A Role for Fer in Prostate and Breast Cancer

by

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Abstract

Phosphorylation of substrates by kinase proteins is one of the most prominent mechanisms by which signal transduction occurs and dysregulation of this activity is implicated as a major underlying cause of a number of diseases including cancer. Much of the current work focuses on developing inhibitors for various kinases in an effort to restore normal function in pathways where this deregulation occurs. However the vast majority of kinases remain uncharacterized and the roles they play in many of these signaling pathways are still unclear. This study examines the role of the non-receptor tyrosine kinase Fer in two malignant cells lines, MDA-MB-231 (breast cancer) and PC3 (prostate cancer). Using a lentiviral based RNA interference approach, the level of Fer was reduced to 6% in MDA-MB-231 and 10% in PC3 cells relative to WT levels. Fer knockdown correlated with enhanced migration in MDA-MB-231 cells, but impaired migration in PC3 cells, suggesting opposing roles for Fer in regulating migration in each cell type. Fer knockdown correlated with enhanced proliferation in MDA-MB-231 cells, but was slightly reduced in the PC3 cells, again suggesting opposing roles for Fer in regulating proliferation between these two cell lines. In Fer knockdown PC3 cells, N-cadherin expression was increased, while E-cadherin levels were decreased; and in a fraction of cells E-cadherin relocalized from a predominantly intracellular vesicular compartment to a plasma membrane localization. In xenografting experiments, Fer knockdown MDA-MB-231 cells showed reduced tumor growth rates compared with tumors established with WT cells, suggesting that Fer may influence tumorigenesis on a tumor cell intrinsic basis. Separate xenografting studies showed that MDA-MB-231 derived tumors grew slower in fer-knockout compared to fer-wild type nude mice. This
trend failed to show any statistical significance however it may still suggest an additional
tumor promoting role for Fer in the stroma.
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<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>AA</td>
<td>Antibiotic/antimycotic</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAR</td>
<td>Bin/Amphiphysin/Rvs</td>
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<tr>
<td>BCR</td>
<td>Break point cluster region</td>
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<tr>
<td>β</td>
<td>Beta</td>
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<tr>
<td>CC</td>
<td>Coiled-coil</td>
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<tr>
<td>CIP4</td>
<td>Cdc42-interacting protein 4</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl terminal</td>
</tr>
<tr>
<td>Ctn</td>
<td>Cortactin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle Medium</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Epithelial-cadherin</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGTA</td>
<td>β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>F-BAR</td>
<td>FCH-containing BAR-like</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FCH</td>
<td>Fes/Fer/CIP4 homology</td>
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<tr>
<td>Fer</td>
<td>Fes related</td>
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<tr>
<td>Fes</td>
<td>Feline sarcoma</td>
</tr>
<tr>
<td>Fps</td>
<td>Fujinami poultry sarcoma</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>Kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
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</tbody>
</table>
mM  Millimolar
N-cadherin  Neural-cadherin
N-terminal  Amino-terminal
PAGE  Poly acrylamide gel electrophoresis
PBS  Phosphate buffered saline
PDGF  Platelet derived growth factor
PI3K  Phophoinositide-3 kinases
PMSF  Phenylmethylsulfonyl fluoride
PTK  Protein tyrosine kinase
PTP1B  Protein tyrosine phosphatase
P120ctn  p120 catenin
Q  Glutamine
RPMM  Roswell Park Memorial Institute
SCL  Soluble cell lysate
SDS  Sodium dodecyl sulfate
SFK  Src family kinase
SHC  Src homology 2 domain containing
Stat 3  Signal transducer and activator of transcription 3
SH2  Src homology 2
VE-cadherin  Vascular endothelial cadherin
WASP  Wiskott-Aldrich syndrome protein
Y  Tyrosine
µg  Micro gram
µL  Micro liter
µm  Micro meter
°C  Degrees celsius
Chapter 1 – Introduction

1.1 General introduction

Protein kinases constitute one of the largest protein families within the human genome. The protein kinase super family is comprised precisely of 518 members, which are defined by a conserved catalytic domain \(^1\). Phosphorylation is one of the most prominent mechanisms by which signal transduction is carried out within the cellular environment. These signals regulate various intracellular processes including: progression through the cell cycle, gene expression, metabolic processes, cell motility, differentiation, morphology, proliferation and apoptosis. The signal transduction pathways that control many of these processes can be disturbed by genetic mutations that disrupt the primary sequences of proteins that lie within a given pathway. Phosphorylation is the driving force behind many of these pathways and thus it is evident that kinases are an attractive target for therapeutic intervention.

Protein kinases mediate the phosphorylation of serine, threonine and tyrosine residues on substrate proteins which also forms the basis for classifying specific kinase subfamilies \(^1\). The protein-tyrosine kinase subfamily consists of two main groups; the receptor tyrosine kinases and cytoplasmic tyrosine kinases. The focus of this study was to examine a possible role for the cytoplasmic tyrosine kinase Fer in breast and prostate cancer.
1.1.2 The history of Fps and Fer

Fps and Fes, orthologous kinases related to Fer, were first isolated as oncogene products encoded by tumor causing avian and feline retroviruses, respectively.\(^2\) Sequence comparisons of the retroviral Fps and Fes proteins revealed multiple cellular orthologs from various species all corresponding to the same gene.\(^3\) Viral Fps and Fes proteins are chimeric versions of the kinase that consist of an N-terminal retroviral Gag peptide fused to all or part of the Fps or Fes protein. From this point on, I will refer collectively to Fps and Fes proteins as Fps. Figure 1.1. The Gag sequence confers unregulated kinase activity to the Fps protein, possibly by interfering with targeting or other possible regulatory elements found in the non-catalytic domain of the protein.\(^4\) Interestingly, fibroblasts transformed with viral Gag-Fps displayed a down-regulation in platelet derived growth factor (PDGF) receptor, suggesting a possible receptor regulatory role for the Fps protein in growth factor receptor levels.\(^5\) In addition, several cellular proteins have shown increased tyrosine phosphorylation in response to transduced Gag-Fps. These proteins include p120RasGAP\(^5\) and associated proteins p190RhoGAP\(^6\) and p62Dok\(^5\), phosphatidylinositol 3- kinase (PI3K)\(^7\), break point cluster region (BCR)\(^8\), SHC\(^9\), connexin 43,\(^10\) and signal transducer and activator of transcription 3 (STAT3)\(^11\). It is still not understood exactly how the Gag-Fps viral protein causes cellular transformation, but it may be acting through the above cellular proteins or through other signaling systems.\(^4\)

To identify the endogenous cellular Fps proteins, antibodies were raised against peptides generated from the viral Gag-Fps sequence.\(^12\) These antibodies identified two cross reactive human and mouse candidate proteins, a 92KDa Fps ortholog and a slightly
larger 94KDa protein which was later characterized as the Fes related protein, Fer.

Sequence comparisons of human and rat cDNAs show that Fps and Fer have significant structural similarity and together comprise the only two members of a distinct subclass of non-receptor PTK’s ¹³.
Figure 1.1. Structures of the fps and fer loci and encoded proteins. The fps proto oncogene consists of 19 exons that span approximately 13 Kb and is localized to human chromosome 15q21.1. The fer locus contains 20 exons and is localized to human chromosome 5q21. The fer and fps exon structures are essentially identical with the exception of an extra 5’ non-coding exon for Fer and an internal testis specific promoter, and two testes-specific exons which allows for tissue specific expressions of a shorter 51KDa Fer-T isoform. T1 T2 and T3 represent the first 3 exons of the fer T transcript. The Fer D743R mouse model was generated by mutating aspartate (D) 743 to arginine (R) (D743R) in exon 19, which encodes the kinase subdomain IX. The fer locus spans approximately 500 Kb with much larger introns indicated by the open lines. The color-coded exons correspond with the structural components of the protein products and are as follows: beginning at the N-terminus; Blue, green and yellow represent the F-BAR domain, purple represents the CC2 motif, light blue represents the SH2 domain and black represents the kinase domain (Adapted from 37)
1.2 Organization of the fps and fer loci

The fps proto oncogene consists of 19 exons that span approximately 13 Kb and is localized to human chromosome 15q26.1\textsuperscript{14}. The Fer locus contains 20 exons and is localized to chromosome 5q.21 (reviewed in \textsuperscript{4}). The fer and fes genes have an essentially identical exon structure with the exception of an extra 5’ non-coding fer exon and internal testis specific promoter and exons which allows for tissue specific expression of a shorter 51KDa Fer-T isoform (Figure 1.1)\textsuperscript{4}. In addition the fer locus is significantly more expansive that the fps locus, spanning approximately 500 Kb, compared to 13 Kb for the complete fps locus\textsuperscript{4}.

Fps expression is tissue specific with significant levels observed in several cell lineages which include vascular endothelial, epithelial and neuronal cells\textsuperscript{15}. Fer on the other hand is ubiquitously expressed, with the exception of the shorter Fer-T isoform which is testis-specific and has been shown to accumulate in primary spermatocytes during meiotic prophase\textsuperscript{16}.

1.3 Structural components of Fps and Fer

Fps and Fer share a unique structure among the various non-receptor kinases, leading to their classification into a distinct subclass\textsuperscript{17,18}. Fps and Fer are 822 and 823 amino acids in length, respectively. They are comprised of four structurally recognized homology regions. Beginning at the N-terminus these sequences include a Fes/Fer/CIP4 homology (FCH)-containing Bin/Amphiphysin/Rvs (BAR) domain, followed by a
distinct coiled-coil region (CC2), a central Src homology 2 (SH2) domain, and lastly a C-terminal kinase domain (Figure 1.1).

