This suggested that myoferlin may play a role in 2D cell protrusion development through receptor stability, exocytosis of growth factors at leading edge, intracellular signaling events, and/or cytoskeletal dynamics (5, 8, 9, 19, 43, 60).

Cell migration was assessed using live-cell imaging platforms to quantify the 2D in vitro migration assays. Migration assay studies demonstrated (Figures 7-12) that myoferlin deficient MDA-MB-231 cells exhibited: 1) an increase in cell coordination, 2) a decrease in cell velocity, 3) a decrease in path-dependent distance traveled, and 4) an increase in directional or persistent migration during wound closure. These affects suggest that myoferlin may play a role in either/both integrin signaling and soluble factor
signaling pathways that influence cell motility. Further studies are necessary to determine myoferlin’s affect on cell migration molecular mechanisms.

Based on these observations, immunofluorescence was performed to determine variations in actin architecture and paxillin-mediated focal adhesion sites (Figure 13). In addition, MDA-MB-231 control cells revealed blebs and depicted a stronger distribution of paxillin, which may provide a rationale for the greater motility (Figure 10). Moreover, myoferlin deficient cells demonstrated that paxillin localized near the nucleus and leading edges (Figure 14). This difference in focal adhesion site distribution and actin cytoskeleton suggests once again that myoferlin may be involved in membrane receptors and/or integrins that produce intracellular signals affecting cytoskeletal remodeling, protrusion formation, and loss of spindle-like morphology. However, the immunofluorescent images require repeating based on background fluorescence. Additional paxillin imaging may also require total internal reflection fluorescence (TIRF) to accurately quantify the focal adhesion differences.

Atomic force microscopy was implemented to image and calculate the elasticity (58, 59) of MDA-MB-231 lentiviral control and myoferlin deficient cells. This biomechanical study demonstrated that myoferlin deficient cells affect cytoskeletal architecture and the cell stiffness (Young’s modulus) of MDA-MB-231 cells. Moreover, such behavior may provide insight into the migratory differences demonstrated in Figures 10-12. This may be due to the importance of cytoskeletal dynamics in motility.

In addition, a cell motility RT-PCR array was completed in order to further elucidate possible gene expression differences between MDA-MB-231 lentiviral control
and myoferlin knockdown cells. The top five fold changes (Table 2) were Calpain 1, Dipeptidyl Peptidase 4, Epidermal Growth Factor, Integrin Beta 2, and Myosin Heavy Chain 9. All the genes provided possible rationales for differences demonstrated in Chapters 3 and 4. Calpain 1, Dipeptidyl Peptidase 4, and Myosin Heavy Chain 9 may represent effectors of myoferlin that adjust cell morphology and migration (62, 64, 69, 86). In addition, Integrin Beta 2 overexpression suggests a possible adhesion and migration signaling pathway (61, 69) that may be highly activated in 231MYOFKD cells. However, additional repetitions are required to determine conclusive differences, albeit this study provided possible insights into myoferlin’s mechanistic effect on cancer cell migration and adhesion.

Myoferlin investigation is still in its infant stages with a lack of understanding its role in cell protrusion, cell migration, and actin dynamics. Nonetheless, collectively the studies presented in this thesis demonstrate that myoferlin depletion in MDA-MB-231 cells: 1) elicits an epithelial morphology, 2) elicits collective and persistent migration, 3) affects actin cytoskeleton architecture, 4) affects distribution and formation of paxillin, 5) alters biomechanical properties, and 6) may regulate several genes. This study’s observations provide an interesting outlook on myoferlin’s involvement in cell mechanics, cell membrane dynamics, and intracellular signaling pathways. Based on these observations, future studies may continue to quantify myoferlin’s physiological affects, e.g. focal adhesion site turnover rates, cell-substrate adhesion, chemotactic polarity, and additional biomechanical properties. Other studies may focus on how myoferlin affects specific molecular cues and mechanisms.
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Appendix A: SEM Morphometrics Images

Figure A 1 SEM images from morphometrics study. This is a sample of 3 images for each cell type: 231WT, 231LTVctrl, and 231MYOFKD. Images were quantified for lamellipodia area, cell area, lamellipodia number, filopodia number, and filopodia length. Scale bars and magnification are included for each individual image.
Appendix B: SEM Wound Assay Images

