THE EZRIN SIGNALLING NETWORK AS A POTENTIAL NOVEL MARKER IN BREAST CANCER METASTASIS

by

HANNAH YUNNLOK MAK

A thesis submitted to the Department of Pathology and Molecular Medicine
in conformity with the requirements for the degree of
Master of Science

Queen’s University
Kingston, Ontario, Canada
April, 2010

Copyright © Hannah Yunnlok Mak, 2010
ABSTRACT

Metastasis is the leading cause of mortality in human breast cancer. However, there are few predictive, prognostic, or therapeutic targets of breast cancer metastasis. Ezrin, a membrane cytoskeletal cross-linker, is frequently over-expressed in human breast cancer and is required for motility and invasion by cultured epithelial cells. Our group has recently shown that ezrin acts co-operatively with the non-receptor tyrosine kinase, Src, in the transformation of epithelial cells, in which ezrin is phosphorylated on specific tyrosines, such as Y477, by Src (91, 93). We therefore examined whether Src/ezrin interaction also regulates invasion and metastasis of breast cancer. This thesis presents the following results: 1) In a murine system, ezrin and Src are differentially localized in nulliparous, lactating mammary glands and PyMT-induced tumours, with pronounced apical expression in nulliparous mammary glands but non-polarized strong cytoplasmic expression in PyMT-induced tumours. 2) Increased expression and activation of ezrin, Src and Met in PyMT-induced tumours compared to normal breast tissues was observed. A concomitant increased expression of activated Stat3 and HGF was also observed in PyMT-induced tumours, consistent with the establishment of an HGF/Met autocrine loop. 3) In invasive human breast tumours, from a premenopausal patient cohort, ezrin showed significantly greater cytoplasmic localization compared to non-neoplastic epithelial ducts in normal mammoplasties. 4) In a mouse breast carcinoma xenograft model, a Y477F ezrin mutant (not phosphorylatable by Src), significantly reduced local invasion of primary tumours and spreading into visceral organs, yet, it did not significantly affect primary tumour growth rate. 5) Y477F ezrin-expressing tumours exhibited focal areas of incomplete membranous ezrin staining which was absent in control tumours. Moderate/strong cytoplasmic ezrin staining was evident in both tumour groups. Thus, ezrin is differentially localized in non-invasive versus invasive mammary tumours. Our study implicates a role of the Src/ezrin pathway in regulating local invasion and metastasis of breast carcinoma cells and provides a clinically relevant model for assessing the Src/ezrin pathway as a potential prognostic marker and treatment target for invasive breast cancer.
ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my supervisor Dr. Bruce Elliott for his guidance, advice, encouragement, support and openness to share his life experiences with me. His enthusiasm and optimism have repeatedly inspired me and has given me confidence.

I also thank everyone in the Elliott Lab, past and present members who made the lab such fun to be a part of and provided me with encouragement and laughter: Colleen Schick, Victoria Hoskin, Esther Carefoot, Jamaica Cass, Blerta Starova, Jalna Meens, Amanda Hyde, Emily Christie, Jenna Pilon, Joe Oliver, Andrea Dias, Joyce Zhou, Zachary King, Jing Jing Ma, Lesley Leung and Manjury Murthy.

Very special thanks for Colleen Schick and Victoria Hoskin. To Colleen, I will never forget the good times we had with the mice and for all the help in the \textit{in vitro} side of the study. Your laughter and cheerfulness radiates everyday making each day enjoyable. Without you, I could not have finished this project. To Victoria, your friendship has made this Masters experience just that much more fun; from conferences to volleyball, from PBS to ping pong.

I thank my supervisory committee and mentors: Dr. Judy-Anne Chapman, Dr. Sandip SenGupta, and Dr. Lois Mulligan for your expert guidance and the many helpful as well as constructive discussions that have helped improve and polish up my project.

I would also like to thank Lee Boudreau, Jalna Meens, Jeff Mewburn, Dr. Peter Truesdell and Dr. Sonal Varma for their technical support and their willingness to share their wealth of experience. To Lee, I also thank you for letting me invade and spend hours upon hours doing IHC in your room. I enjoyed your choice of music.