1.3.1. The F-BAR domain

The F-BAR domain is composed of what was originally referred to as the Fps/Fer/Cdc42-interaction protein 4 (CIP4) homology (FCH) motif together with a directly adjacent CC motif (CC1) (not shown in Figure 1.1). Together these two motifs comprise what is believed to be an F-BAR domain, which spans approximately 300 amino acids at the N-terminus end of the protein \(^19\). The FCH motif, which is comprised of approximately 90 amino acids within the predicted F-BAR domain of Fps and Fer, was originally described as a region of homology between Fps/Fer kinases and a Cdc42 interacting protein called CIP4 \(^20\). This domain has been detected in multiple proteins, many of which have been implicated in vascular transport, endocytosis and cytoskeletal re-arrangements \(^21,22\). The F-BAR domain belongs to a larger BAR domain family which includes other BAR domains such as N-BAR and I-BAR \(^23\). The F-BAR domain is able to bind and promote curvature of membrane by interacting with phospholipids, including phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) \(^24\). In general, BAR domains bind and tubulate membrane, allowing proteins equipped with these domains to act as regulators of membrane processes \(^25\).
1.3.2 *The coiled-coil motif*

The coiled-coil (CC) motif is a unique characteristic of Fps and Fer that separates them from other non-receptor tyrosine kinases. The CC motif consists of a seven amino acid repeat that forms a multi \( \alpha \)-helical structure\(^{26}\). One of the known functional roles for the CC motif is to facilitate the formation of oligomers, homotrimers in the case of Fer\(^{27}\) and pentamers or higher order oligomers for Fps\(^{28}\). Both kinases, despite their close structural similarity, do not interact heterotypically with one another\(^{27}\). It is also evident that the CC motif may serve a different function for Fer compared to Fps. Mutations in Fps made in CC1 (L145P), contained within the F-BAR domain, enhance kinase and transforming capability while a similar mutation made in CC2 (L332P) has no effect\(^{29}\). Combining the L145P and L332P mutations resulted in a decrease in kinase and transforming capability. These observations suggest that CC1 is involved in regulating kinase activity while CC2 may be required for oligomerization\(^{29}\). Conversely, mutations made to Fer in either CC1 (KL134RP), contained within the F-BAR domain, or CC2 (ML322RP) eliminated oligomerization while having no effect on autophosphorylation activity\(^{27}\). These observations suggest that the CC motifs in Fer may serve an alternate function in comparison to Fps. The N-terminal region of Fer, has been shown to interact with the cadherin-binding adherens junction protein p120 catenin (p120ctn)\(^{30}\). Further analysis indicates that this interaction occurs through Fer residues 331-360\(^{30}\). These sequences comprise part of CC2 and are involved in Fer homotypic oligomerization\(^{27}\) as well as interactions with p120ctn\(^{30}\).
1.3.3 SH2 domain

The SH2 domain spans approximately 100 amino acids and was originally described as a non-catalytic domain that could regulate the kinase activity and transforming capability of viral Gag-Fps. The SH2 domain is found in a wide variety of proteins involved in signaling and is thought to function to mediate protein-protein interactions. The SH2 domain interacts with other proteins by binding to a consensus sequence that contains a phosphorylated tyrosine residue. Phosphopeptide library screening using the SH2 domain as an affinity matrix identified pYEXV/I as the binding consensus sequence for the SH2 domain of Fps (where pY represents phosphotyrosine, E represents glutamate, X is any amino acid, V represents valine and I is isoleucine). The SH2 domains of Fps and Fer are thought to mediate phosphotyrosine dependent interactions with putative regulators or substrates. Based on the identified binding consensus sequence for the Fps SH2 domain, there are a number of proteins that contain this sequence and thus could be potential interacting partners for Fps and Fer. These proteins include FAK, FcγRI, γ-adaptin, Hck, LAR-PTP, Lyn, SHP-1, Tec, 3BP2A, Abl, BCR, CD3ε, CD72 and ezrin. Despite this long list of potential interacting partners there is little biochemical evidence to support these interactions. A recent study has shed some light on a mechanism by which the SH2 domain of Fes interacts with the kinase domain. This study suggests that the SH2 domain provides substrate recognition by binding to phosphorylated tyrosine residues on target proteins. The binding of this phosphorylated protein then stabilizes the SH2-kinase interaction allowing the SH2 domain to orient the substrate protein with the active site of the kinase domain to allow for further phosphorylation of the substrate protein.
1.3.4 Kinase domain

The structure for the Fer kinase domain to date remains unsolved. However efforts to model the Fer SH2 and kinase domains based upon the known structures for other related PTKs such as Src \(^{35}\) and more recently Fes \(^{34}\) have provided a basis for exploring Fer’s catalytic domain and intramolecular interactions with the SH2 domain. The kinase domain of Fer contains many of the conserved features that are seen in kinases domains. These features include a conserved ATP binding sequence spanning amino acids 571 – 592 together with two invariant residues; lysine (K)-592 located in sub domain II and a presumptive catalytic base aspartate (D)-685 located in subdomain VI \(^{36,35}\). These residues are essential for ATP binding and contribute to the conformational stability of the catalytic loop \(^{35}\). An interesting feature that sets Fer and Fes apart from other PTK’s such as SRC is that the SH2 domain does not interact with a C-terminal phosphorylation site in order to restrain the kinase domain in an inactive state \(^{37}\). Instead, as mentioned previously, the SH2 domain interacts with the ATP binding lobe of the kinase domain orienting substrate proteins with the active site \(^{34}\).

1.4 Transgenic mouse models of Fer

To investigate the biological function of Fer, a transgenic mouse line with a kinase inactivating mutation was generated \(^{38}\). The mutation was made in exon 19, which encodes the kinase subdomain IX. The mutation targets aspartate (D)-743 which contributes to the conformational stability of the catalytic loop \(^{39}\). Mutating aspartate D743 to arginine (R) (D743R) destabilizes the catalytic loop resulting in the inactivation of the kinase domain \(^{35}\). The D743R mutation not only results in a defective full length
version of Fer but also a defective version of the testis specific Fer T isoform. This mutation not only abolished the kinase activity of Fer, but destabilized the protein leading to its rapid degradation. No defects are observed in homozygous mutant (fer\(^{DR/DR}\)) embryos, suggesting Fer activity is not required for normal development. Both homozygous fer\(^{DR/DR}\) males and females remain viable and fertile showing no overt defects. This observation was surprising given Fer’s ubiquitous expression and its proposed roles in regulating cadherin stability, cortical actin cross-linking, cell growth and axon outgrowth. These observations would suggest that either Fer is not critical to the pathways regulating these cellular functions or that other kinases may serve redundant roles in the absence of catalytically active Fer. Challenging the fer\(^{DR/DR}\) mice with lipopolysaccharide (LPS) revealed a physiological difference with respect to leukocyte recruitment to sites of inflammation. The fer\(^{DR/DR}\) showed a greater ability to recruit leukocytes in response to the same local dose of LPS. Further examination of this phenomenon revealed that Fer may be acting through a mechanism specific to the neutrophils to regulate their recruitment. It was also speculated that with the growing body of evidence that implicates Fer in the regulation of adherens junctions that Fer could be acting in the epithelium to strengthen cell adhesion and with the loss of Fer the integrity of the epithelium is compromised.

1.5 Adherens junctions - Cadherins

Cadherins encompass a large family of calcium dependent cell adhesion proteins that have major roles in tissue morphogenesis and development. The cadherin family is composed of four subfamilies which include; classical cadherins, proto cadherins,
desmosomal cadherins and cadherin-like proteins which fail to fit the criteria for the other subfamilies \(^{44}\). The most extensively studied subfamily of cadherins is the classical cadherins which are named according to the tissue type from which they were first isolated. For example E, N and VE cadherins where first isolated from epithelial, neuronal and vascular endothelial cells, respectively; however their expression is not exclusively limited to these tissues \(^{44}\). The classical cadherin structure is composed of three domains; an extra-cellular domain which forms calcium-dependent interactions with other cadherins on adjacent cells, a single transmembrane domain and a very highly conserved cytoplasmic domain which interacts with a number of signaling proteins to establish a physical link to the actin cytoskeleton (Figure 1.2) \(^{30}\). As adhesion molecules, cadherins maintain tissue morphology; for example the E-cadherin present in adherens junctions between epithelial cells forms a transport barrier between the intestine and blood stream isolating bacteria and other toxins in the intestine \(^{44}\). At the same time cadherins must be dynamic; take for example VE-cadherin which maintains the barrier between the blood stream and surrounding tissues. This barrier must remain strong, but at the same time be sufficiently plastic to rapidly remodel to allow immune cells to move through the barrier to the surrounding tissues \(^{44}\). Cadherins also play roles in the establishment of distinct tissue interfaces during development which is attributed to specific recognition between cadherins on adjacent cells \(^{44}\). It is believed that the formation of these junctions is dependent upon specific matchmaking, whereby one type of cadherin on one cell will adhere to that same type of cadherin on an adjacent cell (for example N-cadherin to N-cadherin and E-cadherin to E-cadherin). Lastly, cadherins can
Figure 1.2 Theoretical diagram of Fer interaction with N-cadherin. Fer (Green) interacts with the AJ proteins, p120ctn (yellow), PTP1B (dark blue) and β-catenin (red). Fer’s presence in the AJ is mediated through binding to p120ctn which itself binds directly to a membrane proximal sequence of the cadherin. From this location, Fer can then phosphorylate PTP1B at tyrosine 152 (Y152) which is required for its association with cadherin. From this position PTP1B can then continuously dephosphorylate β-catenin on Y654, which is crucial to maintain its binding to the cadherin cytoplasmic domain. β-catenin, though its interaction with α-catenin, (light blue) forms a bridge linking the cadherin stabilization complex to the actin cytoskeleton. Phosphorylation of β-catenin at Y654 correlates with a loss of its association with cadherin leading to disruption of AJ’s.
also act as signaling proteins influencing properties such as cell differentiation, migration and apoptotic signaling \(^{44}\).

\[ \text{1.5.1 Fer in migration and cell adhesion} \]

Stabilization of the cadherins at the cell surface requires both extracellular and intracellular protein-protein interactions. From the extracellular side the 5 ectodomains interact with other ectodomains on adjacent cells to form adhesive protein complexes. On the intracellular side the cytoplasmic domain interacts with a number of signaling and scaffolding proteins to regulate the cadherins stability and anchorage to the actin cytoskeleton. The linkage to the actin cytoskeleton is made through a direct interaction between two catenins, \(\beta\)-catenin and \(\alpha\)-catenin (Figure 1.2). These proteins are referred to as the ‘core’ proteins of the stabilization complex because they are essential to classical cadherin mediated adhesion \(^{30}\). Other proteins which bind directly or indirectly to the cytoplasmic domain of the cadherin, such as p120ctn and the phosphatase PTP1B, are thought to regulate the stability of the cadherin by modifying the activity of these core proteins \(^{45}\). Phosphorylation of specific tyrosine residues on \(\beta\)-catenin is known to modify its affinity to bind to the cytoplasmic domain of cadherins. In particular, the phosphorylation status of tyrosine \((Y)\) 654 appears to regulate \(\beta\)-catenin’s affinity with N-cadherin; such that phosphorylation reduces its affinity for the cadherin cytoplasmic domain \(^{46, 47}\). Phosphorylation of another tyrosine residue, Y142 on \(\beta\)-catenin, reduces its affinity for \(\alpha\)-catenin, providing yet another way to disrupt the linkage to the actin cytoskeleton \(^{30}\). It has previously been assumed that this cadherin-catenin complex is linked to the actin cytoskeleton through a direct interaction between \(\alpha\)-catenin
and actin fibers. A recent study however has cast some doubt on this model demonstrating that α-catenin is unable to simultaneously bind to actin and β-catenin. This observation was reconciled with the identification of EPLIN as a novel α-catenin binding partner that links α-catenin to the actin fibers.