Figure B 1 Post-wound SEM images (10X) at 2 hrs and 10 hrs showing the morphology and migration of MDA-MB-231 WT and MYOFKD cells during closure. The MDA-MB-231 MYOFKD cells exhibit a more epithelial morphology than the MDA-MB-231 WT cells. Similar wound closure rates are present when comparing the latter time point. The early time point of 2 hrs reveals more active MDA-MB-231 WT cells.
## Appendix C: Genes of PCR Array

### Chemotaxis:
- FGF2, ITGB2, MAPK1 (ERK2), MYH10, MYH9, PLAUR (uPAR), PLD1, PRKCA, RAC2, TGFB1, VEGFA, WASF2, WIPF1.

### Receptors:
- EGFR, IGF1R, ITGA4, ITGB1, ITGB2, ITGB3, MET, PLAUR (uPAR), RHO.

### Growth Factors:
- CSF1 (MCSF), EGF, FGF2, HGF, IGF1, TGFB1, VEGFA.

### Rho Family GTPases:
- **Rho Signaling:** ACTR2, ACTR3, ARHGDI1, LIMK1, MSN, MYL9, MYLK, PLCG1, PLD1, PRKCA, Pten, PTPN1, RHO, RHOA, RHOB, RHOC, RND3, ROCK1, VIM.
- **Rac Signaling:** ACTR2, ACTR3, BAIAP2, CFL1, CRK, PAK1, PAK4, PLD1, PRKCA, RAC1, RAC2, STAT3, WASF1, WASF2, WASL.
- **Cdc42 Signaling:** ACTR2, ACTR3, CDC42, PPN1, WASF1, WASF2, WASL.

### Adhesion:
- **Cell-Cell Adhesion:** DPP4, EGFR, EZR, ITGA4, ITGB1, ITGB2, MSN, MYH9, ROCK1, TGFB1.
- **Cell-Matrix Adhesion:** ACTN1, ACTN3, CSF1 (MCSF), ILK, ITGB1, ITGB2, ITGB3, MMP14, PTEN, PTX2B, PXN, RASA1, RHOA.
- **Focal Adhesion:** ACTN1, ACTN3, ARHGFE7, BCAR1, CAPN2, CAV1, ENAH, ILK, ITGB1, MYL9, PTK2, PTK2B, PXN, TNN1, VASP, VCL.
- **Leukocyte Adhesion & Rolling:** EZR, ITGA4, ITGB1, ITGB2, MSN, ROCK1.

### Integrin-Mediated Signaling:
- BCAR1, ILK, ITGA4, ITGB1, ITGB2, ITGB3, MYH9, PTK2.

### Cellular Projections:
- **Filopodia:** BAIAP2, CDC42, DIAPH1, EGFR, ENAH, EZR, MSN, RDX, SVIL, VASP, WASF1, WASF2, WASL.
- **Lamellipodia:** CTTN, DPP4, EGFR, ENAH, FAP, PIK3CA (p110a), PLD1, PTK2, PXN, RDX, SVIL, VASP, VCL.
- **Stress Fibers:** ACTN4, DIAPH1, MYH10, MYH9, RHOA, RHOB, RHOC.
- **Membrane Blebs:** ACTN1, ACTN3, ACTN4, EZR, MYH10, MYH9, MYLK, RND3, ROCK1.
- **Invasive Projections:** ACTR2, ACTR3, ARF6, CDC42, CFL1, CTTN, DPP4, EGFR, EZR, FAP, MMP14, MMP2 (Gelatinase A), MMP9 (Gelatinase B), MSN, MYH9, PLAUR (uPAR), RAC2, RASA1, SH3PXD2A, SRC, SVIL, TGFB1, VEGFA, WASL, WIPF1.

### Growth Cones:
- ARHGEF7, CDC42, CFL1, PTK2B.

### Membrane Ruffles:
- ACTR2, ACTR3, ARF6, BAIAP2, BCAR1, CTTN, DIAPH1, EZR, ITGB1, MYH9, RAC1, RAC2, RDX, RHOA, TNN1, WASF2.

### Cell Polarity:
- CDC42, CFL1, EZR, IGF1R, ILK, MYH9.

### Proteolysis:
- AKT1, CAPN1, CAPN2, DPP4, FAP, HGF, MMP14, MMP2 (Gelatinase A), MMP9 (Gelatinase B), MYH9, PLAUR (uPAR), TIMP2.

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*Figure: List of all the PCR array genes. The 84 genes are listed from SABiosciences website by their corresponding signaling effect.*