There are several tyrosine kinases that could potentially phosphorylate β-catenin which include epidermal growth factor receptor (EGFR), Abl, Src-family kinases (SFKs) and Fer. Of these tyrosine kinases, Fer is the only one that has been found to associate, through p120ctn with the cytoplasmic domain N-cadherin and E-cadherin. This interaction is mediated by Fer binding to p120ctn which itself binds to the juxtamembrane sequence of the cadherin cytoplasmic domain. Additionally, over-expression of Fer or activation correlates with increased phosphorylation of Y142 on β-catenin, which would suggest that Fer plays a role in destabilizing the adherens junction. However, the constitutive presence of Fer in association with the cytoplasmic domain of the cadherin would suggest otherwise. Studies have shown that disrupting Fer’s association with the cytoplasmic domain of N-cadherin causes a loss of N-cadherin mediated adhesion. Mouse embryonic fibroblasts (MEFs) from ferDR/DR mice also show a defect in N-cadherin based cell-cell adhesion in the absence of functional Fer. The endogenous N-cadherin association in ferDR/DR MEFs is decreased by 4 fold and intracellular adhesion strength by 2 fold in comparison to WT MEF’s.

To examine Fer’s interaction with the cytoplasmic domain of N-cadherin more closely, cell-permeable peptides that mimic the binding sequences of Fer and p120ctn were incubated with chick neural retinal cells. These experiments revealed that Fer plays an indirect role in maintaining the dephosphorylation of Y654 of β-catenin. As presented
by Xu and colleges, this model proposes that Fer binds to the juxtamembrane proximal sequence of N-cadherin through its interaction with p120 catenin. From this position Fer is then able to phosphorylate the phosphatase PTP1B at Y152, which is a crucial residue for regulating its ability to bind to the N-cadherin cytoplasmic domain. PTP1B when bound to the cytoplasmic domain of N-cadherin is then positioned to dephosphorylate Y654 on β-catenin (Figure 1.2). Other studies have shown that in addition to a loss of N-cadherin mediated adhesion there is an accompanied loss of integrin mediated adhesion.

Integrins consist of two transmembrane glycoprotein subunits that form non-covalent heterodimers composed of α and β subunits. To date there are 16 different types of α and 8 types of β subunits that have been identified. The different combinations formed from these α and β subunits bind various extra cellular matrices with differing affinities, thus the expression patterns of these subunits will ultimately determine how well the cell can bind and migrate on specific types of extra cellular matrix.

Studies have revealed that interfering with Fer’s ability to bind to the cytoplasmic domain of N-cadherin correlated with an increase in the amount of Fer bound with integrin-associated FAK. In addition there was a 30-50% decrease in the tyrosine phosphorylation of integrin–associated adapter protein p130Cas. This observation coupled with the loss of integrin function was in agreement with previous reports that phosphorylation of p130Cas is essential for its assembly into the focal adhesion complex, where it is a key regulator of various effector molecules that regulate integrin function. It is however still unclear how exactly Fer’s presence at the integrin complex causes
this reduction in p130Cas phosphorylation, but it is speculated that it may act in an indirect way by phosphorylation of other binding partners for p130Cas—such as PTP-PEST, PTP1B and FAK 54.

1.6 Fer and cytoskeletal regulation

Changes to the cortical actin cytoskeleton are essential to a wide variety of cellular processes including motility, adhesion, movement of vesicles, anchorage of transmembrane proteins, endocytosis and phagocytosis 60. In motile cells the actin cytoskeleton that is close to the cell periphery consists of a highly organized mesh-work of filamentous actin (F-actin) which closely associates with the overlying cell membrane 61. Two types of protrusive membranes are formed as a result of this closely associated F-actin. The first type are lamellipodia which are composed of short filaments of F-actin linked into orthogonal arrays 62. The second type are filopodia, comprised of bundled and cross linked actin filaments 62.

Rac and Cdc42, both members of the Rho family of GTPases, play essential roles in the signaling pathways that induce the necessary cortical actin arrangements that lead to the formation of lamellipodia and filopodia. Rac and Cdc42 both bind to and activate members of the WASP family of proteins. This modifies the activity of the Arp2/3 complex whose activity is essential to the formation of the leading edge of these structures during cell migration 63. The activation of Rac and Cdc42 is closely tied with the tyrosine kinase pathways of the EGFR and PDGFR. Ligand engagement of both
receptors has been shown to drive the cortical actin rearrangements needed for cell motility, which is mediated by the downstream activation of Rac and Cdc42 \(^{64}\). Activation of the EGFR and PDGFR also results in the activation of Src and other related non-receptor tyrosine kinases such as Fer \(^{60}\). Similarly to Rac and Cdc42, the activity of Src and other related kinases are responsible for activating, through tyrosine phosphorylation, many actin bound proteins which effect cortical actin rearrangements \(^{65}\).

Cortactin (Ctn) is an actin bound protein that was initially identified as a tyrosine phosphorylated protein in v-Src transformed chicken embryo fibroblasts \(^{66}\). Ctn has been implicated in regulating cell motility through stabilization of the cortical actin cytoskeletal; and it functions as a key regulator of the actin remodeling that is required for membrane dynamics \(^{67,68}\). Ctn promotes the polymerization of actin by activating the Arp2/3 complex indirectly through the actin-nucleation regulator N-WASP \(^{69}\), or directly through Arp2/3’s interaction with the Ctn N-terminal domain \(^{70}\). The activity of Ctn is regulated by the phosphorylation of three tyrosine residues, Y421, T466 and Y482 \(^{67}\). The phosphorylation of these residues correlates with a decrease in F-actin cross linking activity and with an increase in cell motility, invasion and metastasis \(^{67}\). Much of the tyrosine phosphorylation of Ctn is attributed to Src family kinases, however Fer has been also shown to interact directly with and phosphorylate Ctn in response to growth factor stimulation and integrin engagement \(^{38}\) and \(^{71}\). MEF \(fer^{DR/DR}\) treated with PDGF showed markedly reduced phosphorylation of Ctn \(^{38}\). In addition \(fer^{DR/DR}\) MEF also displayed impaired integrin mediated migration which was correlated with decreased integrin mediated Ctn phosphorylation \(^{38}\) and \(^{71}\). These observations implicate Fer in the
regulation of Ctn phosphorylation downstream of growth factor and integrin signaling pathways.  

1.7 Fer and breast cancer

Clinical studies have found a region within chromosome 11 designated q13 which is amplified in 14-16% of breast cancers with lymph node metastasis. The EMS locus within this region encodes for Ctn; and its amplification correlates with reduced survival in patients diagnosed with lymph node metastasis. Amplification of the EMS1 locus results in the over-expression of Ctn which is thought to increase the invasive and metastatic phenotype of breast cancer. Fer’s implication in the regulation of Ctn activity may provide a way to reduce the activity of the over-expressed cortactin in these breast cancers.

1.8 Fer and malignant cell proliferation

There are several lines of evidence which support a role for Fer in the proliferation of malignant cells. When comparing the levels of Fer in malignant prostate tumors to benign tumors, studies have shown that significantly high levels of Fer are detected in malignant compared to benign tumors. In addition, down-regulation of Fer using RNAi methods impairs the proliferation of PC3 (prostate carcinoma) and MDA-MB-231(breast cancer) cells in culture. This impairment to proliferation was caused by the arrest of these cells in the G0/G1 phase of the cell cycle, suggesting that perhaps Fer was interacting directly or indirectly with proteins that modulate the cell cycle. In both the PC3 and MDA-MB-231 cell lines, Fer was identified as a key
supressor of PP1α, a phosphatase that activates the tumor suppressor pRB. Thus in the absence of Fer, PP1α activity is no longer suppressed and the normal tumor suppressive signals that bring about the cell cycle arrest can resume. This interaction appears to be specific to malignant cells due to the inability to demonstrate a similar Fer cell cycle dependency in primary fibroblasts.

1.9 Fer and prostate cancer

The implication of Fer in the proliferation of malignant cells could prove to be a useful avenue to developing a new targeted therapy for prostate cancer. The most common and effective form of therapy to treat advanced prostate cancer is androgen ablation. This treatment involves the removal of testosterone from a patient’s body through surgical castration or through the uses of androgen blocking agents. This therapy is often effective at first, but the majority of cases relapse with an androgen independent form of the disease. This presents the obvious challenge to determine what signaling pathways promote the growth of the androgen independent cancer cells. It was previously reported that the expression level of Fer in prostate tumors was higher in malignant vs benign ones. In addition, down-regulation of Fer impaired the proliferation of PC3 cells in vitro. The PC3 cell system represents an androgen independent form of prostate cancer and thus serves as an adequate representation of the androgen insensitive form of prostate cancer that reoccurs after androgen ablation treatment. Fer’s affect on the proliferation of this cell line may implicate Fer in participating in the signaling pathways that allow the prostate cancer cells to progress in the absence of androgens.
1.10 Hypothesis and research objectives

Many of the studies to date have implicated Fer as a participant in many of the signaling pathways that regulate adhesion, migration, cytoskeletal rearrangements and proliferation. All four of these cellular processes are altered in one way or another as a cell progresses from a differentiated state to a malignant cancer cell; and Fer’s participation in this wide array of cellular processes may make it an attractive target for therapeutic intervention. However further investigation to determine the direct effect of targeting Fer in cancer cells is required to warrant the development of an inhibitor. The purpose of this study was to examine the effect of targeting Fer in a prostate cancer and a breast cancer cell line using short hairpin RNA interference, shRNA, to target the expression levels of Fer. The PC3 cell line was used as a prostate cancer cell culture system which represents a human androgen independent prostate cancer cell with a high metastatic potential. It consists of a heterogeneous population of cells which display both epithelial and fibroblastic phenotypes. The MDA-MB-231 cell line was used as a breast cancer cell culture system which represents a highly metastatic human breast cancer cell with fibroblastic like morphology. The MDA-MB-231 is an established cell line for use in mouse xenografts, which allowed us to examine the effect of targeting Fer expression levels in vitro as well.

The first objective of my study was to establish an effective method to knock down Fer expression in the PC3 and MDA-MB-231 cells using a lentiviral shRNA delivery system. The second objective was then to characterize the effect of knocking down Fer in terms of integrin mediated migration and proliferation. The purpose here was
to develop an effective method to assay migration and proliferation that could be applied to both the PC3 and MDA-MB-231 cell lines.

The third objective was to examine the effect of knocking down Fer on cadherin expression and localization in terms of E- and N-cadherin in the PC3 cells. Fer is implicated in the stabilization of both E- and N-cadherin and may influence which cadherin predominates at the cell membrane. The expression levels of both E- and N-cadherin were first examined by western blot analysis to evaluate any changes to expression levels in the absence of Fer. This was followed immunofluorescence microscopy to evaluate the localization of each cadherin.

The fourth and final objective was to develop two mouse models to evaluate the role for Fer in breast cancer tumorigenesis. The first mouse model was designed to assess the role of Fer on a tumor cell intrinsic basis. This model involved a xenograft of a stable MDA-MB-231 Fer knockdown line into the mammary fat pad of athymic nude mice. The purpose of this model was to determine how lowering the levels of Fer could affect growth of the MDA-MB-231 cells in vivo.

The second mouse model was designed to assess the effect that stromal cells that surround the tumor may have on the growth of MDA-MB-231 cells in a Fer-deficient background. This model involved a xenograft of WT MDA-MB-231 cells into the mammary fat pad of ferDR/DR athymic nude mice. The purpose of this model was to determine if a Fer-deficient background in the stromal cells could affect growth of the MDA-MB-231 cells in vivo.
Chapter 2 - Materials and Methods

2.1 Preparation of shRNA-encoding lentivirus

293T cells were plated at 2.5 x 10^6 cells per 100mm plate in 10mL of pre-warmed (37°C) complete media - Dulbecco’s modified Eagle Medium (DMEM; Gibco) supplemented with 10% [v/v] FBS, 1% [v/v] antibiotic/antimycotic (AA; Gibco) and 1% [v/v] glutamine (Q; Gibco) - the night before the transfection. The cells were allowed to reach 60% confluence before the media was aspirated and replaced with 10mL of fresh pre-warmed complete media. 0.5mL of sterile distilled water was added to a 1.5mL eppendorf tube followed by 20μg of transfer vector (pLKO-F1, -F2, -F3, -F4, -F5 or empty vector control; Open Biosystems), 15μg of packaging plasmid (CMVΔR8.91; Open Biosystems) and 6μg of envelope plasmid (pMD2G; Open Biosystems). 0.5mL of 2X HBS-( 21mM Hepes, pH = 7.05, 270mM NaCl, 10mM KCl, 1.5mM Na₂PO₄, 0.002% [w/v] Glucose) solution was added to the mixture in the eppendorf tube and vortexed briefly, followed by the edition of 50μL of 2.5M CaCl₂ and again vortexed briefly. The solution was incubated at room temperature for 5 minutes before being added drop-wise to each 100mm plate of 293T cells and mixed gently. The 293T cells were incubated with the solution at 37°C in 5% CO₂ for 16 hours. The media was aspirated and the plates were rinsed in pre-warmed phosphate buffered saline (PBS; 136.9mM NaCl, 2.7mM KCl, 4.3mM Na₂HPO₄, 1.5mM KH₂PO₄). Six mL of prewarmed complete media was added to each 100mm plate of 293T cells which were then incubated for 24hours at 37°C in 5% CO₂. Following the 24 hour incubation, 6mL of media was collected from each 100mm plate of 293T cells and filtered through a 0.45μm filter into a fresh 50mL falcon tube and stored at 4°C. Six mL of fresh pre-warmed media complete was added to each 100mm
plate of 293T cells which were incubated for another 24 hours at 37°C in 5% CO₂. Six mL of media was again collected from each 100mm plate of 293T cells, filtered through a 0.45μm filter and combined with the previous day’s collection of media, totaling 12 mL, and frozen at -80°C in 2mL aliquots.

2.2 Lentiviral infection and selection of transduced cell lines

MDA-MB-231 or PC3 cells were plated at 500,000 cells per 35mm plate in 2.0mL of pre-warmed complete media consisting of DMEM complete (Gibco) for MDA-MB-231 and RPMI complete (Gibco), each supplemented with 10% [vol/vol] FBS, AA and Q. The cells were grown to approximately 60% confluence at which point the media was aspirated and replaced with 2mL of freshly thawed complete media (DMEM) containing the virus. One plate was left in the original media to act as a control for the selection process while the rest were incubated in the virus containing media at 37°C in 5% CO₂ for 3 days. Following the 3 day incubation the virus containing media was aspirated from the cells and replaced with fresh complete media. Two μL of a 2.0 mg/mL stock solution of puromycin (Sigma) was added to each plate and cells were incubated for 4 days or until the control had completely died off. The media containing puromycin was aspirated and cells were washed in pre-warmed PBS. The cells were then detached from the plates with 0.5mL of trypsin solution (Gibco) at 37°C for 5 minutes. Two mL of media was added to the plates to re-suspend the cells and the suspension was transferred to a 100mm plate. Seven and one half mL of complete media was added to bring the final volume to 10mL. The cells were allowed to recover and grow to approximately 80% confluence before being used for experiments 2.3, 2.4, 2.5 and 2.6.
2.3 Preparation of soluble cell lysates and western blotting

Cells were rinsed in 5.0mL of cold PBS, placed on ice and lysed in 0.650mL of cold kinase lysis buffer (KLB; 20mM TRIS- HCl, PH7.5, 150mM NaCl, 1mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), 1% [vol/vol] nonident-p40, 0.5 % [w/v] sodium Deoxchloric Acid, 1mM sodium orthovanadate,1 mM phenylmethylsulfonyl fluoride (PMSF), 10μg/mL leupeptin, 10μg/mL aprotinin) for 20 minutes before being scraped and transferred to a 1.5ml eppendorf tube. The tubes were spun at 13000 rpm at 4°C for 15 minutes to pellet cell debris, after which 600µL of the soluble cell lysate (SCL) was removed and placed in a new eppendorf tube. The concentration of protein in the SCL was determined using a Biorad Bradford assay and standardized to 1µg/µL. One hundred µL of 6X SDS sample buffer- (350mM Tris-HCl pH6.8, 10.28% [w/vol] SDS, 30% [vol/vol] glycerol, 0.6M dithiothreitol, 0.012% [mass/vol] bromophenol blue) was added to 500µL of each standardized SCL and heated for 5 minutes at 100°C.

The SCLs were resolved on a 7.5% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore) in transfer buffer- (48mM Tris, 39mM glycine, 1.3mM SDS, 20% [v/v] methanol) using a semi-dry transfer apparatus (Bio-Rad). Membranes were then rinsed in Tris buffered saline-0.1%Tween20 (TBS-T; 10mM Tris pH7.5, 150mM NaCl, 0.1% [vol/vol] Tween20) for 10 minutes before being blocked in either 5% [w/v] bovine serum albumin, BSA; Gibco) in TBS-T or 5% [w/v] skim milk powder in TBS-T. Membranes were then incubated in a primary antibody diluted in blocking solution overnight at 4°C. The primary antibodies used included; anti-FerLA
(rabbit polyclonal, 1:500), anti-RasGAP (rabbit polyclonal, 1:500), anti-N-cadherin (mouse monoclonal, 1:200; Transduction Labs), anti E-cadherin (mouse monoclonal, 1:1000; Transduction Labs), anti-Cortactin (Mouse monoclonal, 1:1000, Sigma), anti-P120ctn (Mouse monoclonal, 1:1000; Transduction Labs) and anti- β-catenin (mouse monoclonal, 1:1000; Transduction Labs). Membranes were washed in TBS-T three times at room temperature for 15 minutes each time with shaking. Membranes were then incubated in the appropriate secondary antibody diluted in blocking solution for 1 hour at room temperature. The secondary antibodies used included; horseradish peroxidase-conjugated goat anti-rabbit (1:10000; Vector Laboratories) and horseradish peroxidase-conjugated sheep anti-mouse IgG (1:5000; Amersham Pharmacia). Membranes were then washed in TBS-T 3 times at room temperature for 15 minutes each time with shaking. The membranes were incubated with chemiluminescence reagents according to the manufactures instructions (PerkinElmer) and imaged with X-ray film (Kodak).

2.4 Boyden chamber migration assay

A stock fibronectin solution (Sigma) at a concentration of 1mg/mL was diluted with sterile water to 10μg/mL and was used to coat the Boyden chamber cell culture inserts (Falcon). The cell culture inserts were coated by placing 500μL of diluted fibronectin solution in the bottom and top chambers to ensure a thorough coating. The plates containing the cell culture inserts were then sealed in parafilm and placed at 4°C overnight. The plates were removed from 4°C storage and the diluted fibronectin solution was aspirated from the bottom and top chambers. The cell culture inserts were dried in a laminar flow hood for 20 minutes before seeding the cells. Cells were detached from 100mm plates with 1.5mL of trypsin solution for 5 minutes at 37°C. Two mL of complete
media was added to each plate and cells were carefully re-suspended by pipetting gently to avoid the formation of bubbles. The solutions from each plate were then transferred to 15mL falcon tubes and the concentration of the cells in each solution was determined using a coulter counter (Beckman). Using base media without FBS (DMEM supplemented with AA and Q for MDA-MB-231 cells and RPMI supplemented with AA and Q for PC3 cells) diluted cell solutions were prepared such that 250μL contained 100,000 cells. Two hundred and fifty μL of each loading cell solution was then added to the top chamber of the cell culture inserts such that each independent cell line was represented by 6 inserts each. Five hundred μL of base media was added to the bottom chamber and the inserts where incubated at 37°C in 5% CO₂ for one hour to allow the cells to adhere to the membranes. The media was then removed from both chambers and replaced with 500μL of base media (without FBS) in the top chamber and 500μL of complete media (with 10% FBS) in the bottom chamber. For the control chambers 500μL of base media was added to the bottom chamber. The 10% FBS contained within the complete media acted as our chemo-attractant to confer directionality of migration in our experiments. In control wells, base media which does not contain any FBS was used in the bottom chambers. The Boyden chambers were incubated for 8 hours at 37°C in 5% CO₂ at which point the media was aspirated from the top and bottom chamber and replaced with 500μL of 3% [w/v] paraformaldehyde in PBS for 30 minutes to fix the cells. The solution was then aspirated from the bottom and top chamber and replaced with 500μL of 0.2% [w/v] crystal violet in 20% [w/v] methanol in the bottom and top chambers. The cells were stained with this solution for 2 hours, after which the solution was aspirated and the inserts were washed 5 times with PBS. Using a cotton swab the top
of each membrane was wiped to remove non migrating cells. The membranes were then removed using a scalpel and mounted on microscope slides for viewing. Three pictures were taken under bright field microscopy, at 20X magnification, for each membrane in a triangular pattern and migrated cells were counted qualitatively by eye. One way anova statistical analysis was performed on the data generated.

2.5 MTT proliferation assay

Cells were detached from 100mm dishes with 1.5mL of trypsin solution for 5 minutes at 37°C. Two mL of complete media was added to each plate and carefully re-suspended by pipetting gently to avoid the formation of bubbles. The solutions from each plate were then transferred to 15mL falcon tubes and the concentration of the cells in each solution was determined using a Coulter counter (Beckman). Diluted cell solutions were prepared such that 100μL contained 5,000 cells. Using a multi-channel pipette, 100μL of each diluted cell solution was added to each well of a 96-well dish such that each cell line was represented by 24 wells on each plate. Four plates were prepared for the 0, 24, 48 and 72 hour time points and placed in an incubator at 37°C in 5% CO₂. At each time point a plate was removed from the incubator and 25μL of MTT solution (Sigma) was added to each of the wells. The plate was then incubated for another 3 hours at 37°C in 5% CO₂. The plate was then removed from the incubator and 100μL of MTT stop solution - (1N HCL in absolute isopropanol) was added to each well with mixing to dissolve the contents of the well. The plate was incubated for another 30 minutes at 37°C in 5% CO₂ before being analyzed on a UV spectrophotometer set to read 590nm to detect the concentration of the MTT product. Two way anova statistical analysis was performed on the data generated.
2.6 Immunofluorescence analysis

A stock fibronectin solution (Sigma) at a concentration of 1mg/mL was diluted with sterile water to 10μg/mL. Each cover slip was submerged in 1mL of diluted fibronectin solution in separate wells of a 12 well plate, sealed with parafilm, and stored at 4°C overnight. The diluted fibronectin solution was aspirated from each well and the cover slips were left to dry for 20 minutes in a laminar flow hood. PC3-WT or PC3-F2 cells were plated on the top side of each cover slip at 2.5 x 10^5 cells per well. The 12 well plates containing the cover slips were incubated for 24 hours at 37°C in 5% CO₂. The plates were then placed on ice and the media was aspirated followed by the addition of 1mL of PBS* (*: 0.1mM CaCl₂, 0.1 mM MgCl₂) to rinse cells. The PBS* was aspirated from wells and replaced with 1mL of 3% [v/v] paraformaldehyde in PBS* and incubated at room temperature for 15 minutes. The fixative solution was aspirated from the wells and the cells were rinsed with PBS* three times with shaking for 5 minutes at a low speed for each wash. One mL of 0.2% [v/v] Triton-X in PBS* was then added to each well and incubated at room temperature for 10 minutes. The solution was then aspirated from the wells and the cells were rinsed with PBS* three times with shaking for 5 minutes at a low speed for each wash. One mL of blocking solution – (3% [w/v] BSA + 5% [v/v] goat serum (Sigma) in PBS* filtered through a 0.45 μm filter) was then added to each well and incubated for 30 minutes with shaking at low speed. Each cover slip was incubated in 35μL of the indicated primary antibody diluted in blocking solution face down on parafilm in a moisture chamber over night at 4°C. The primary antibodies used were anti-E-cadherin (mouse monoclonal, 1:50; Transduction Labs) and anti-N-cadherin (mouse monoclonal, 1:50; Transduction Labs).
Cover slips were removed from the moisture chamber and returned to the original wells and rinsed with 1mL PBS* three times with shaking for 5 minutes. Each cover slip was then incubated in 35µL of the indicated secondary antibody diluted in blocking solution face down on parafilm in a moisture chamber at room temperature for 1 hour. The secondary antibody was Alexa546 fluor-conjugated (goat anti-mouse IgG F(ab)2,1:200). Phallodin-Alexa-488 conjugate (1:200) was used to image F-actin. The remainder of the incubations and washes were performed in dimly lit or dark conditions when possible due to the light sensitive nature of the fluorochrome conjugates. The cover slips were removed from the moisture chamber and returned to their original wells where 1mL of 0.01% [v/v] Triton- X, 0.3% [w/v] BSA and 0.5% [v/v] Hoechst 33258 in PBS* was added and incubated at room temperature for 5 minutes with shaking at a low speed. The solution was then aspirated from each well and rinsed with 0.01% [v/v] Triton- X, 0.3% [w/v] BSA and 0.5% [v/v] in PBS* with shaking for 5 minutes at a low speed for each wash. The cover slips were then mounted onto microscope slides with 30µL of Mowiol (Sigma) and stored in the dark at 4°C.

2.7 MDA-MB-231 breast cancer xenograft model

*Foxn1*^Nu/Nu^ (BALB/C background) mice obtained from Charles River were bred with *fer^{DR/DR}* mice (129SvJ background) to obtain the nude mouse phenotype based upon the absence of hair. These mice were then genotyped by PCR and restriction endonuclease digestion, as previously described, to identify mice that were either wild type (*fer*^DR/+^) or homozygous mutant (*fer*^DR/DR^) with respect to the *fer* gene. The *fer*^DR/DR^ and *fer*^+/+^ mice were then compared in the context of a mammary fat pad xenografts of MDA-MB-231 cells to evaluate how a
Fer-deficient background in the stromal cells may affect tumor growth. A similar xenograft of MDA-MB-231-WT and MDA-MB-231-F1 cells was performed in Foxn1^{Nu/Nu} to evaluate how a Fer-deficient background with respect to the tumor cells affects the growth of the tumor.

MDA-MB-231 cells were thawed from cryo freeze approximately one week before transplantation. Cells were cultured in complete media at 37°C in 5% CO2 and were continually passaged to maintain a 60% confluence prior to harvesting for transplantation. Prior to transplantation the media was aspirated from each plate and replaced with 5mL of pre-warmed PBS in order to rinse cells. The PBS was aspirated and the cells were detached from 100mm dishes with 1.5mL of trypsin solution for 5 minutes at 37°C. Two mL of complete media was added to each plate and carefully re-suspended by pipetting gently to avoid the formation of bubbles. The solutions from each plate were then transferred to a 50mL falcon tube and the concentration of the cells in the total solution was determined using a coulter counter (Beckman). Once the concentration was determined the appropriate amount of media was transferred to a new 50mL falcon tube that contained the desired amount of cells and spun at 700 rpm in a swinging bucket centrifuge for 5 minutes to pellet the cells. The media was then aspirated from the falcon tube leaving the pellet behind. The pellet was then re-suspended in the appropriate amount of PBS so that 25μL of the suspension would contain approximately 2 x 10^6 cells. An equal volume of Matrigel (Sigma) was added to the suspension of cells which was then placed on ice until transplantation.

Mice were anesthetized by intraperitoneal injection of 0.5mL avertin (12.5mg/mL). A small incision, approximately 1cm, was then made at a 45° angle to fat
pad (D) and the linea alba of the mouse (Figure 2.1). Forceps were then used to separate the fat pad from the abdominal wall and two 18 gauge needles were used to hold the flap open for the injection. The Hamilton syringe was rinsed 3 times with sterile PBS before being filled with 50μL of the cell suspension. Using the lymph node as a landmark the tip of the Hamilton syringe was then inserted away from the lymph node and the suspension was injected in one smooth motion. The wound was then closed with 2 staples and the mouse received a subcutaneous injection of 0.1mL of buprenorphine before being placed in a clean cage. Mice received another 3 injections of 0.1mL buprenorphine at 12 hour intervals following surgery. The staples were removed 7 days after surgery prior to the first ultrasound measurement. Measurements were taken using an ultrasound machine (Visual Sonics). Measurements were performed every week for 6 weeks total. The tumors were then resected from each mouse and stored in paraformaldehyde. The incision was closed with staples as before and mice received a subcutaneous injection of 0.1mL of buprenorphine before being placed in a clean cage. The mice received another 3 injections of 0.1mL buprenorphine at 12 hour intervals following the surgery. The mice were kept alive for 90 days at which point the mice were sacrificed and internal organs were removed. The liver, lungs, spleen and intestines were all visually inspected for potential metastatic lesions. Two way anova statistical analysis was performed on the data generated.
Figure 2.1. Lateral cross section of mouse mammary fat pads. (Adapted from Dr Bruce Elliot AC2M2 injections – general protocol)
Chapter 3 - Results

3.1 Knockdown of Fer in PC3 cells

In order to investigate the role of Fer in the prostate cancer cell line PC3, I first developed a RNA interference approach to knock down fer expression. Five different lentiviral shRNA constructs designed to target the fer mRNA, designated F1 – F5, along with an empty vector, designated pLKO, were purchased from Open Biosystems. After generating virus for each construct I next determined which shRNA construct caused the maximal knockdown of Fer in the PC3 cell system. The PC3 cells were transduced with each of the five different lentiviruses and then subjected to western blotting analysis to evaluate the expression level of Fer (Figure 3.1A). Using densitometry analysis it was determined that the F2 construct caused the maximal knockdown of Fer, reducing levels to approximately 10% compared to WT levels (Figure 3.1B). F1, F3 and F5 were also effective at reducing Fer levels to 25%, 50% and 20%, respectively, of WT levels (Figure 3.1B). It should be noted that cells transduced with the virus generated from the F4 shRNA construct were not viable after selection. The PC3-F2 cell line was used for all subsequent analysis.

3.2 Integrin mediated migration defect in PC3-F2 cells

A modified transwell assay was performed using 8 µm Boyden chamber inserts that had been coated with fibronectin on both the top and bottom of the membrane. The PC3 cells were subjected to a migration assay using 10% FBS as a chemo attractant. The PC3-F2 cells along with WT PC3 (PC3-WT), and PC3 cells transduced with the empty
vector control, (PC3-pLKO), were seeded and allowed to migrate through the membrane for 16 hours, at which point the cells which had not migrated were removed and the membranes were stained with crystal violet and imaged at 20X magnification to evaluate the amount of cells that had migrated through to the other side of the membrane. The results showed a significantly reduced number of migrated PC3- F2 cells in comparison to PC3-WT and PC3-PLKO (Figure 3.2). The PC3-F2 cells displayed a statistically significant 35 % reduction in the amount of migrated cells compared to both the PC3-WT (P<0.001) and PC3-pLKO cells (P<0.001).
Figure 3.1. Immunoblotting analysis of RNAi-mediated Fer knockdown in PC3 cells. PC3 cells were transduced with lentiviruses expressing four different fer-targeted shRNAs. A) Immunoblotting (IB) analysis of soluble cell lysates (SCL) for Fer (upper panel - anti-FerLA antibody) or the loading control (lower panel – anti-Ras-GAP) was performed on WT cells or the four stably transduced lines. B) Densitometry analysis of relative expression levels of Fer normalized to p120-RasGAP ratios. These data are representative of 4 independent experiments.
Figure 3.2. Fer knock-down reduces PC3 cell migration. PC3 cells were subjected to a Boyden chamber migration assays with 10% FBS used as a chemo attractant. Cells were allowed to migrate for 16 hours at which point membranes were imaged to evaluate the number of migrated cells. PC3-F2 cells showed a statistically significant 35% reduction in the number of migrated cells compared to either PC3-WT cells (* P value < 0.001) or PC3-pLKO cells (** P value < 0.001). (n =3)
3.3 Reduced proliferation in PC3-F2 cells

It has previously been reported that knocking down Fer expression impairs the proliferation of PC3 cells in vitro. These studies utilized a Fer siRNA transient transfection method to reduce the expression levels of Fer. In this current study instead employed a lentiviral shRNA and established a stable line of PC3 cells with reduced expression levels of Fer. The purpose of this experiment was to develop a Fer knock-down cell system suitable for longer term studies, starting with proliferation and migration analysis. An MTT proliferation assay was used to measure the amount of cells present at 0, 24, 48 and 72 hours after seeding PC3-F2, PC3-WT and PC3-pLKO cells. The results indicated that there were significantly fewer PC3-F2 cells at the 24, 48 and 72 hour time points compared with PC3-WT and PC3-pLKO cells (Figure 3.3). This observation suggests that Fer promotes proliferation in PC3 cells.

3.4 Changes to E- and N-cadherin expression levels in PC3 F2 cells

The PC3 cell system is a unique cell line consisting of heterogeneous population of cells expressing both E- and N-cadherin. To explore a possible role for Fer in regulating the expression levels of E- and N-cadherin in PC3 cells, western blotting analysis was performed on soluble cell lysates prepared from PC3-F2 and PC3-WT cells (Figure 3.4A). This analysis revealed a slight decrease in the expression level of E-cadherin in PC3-F2 cells compared to PC3-WT cells. In addition there was a slight increase in the expression level of N-cadherin in PC3-F2 cells compared to PC3-WT cells. To confirm that the observed decrease in E-cadherin
Figure 3.3. Fer knockdown reduced PC3 cell proliferation. PC3-WT, PC3-pLKO and PC3-F2 cells were seeded at approximately 5000 cells per well in triplicate and a quantitative MTT assay was used to assess the amount of viable cells at 0, 24, 48 and 72 hours after plating. PC3-F2 cells showed a significantly lower absorbance compared to PC3-WT cells and PC3-pLKO at the 24 hour (* P value < 0.05), 48hour (** P value < 0.05) and 72 hour (*** P value < 0.05) time points. (n=3)
Figure 3.4. Changes to E- and N-cadherin expression levels in response to Fer knockdown. **A** Immunoblotting (IB) analysis of soluble cell lysates (SCL) for E cadherin (upper panel – anti-E-cadherin antibody), N-cadherin (middle panel – anti-N-cadherin antibody) or the loading control (bottom panel – anti-Ras-GAP) was performed on PC3-WT or PC3-F2 cells. **B** Immunoblotting (IB) analysis of soluble cell lysates (SCL) for E-cadherin (upper panel – anti-E-cadherin antibody), or loading control (lower panel – anti-Ras-GAP) was performed on PC3-WT, PC3-F1, PC3-F2, PC3-F3 and PC3-F5.
expression was not specific to the F2 Fer shRNA construct, western blotting analysis was performed to measure E-cadherin expression in PC3 cells transduced with lentivirus expressing three other fer-targeted shRNA constructs (F1, F3 and F5). This analysis revealed that there was a decrease in E-cadherin expression levels in PC cells transduced with all four fer-targeting shRNA constructs (Figure 3.4B).

3.5 Localization of E- and N-cadherin in PC3-F2 cells

The change in E- and N-cadherin steady-state levels observed in the PC3-F2 cells suggests that Fer may participate in pathways that regulate cadherin expression or turnover. Other studies have reported that Fer associates with the cytoplasmic domain of both E- and N-cadherin and may regulate cadherin stability and function at adherens junctions 30 and 53. Therefore, the next step in my analysis was to examine if a loss of Fer expression could influence the localization of E- and N-cadherin. The localization of E- and N-cadherin was visualized by immunofluorescence microscopy to determine if there was a difference between the PC3-F2 and PC3-WT cells. This analysis revealed that N-cadherin remains predominantly at the plasma membrane with some punctate cytoplasmic staining in both PC3-F2 and PC3-WT cells (Figure 3.5, panels D and A). F-actin shows cortical localization in both PC3-F2 and PC3-WT cells (Figure 3.5, panels E and B). A merge of the two sets of images that the N-cadherin co-localizes with F-actin at the plasma membrane, which is evident due to the overlap of Alexa-546 and Alexa-488 signals resulting in a yellow color (Figure 3.5, panels C and F). Interestingly, the PC3-F2 cells displayed more of what appeared to be hair-like extensions in a sub population of cells compared to PC3-WT cells.
When this analysis was extended to E-cadherin, I observed a more striking difference in localization between PC3-F2 and PC3-WT cells. E-cadherin displayed prominent punctuate perinuclear localization in the majority of PC3-WT cells (Figure 3.6, panel A), which is consistent with the endocytosis and down regulation or intracellular storage of E-cadherin that is reported in many epithelial derived malignancies \(^8\). In PC3-F2 cells however, a sub population of cells displayed a more prominent plasma membrane localization (Figure 3.6, panel D). This observation that E-cadherin returns to the surface in a sub population of cells within the PC3-F2 cells suggests that Fer may play an important role in the down regulation and trafficking of E-cadherin in this cell system. F-actin shows cortical localization in both PC3-F2 and PC3-WT cells (Figure 3.6, panels E and B). A merge of the two sets of images shows that the E-cadherin co-localizes with F-actin at the plasma membrane only in this sub population of PC3-F2 cells, which is evident due to the overlap of Alexa-546 and Alexa-488 signals resulting in a yellow color. The co-localization of E-cadherin with cortical F-actin reinforces the observation that E-cadherin is localized to the plasma membrane in the PC3-F2 cells. Plasma membrane localization of E-cadherin could contribute to a situation in which the PC3 cells were more likely to associate with one another and less likely to migrate.
Figure 3.5. Confocal immunofluorescence microscopy analysis of N-cadherin localization in PC3 cells. A, D) N-cadherin was detected by indirect immunofluorescence using an Alexa-546 conjugated secondary antibody (Red). B, E) F-actin was visualized using a phallodin-Alexa-488 conjugate (Green). C) Depicts a merge between Panels A) and B) while F) depicts a merge between panels D) and E), the nucleus of the cells was visualized using Hoechst 33258 (Blue). The upper row of panels A), B), C) are PC3-WT cells while the lower row of panels D), B), C) are PC3-F2 cells.
Figure 3.6. Confocal immunofluorescence microscopy analysis of E-cadherin localization in PC3 cells. 

A, D) E-cadherin was detected by indirect immunofluorescence using an Alexa-546 conjugated secondary antibody (Red). B, E) F-actin was visualized using a phallodin-Alexa-488 conjugate (Green). C) Depicts a merge between Panels A) and B) while F) depicts a merge between panels D) and E), the nucleus of the cells was visualized using Hoechst 33258 (Blue). The upper row of panels A), B), C) are PC3-WT cells while the lower row of panels D), B), C) are PC3-F2 cells.
Chapter 4 – Results

4.1 Knockdown of Fer in MDA-MB-231 cells

To determine which shRNA construct causes the maximal knockdown of Fer in the MDA-MB-231 cell system, these cells were transduced each with each of the five different fer mRNA targeting shRNA lentivirus constructs. The resulting stably transduced cell lines were then subjected to western blotting analysis to evaluate the expression levels of Fer (Figure 4.1A). Using densitometry analysis it was determined that the F1 shRNA construct causes the maximal knockdown of Fer, reducing levels to approximately 6% compared to WT levels (Figure 4.1B). F2, F3, F4 and F5 were also effective at reducing Fer levels to 20%, 10%, 30% and 12%, respectively, compared to WT levels (Figure 4.1B). The MDA-MB-231-F1 cell line was used for all subsequent analysis.

4.2 Integrin mediated migration enhancement in MDA-MB-231–F1 cells

A modified Boyden chamber transwell assay was performed using 8 µm pore size membrane inserts that had been coated with fibronectin on both the top and bottom. MDA-MB-231 cells were induced to migrate using 10% FBS as a chemo attractant. The MDA-MB-231-F1 cells along with parental cells (MDA-MB-231-WT) and cells transduced with the empty vector control (MDA-MB-231-pLKO) were seeded and allowed to migrate through the membrane for 8 hours at which point the un-migrated cells were removed and the membranes were stained with crystal violet and imaged at
Figure 4.1. Immunoblotting analysis of RNAi-mediated Fer knockdown in MDA-MB-231 cells. MDA-MB-231 cells were transduced with lentiviruses expressing five different fer-targeted shRNAs. A) Immunoblotting (IB) analysis of soluble cell lysates (SCL) for Fer (upper panel - anti-FerLA antibody) or the loading control (lower panel – anti-Ras-GAP) was performed on WT cells or the five stably transduced lines. B) Densitometry analysis of relative expression levels of Fer normalized to p120-RasGAP ratios. These data are representative of 4 independent experiments.
Figure 4.2. Fer knock-down enhances migration of MDA-MB-231 cells. MDA-MB-231 cells were subjected to a Boyden chamber migration assays with 10% FBS used as a chemo attractant. Cells were allowed to migrate for 16 hours at which point membranes were imaged to evaluate the number of migrated cells. MDA-MB-231-F1 cells showed a statistically significant 100% increase in the number of migrated cells compared to either MDA-MB-231-WT cells (* P value < 0.001) or MDA-MB-231-pLKO cells (** P value < 0.001). (n =3)
20X magnification to evaluate the amount of cells that had migrated to the other side of the membrane. The results showed a significantly reduced number of migrated MDA-MB-231-F1 cells in comparison to MDA-MB-231-WT and MDA-MB-231-PLKO (Figure 4.2). The MDA-MB-231-F1 displayed a statistically significant 100% increase in the amount of migrated cells compared to both the MDA-MB-231-WT cells (P<0.001) and MDA-MB-231-pLKO cells (P<0.001) (Figure 4.2).

4.3 Increased rate of proliferation in MDA-MB-231-F1 cells

Previous studies have demonstrated that transiently knocking down Fer expression in MDA-MB-231 cells impairs their proliferation in vitro, similarly to what is seen in the PC3 cell system. The purpose of this experiment was to determine if a stable Fer knock-down cell line could produce a similar result as what is seen in the siRNA knockdowns in other studies. An MTT proliferation assay was used to measure the amount of cells present at 0, 24, 48 and 72 hours after seeding MDA-MB-231-F1, MDA-MB-231-WT and MDA-MB-231-pLKO cells. The results indicate that there were significantly more MDA-MB-231-F1 cells present at the 24, 48 and 72 hour time points compared with both MDA-MB-231-WT and MDA-MB-231-pLKO cells (Figure 4.3). These observations suggest that Fer knock-down promotes a higher proliferation rate in the MDA-MB-231 cell system.

4.4 Decreased xenograft tumor growth rate by MDA-MB-231-F1 cells

In vitro it was found that the MDA-MB-231-F1 cells had a higher rate of proliferation compared to that of MDA-MB-231-WT cells. However this enhanced proliferative characteristic may be specific to the cell culture system and may not reflect
accurately how these cells would behave in vivo. To explore this question, breast fat pad orthotopic injections of MDA-MB-231-WT and MDA-MB-231-F1 cells were performed on athymic nude mice. Tumorigenesis was evaluated by monitoring the growth of the primary tumor using ultrasound to generate a 3D image which was then used to determine tumor volume. Tumor volumes were monitored at 1 week intervals for 6 weeks to generate an average growth curve for each group. The growth of MDA-MB-231-WT tumors was significantly faster compared with tumors established with MDA-MB-231-F1 cells (Figure 4.4). The volumes of tumors at 4, 5 and 6 weeks were significantly higher for tumors established from MDA-MB-231-WT compared to MDA-MB-231-F1. This in vivo observation stands in contrast to the enhanced proliferation of MDA-MB-231-F1 cells in vitro.

4.5 Decreased MDA-MB-231 xenograft tumor growth rate in Fer-deficient mice

An ongoing unpublished study in the lab has established genetic evidence for a tumor promoting role for Fer in an activated ErbB2-driven transgenic breast cancer model (Sangrar, personal communication). This experiment was performed by crossing mice with a targeted kinase-inactivating mutation (D743R) in fer with a transgenic mouse line that expresses activated ErbB2 (actErbB2) in the breast epithelium. Among the progeny from this cross, actErbB2 transgenic female mice homozygous for the fer mutation (ferDR/DR; actErb2) displayed delayed tumor onset relative to fer+/+; actErb2 mice (Sanger, personal communication). This model suggests that Fer may play a role in the mechanisms that underlie ErbB2 mediated signaling; however, it does not address whether this apparent tumor promoting effect of Fer is intrinsic to the developing tumor cells, is a result of stromal effects on the progression of the tumors, or is a combination of
both. To determine if Fer functions within the stromal compartment to influence the progression of breast cancer tumors we compared the in vivo growth of MDA-MB-231 cells engrafted into either Fer-deficient (fer<sup>DR/DR</sup>) or Fer-wild type (fer<sup>+/+</sup>) athymic nude mice. Tumorigenesis was evaluated by monitoring the growth of the primary tumor using ultrasound. The tumor volume was monitored at 1 week intervals for 6 weeks to generate an average growth curve for each group. Tumors in the fer<sup>+/+</sup> mice displayed a clear trend toward faster growth compared with those in fer<sup>DR/DR</sup> mice. However, this relation failed to show any statistical significance at any of the time points measured.
Figure 4.3. Fer knock-down enhances proliferation of MDA-MB-231 cells. MDA-MB-231-WT, MDA-MB-231-pLKO and MDA-MB-231–F1 cells were seeded at approximately 5000 cells per well in triplicate and a quantitative MTT assay was used to assess the amount of viable cells at 0, 24, 48 and 72 hours after plating. MDA-MB-231-F1 cells showed a significantly higher absorbance compared to MDA-MB-231-WT cells and MDA-MB-231-pLKO at the 24 hour (* P value< 0.05), 48 hour (** P value< 0.05) and 72 hour (*** P value< 0.05) time points. (n=3)
Figure 4.4. Fer knock-down reduces *in vivo* tumorigenesis of MDA-MB-231 cells. 2x10^6 cells were injected into the mammary fat pad of athymic nude mice and tumor growth was assessed by ultrasound imaging at 1 week intervals for a total of 6 weeks post injection. The ultrasound data provided a 3D reconstruction of the growing tumors that was used to calculate the tumor volume in mm^3^. The growth curve generated for the MDA-MB-231-WT is significantly faster compared with the growth curve from MDA-MB-231-F1. There was a statistically significant difference between average volumes of tumors at 4, 5 and 6 weeks, (*P value<0.05), (** P value<0.001), (**P value<0.001) (n=6 mice for each cell type)
Figure 4.5. MDA-MB-231 tumor xenografts display apparently reduced growth in Fer-deficient athymic mice. 2x10^6 MDA-MB-231-WT cells were injected into the mammary fat pad of fer^{+/+} and fer^{DR/DR} athymic nude mice. Tumor growth was assessed by ultrasound imaging at 1 week intervals for a total of 6 weeks post injection. The ultrasound data provided a 3D reconstruction of the growing tumors that was used to calculate the tumor volume in mm^3. The growth of tumors the tumors in the fer^{+/+} mice displayed an apparently faster rate relative to those in the fer^{DR/DR} mice, however this relation failed to show any statistical significance at any of the time points measured. (N=5 mice from each genotype)
Chapter 5 - Discussion

Research has come a long way in its efforts to understand the underlying causes of cancer. The focus of current work looks to better understand how the individual signaling pathways, and their constituents, influence the progression of this disease. Phosphorylation of substrates by protein kinases is one of the most significant mechanisms by which signal transduction pathways regulate crucial cellular processes. Deregulation of kinase activity is implicated as a cause in a number of major diseases including cancer. Targeting these kinases with inhibitors to restore the normal levels of kinases activity may prove to be a useful therapy to treat many of these diseases. However the majority of kinases still remain uncharacterized and the roles they play in many of these signaling pathways are still unclear. The cytoplasmic protein-tyrosine kinase Fer has been implicated in a number of signaling pathways that mediate cell adhesion, migration and proliferation. Much of this evidence examines the function of Fer in the context of normal cell lines with little evidence of Fer’s function in the context of transformed cell lines.

The objective of this study was to examine the effect of knocking down the expression of Fer in two malignant cell lines, one representing a highly metastatic breast cancer cell (MDA-MB-231) and the other representing a highly metastatic prostate cancer cell (PC3). Using a lentiviral shRNA delivery system we tested 5 different shRNA constructs and evaluated the percent reduction in Fer expression levels for each one. In the MDA-MB-231 cell line the F1 construct showed the greatest percent knockdown of Fer expression to 6% relative to WT levels, (Figure 4.1), therefore we
used MDA-MB-231-F1 cells for all further experiments. A common practice when generating a stable cell line with shRNA is to select and grow out clones from the population in effort to isolate and concentrate cells that have the lowest expression for the gene of interest. Previous studies have shown that knocking down Fer in the MDA-MB-231 cell line inhibits their proliferation in vitro which could potentially hinder the selection process. Furthermore clones selected form a heterogeneous population are genetically unique and may not behave in the same manner as every other cell in the population. Working with the heterogeneous population gives us a more accurate readout on how the majority of the cells behave in response to the Fer knockdown. We therefore opted to perform our experiments on the heterogeneous population that is generated from the initial knockdown and selection.

In the PC3 cell line the F2 construct showed the greatest percent knockdown of Fer expression to 10% of WT levels (Figure 3.1). The PC3-F2 cell line was therefore studied for all further experiments. Again, with the PC3 cells we did not pursue a method to select for clones based on the previously mentioned reasoning. A further complication also arises from the innate heterogeneity of the PC3 cell line which is composed of a heterogeneous population comprised of two distinct cell types. One type resembles an epithelial cell in culture forming tight associations with neighboring cells and grows the classical cobble stone pattern seen in many epithelial derived cell lines. The other type has more fibroblastic like morphology forming little or no associations with any neighboring cells presenting as a more spindle shaped cell. Choosing clones would inevitably select one morphology over the other and the resulting population grown out would not accurately represent how the original cell line behaves. Therefore we again
opted to perform our experiments on the heterogeneous population that is generated from the initial knockdown and selection

*Migration and proliferation*

The PC3-F2 cell line displayed a significant 35% impairment in integrin mediated migration relative to PC3-WT and PC3-pLKO cell lines (Figure 3.2). This observation is consistent with previous studies that also described a migration defect in the context of Fer deficient MEFs\(^{38, 71}\). Surprisingly, the Fer knockdown MDA-MB-231-F1 cells displayed a highly significant 100% increase in migration relative to MDA-MB-231-WT and MDA-MB-231-pLKO cell lines (Figure 4.2). These differential responses with respect to migration between these two cell lines may indicate that Fer is acting in different signaling pathways. The abundant expression of E- and N-cadherin in the PC3 cells may be a factor to consider. Studies have shown that Fer associates with the cytoplasmic domain of both E- and N-cadherin through p120ctn and functions to maintain the stability of the cadherin complex\(^{30}\). Furthermore it has also been demonstrated that Fer participates in a possible cross talk mechanism between N-cadherin and the integrin complex\(^ {54}\). If Fer is functioning within the context a cross talk mechanism that acts on the integrin complex then the impaired migration could be as a result of a disruption of this mechanism. These previous studies showed that Fer’s presence at the integrin complex correlates with a decrease in phosphorylation of a core stabilizing protein, p130cas, and destabilization of the integrin complex. It could be speculated that Fer is part of a key mechanism that regulates the disengagement of integrins; and loss of Fer might result in a situation where the cell can no longer disengage the integrins from the extracellular matrix in order to efficiently migrate.
Future studies to confirm that Fer participates in such a mechanism might include immunoprecipitation and western blot analysis to determine if Fer directly associates with E- and/or N-cadherin and the integrin complex in the PC3 cell system.

The MDA-MB-231 cells do not express E- or N-cadherin, thus a cross talk mechanism between N-cadherin and the integrin complex would not be possible in this cell system. Fer may instead act through another signaling pathway to influence migration. Studies have shown Fer can also influence cell motility by regulating the phosphorylation status of the actin bound protein Ctn which acts to regulate reorganization of that actin cytoskeleton that is necessary for cell migration\textsuperscript{81, 68}. MDA-MB-231 cells express Ctn and its over-expression in these cells has been shown to promote tyrosine phosphorylation dependent metastasis\textsuperscript{82}. Furthermore, Fer-deficient MEF cells also displayed reduced integrin mediated Ctn phosphorylation which was correlated with impairment to migration implicating Fer as a regulator of Ctn activity\textsuperscript{38} and\textsuperscript{71}. Based on these previous findings, if Fer is acting to regulate the activity of Ctn, in the context of a Fer knockdown we would expect to see impairment to migration. The enhanced migration we observed in response to the Fer knockdown contradicts with these previous findings in the Fer-deficient MEF cells. Another factor which could influence the different effects observed on migration rates in response to the Fer knockdown could be a difference in the expression of integrin subtypes of the $\alpha$ and $\beta$ subunits. The migration assay used fibronectin exclusively as the extra cellular matrix to promote migration and could possibly bias the experiment to suit one integrin subtype expression pattern over another. Further investigation will need to be performed in future studies to elucidate the mechanism by which Fer acts to regulate migration in this cell line.
The next step in our analysis was to determine if knocking down Fer using the lentiviral shRNA approach could affect the proliferation of these cells in a similar manner as to what has been previously reported. The PC3-F2 cells displayed a slight, but significant decrease in the rate of proliferation relative to PC3-WT and PC3-pLKO cell lines (Figure 3.3) which is consistent with previous reports \(^7_5\) and \(^8_3\). However our observation is less compelling when compared to the drastically reduced proliferation demonstrated in these other studies \(^8_3\). One possible explanation could be because of differing conditions under which the PC3 cells were grown. A recent study reports that Fer cooperates with interleukin 6 (IL-6) to activate STAT-3 in IL-6 mediated PC3 growth \(^8_3\). In our study we supplemented our growth media with FBS which contains multiple growth factors that could possibly engage other growth receptor pathways potentially masking a growth dependency on Fer. The Fer knockdown MDA-MB231-F1 cells displayed a significant increase in the rate of proliferation relative to MDA-MB-231-WT and MDA-MB-231-pLKO cell lines (Figure 4.3) which is contradictive to previous reports. Very few studies have examined a possible role for Fer in regulating the proliferation of the MDA-MB-231 cell line. One study reports that knocking down Fer in MDA-MB-231 cells results in their arrest in the G1/G0 phase of the cell cycle, effectively inhibiting proliferation \(^7_5\). They described that Fer acts as a suppressor of PP1α, a phosphatase that activates the tumor suppressor pRB and that in the absence of Fer, PP1α activates tumor suppressor signaling through pRb to halt the progression of the cell cycle. While our data appears to conflict with their findings further analysis to assess the phosphorylation status of PP1α and pRB may reveal why we observed an opposite response. The MDA-MB-231 cell line is also reported to have a highly metastatic
phenotype in vivo, however no metastasis was observed in our mouse models. This could indicate that the MDA-MB-231 cell line used for our studies may not behave in the same way as the cell line that was used in previous studies that reported proliferation data that is in conflict with our findings.

Changes to E- and N-cadherin expression and localization in PC3 cells

Changes to the relative total levels of E- and N-cadherin were observed in Fer knock down PC3-F2 cells relative to the PC3-WT cells. PC3-F2 cells displayed a slight decrease in the level of E-cadherin coupled with a slight increase in the level of N-cadherin relative to the PC3-WT cells (Figure 3.4). Studies have implicated Fer as having a role in the phosphorylation of plakoglobin, a component of the cadherin stabilization complex, and stabilization of E-cadherin junctions. A Fer knockdown in this context could result in an increase in the amount of endocytosed E-cadherin which could lead to degradation, reducing the overall levels of E-cadherin in the cell. Immunofluorescence in the PC3 cells reveal what appear to be vesicular structures where E-cadherin localizes. A down regulation of E-cadherin is often correlated with an up regulation of N-cadherin which would be consistent with our observations. Another possibility may be a result of a shift in the heterogeneous population to favor one morphology over the other. The PC3 cell system as previously mentioned consists of a heterogeneous population composed of 2 morphologically different types of cells, which may have differential expression relative to E- and N-cadherin. Therefore, in the context of a Fer knockdown, these cells may behave differently with respect to proliferation, potentially shifting the proportion of one cell type over the other.
Using immunofluorescence microscopy we found that N-cadherin localizes predominantly at the plasma membrane with some punctate cytoplasmic staining in both PC3-WT and PC3-F2 cells (Figure 3.5, A and D). Interestingly, the PC3-F2 cells displayed what appeared to be hair-like extensions where F-actin and N-cadherin co-localized (Figure 3.5, D and F). E-cadherin displayed prominent punctuate perinuclear localization in the majority of cells in both the PC3-WT and PC3-F2 cells. However, a sub population within the PC3-F2 cells displayed plasma membrane localization (Figure 3.6 A and D). The observation that E-cadherin returns to the surface of the cell in the context of a Fer knockdown contradicts the previously observed decrease in E-cadherin expression seen in the PC3-F2 cells. The localization data suggests that Fer participates in a mechanism that may regulate the destabilization and/or endocytosis of E-cadherin, which is also in direct opposition to some previous reports.\textsuperscript{84, 53} Despite this contradiction, the presence of E-cadherin at plasma membrane may explain the observed impairment to migration in the PC3-F2 cells. More E-cadherin at the surface of the cell could potentiate stronger cell to cell contacts which may perturb a cells ability to migrate. Future studies need to address the issue of heterogeneity with respect to the knockdown by staining for Fer expression to confirm that the plasma membrane localization of E-cadherin correlates with decreased Fer expression.

\textit{MDA-MB-231 breast cancer mouse model}

The growth of MDA-MB-231-WT tumors was significantly faster compared with tumors established with MDA-MB-231-F1 cells (Figure 4.4). This \textit{in vivo} observation stands in contrast with the enhanced proliferation of the MDA-MB-231-F1 cells observed \textit{in vitro}. This apparent contradiction may be explained by the differing conditions that
the cells experienced between *in vitro* to *in vivo* methods. For instance MDA-MB-231 cells grown *in vitro* are exposed to a rich mixture of growth factors within the culture medium; furthermore, they are grown on a 2D matrix and interact very little with neighboring cells. Cells grown *in vivo* in the context of a xenograft experience presumably different growth factors and must interact with the extracellular matrix, one another and the surrounding stromal cells to influence the growth of blood vessels required to nourish the growing tumor. Another factor which may have added to the reduced growth of the MDA-MB-231-F1 cells *in vivo* could be as a result of the enhanced migration that was observed *in vitro*. In order to form the solid tumor mass, the MDA-MB-231 cells must interact with one another to form cell–cell contacts. It could be speculated that an overly motile cell could be less likely to form these interactions and instead migrate away from one another leading to a slower growth rate in the primary tumor mass. Further histopathological analysis will need to be performed on tumors that were resected from the mice to look for possible differences in the relative extent of proliferation and apoptosis, and to assess the tumor front interactions with the stroma. The mice displayed no signs of metastasis in any major organs after 90 days post tumor resection, which is interesting given the highly metastatic nature of the MDA-MB-231 cell line.

The growth of MDA-MB-231 tumors generated in the *fer<sup>+/+</sup>* mice displayed a clear trend toward faster growth compared with those in *fer<sup>DR/DR</sup>* mice (Figure 4.5). However, this relation failed to show any statistical significance. A possible reason as to why the data was so variable could be as a result of background mutation from the crossing the BALB/C and 129svJ backgrounds. Mice were interbred to obtain the nude
mice phenotype based on the absence of hair and further genotyped for the $fer^{DR/DR}$ mutation. These mice most likely have variable genomes with respect to other alleles and this variability makes each mouse genetically unique from one another which can also have an impact on the growth of the tumor in addition to the $fer^{DR/DR}$ genotype. While this experiment suggests that a Fer-deficient background in the context of the stomal cells may slow the rate of tumor growth, this study must be repeated in mice that are more similar with respect to background mutation to reduce variability. Despite the variability, this is an important observation which may support a stromal role for Fer in tumorigenesis. Given the previous reports that the $fer^{DR/DR}$ mice show a greater ability to recruit leukocytes in response to localized challenge with LPS it may be worthwhile to evaluate the tumors and adjacent stroma for signs of increased immune cell recruitment. A greater immune response mounted by the $fer^{DR/DR}$ mice may have provided them with an additional advantage to fight off the xenograft of human tumor cells. The mice from this study again displayed no signs of metastasis in any major organs after 90 days post tumor resection.

**Future directions**

This study has provided some interesting preliminary data that may implicate Fer in signaling pathways that possibly regulate the migration and proliferation of MDA-MB-231 and PC3 cells. We have demonstrated that a stable shRNA knockdown is effective at reducing Fer expression and have developed adequate assays to test for changes in proliferation and migration in response to the Fer knockdown. Despite this success, one weakness of the experimental design was the possibility that the changes we observed were due to off target effects from the $fer$ shRNA construct and not a result of the Fer
knockdown. Future studies will need to validate our findings in the context of the other
fer shRNA constructs to clarify that the differences observed were in fact a response to
the reduced Fer expression. It would be particularly satisfying to see a dose-response
relationship between the level of Fer knockdown with different shRNA constructs and the
magnitude of the phenotypes observed.

One advantage to using the shRNA approach in this study as compared with a
transient siRNA method to target the expression of fer is the ability to generate stable cell
lines suitable for in vivo studies. In the case of the MDA-MB-231 cells, the stable Fer
knockdown cell line allowed us to evaluate the Fer knockdown in the context of a
mammary fat pad xenograft which would not have been possible by a transient approach.
An unfinished goal was to develop a mouse xenograft model for the PC3 cell system. In
addition to the established subcutaneous injection method, I briefly attempted orthotopic
injections into the prostate of male mice using ultrasound guided injections. While the
preliminary studies showed promise, development of this model was not pursued. This
effort was also complicated by difficulty maintaining long term knockdown of Fer in the
PC3 cell line. One down side to generating a stable cell line is the possibility of growing
out a variant cell line through subsequent passages. By targeting the expression of a
protein that is crucial to a certain cellular process you will invariably select for a variant
cell within that population that is insensitive to the knockdown. If for instance targeting
this protein inhibits, as seen in the PC3 cells (Figure 3.3), an insensitive variant that
continues to proliferate normally could potentially dominate in the subsequent
generations. This variant line would no longer adequately represent the earlier
population and would likely behave differently in the experiments. In our experiments
we observed that the reduced proliferation rate that the early passage PC3-F2 cells exhibited returned to normal after longer term passages; which may suggest the generation of a variant line. In order to address this issue we performed all of our experiments on early passage cells following the knockdown. An alternative method that could address this issue in future studies would be the development of an inducible Fer knockdown. By transferring the shRNA constructs that were used in this study into a tetracycline inducible system, we would have the ability to turn off and on Fer expression. Controlling the expression of Fer in this manner would allow us to more accurately simulate the effect of a Fer targeting inhibitor.

In conclusion, this study has provided some compelling preliminary data that with further study may reveal Fer as a novel target for future therapies.
References