I would also like to thank Tim for the love, support and understanding you’ve given me throughout the project and for the difficult times you’ve helped me get through.

Finally and most importantly, I would like to thank my family: my mom, Kathy; my dad, Michael and my grandmother for your constant love, prayers, encouragement and support. I feel truly blessed.

Funding for this study has been generously provided by the Canadian Breast Cancer Research Alliance and by the Canadian Institutes of Health Research. I received graduate training fellowships from the Canadian Breast Cancer Foundation and the Terry Fox Foundation Training program in Transdisciplinary Cancer Research in Partnership with CIHR.
CONTRIBUTIONS

Colleen Schick and Jalna Meens injected AC2M2 cells in the mouse xenograft model. Colleen also carried the AC2M2 cell line and provided Figure 22 and Appendix 1 & 2.

Dr. Monique Arpin (Curie Institute, Paris, France) provided the pCB6 vector and Y477F ezrin mutant constructs, and assisted in transfection of AC2M2 cells.

Dr. William Muller (McGill University, Montreal, Quebec) provided the PyMT tumour tissue sections and frozen samples.

Dr. Peter Truesdell (Queen’s Cancer Research Institute, Kingston, Ontario) provided nulliparous and lactating mouse tissue blocks.

Dr. Judy Anne Chapman and Alex Perry provided the statistical analyses for the TMA portion of the study.

Dr. Sonal Varma, Dr. Ashish Rajput and Dr. Jerry Chen manually acquired H-scores for the TMA portion of the study. Dr. Sonal Varma also contributed to the confirmation of histopathological analysis and overall pathology of the study.

Jing Jing Ma ran the Western blot for Figure 24.

Lee Boudreau stained tissue sections on the Ventana for figures in Appendix 3.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii

ACKNOWLEDGEMENTS ........................................................................................................... iii

CONTRIBUTIONS ...................................................................................................................... iv

LIST OF FIGURES ..................................................................................................................... viii

LIST OF TABLES ....................................................................................................................... x

LIST OF ABBREVIATIONS ......................................................................................................... xi

CHAPTER 1—INTRODUCTION ................................................................................................. 1

1.1 The Breast .......................................................................................................................... 1
    1.1.1 Breast Cancer ............................................................................................................. 1
    1.1.2 Mammary Gland Development .............................................................................. 4
    1.1.3 Epithelial-Mesenchymal Transition and Metastasis ............................................. 5

1.2 Ezrin ................................................................................................................................ 7
    1.2.1 Biochemical Features and Ezrin Activation ............................................................ 8
    1.2.2 Other Mechanisms of Ezrin Regulation ................................................................. 12
    1.2.3 Ezrin in Normal Development ............................................................................... 14
    1.2.4 Ezrin in Cancer ....................................................................................................... 15

1.3 Src .................................................................................................................................. 16
    1.3.1 Biochemical Features and Src Activation .............................................................. 16
    1.3.2 Src in Normal Development .................................................................................. 17
    1.3.3 Src in Cancer ......................................................................................................... 19

1.4 Met .................................................................................................................................. 21
    1.4.1 Biochemical Features and Met Activation .............................................................. 21
    1.4.2 Met in Normal Development .................................................................................. 23
    1.4.3 Met in Cancer ......................................................................................................... 24

1.5 Rationale, Hypothesis and Objectives .......................................................................... 25

CHAPTER 2—MATERIALS AND METHODS ........................................................................... 29

2.1 Reagents ........................................................................................................................... 29

2.2 Cell Lines and Tissue Culture ........................................................................................ 30
    2.2.1 Cell lines and tissue culture .................................................................................. 30
    2.2.2 DNA Constructs and Transfection of AC2M2 cells with pCB6 expression vector .. 30

2.3 Mouse Strains ................................................................................................................. 32
    2.3.1 Polyomavirus Middle T (PyMT) transgenic mouse ................................................. 32
    2.3.2 Nulliparous and Lactating Mice ......................................................................... 34

2.4 Staining ............................................................................................................................ 34
    2.4.1 Antibody Control and Validation ...................................................................... 34
2.4.2 Immunohistochemistry ............................................................................................. 35
  2.4.2.1 Manual ............................................................................................................... 35
  2.4.2.2 Automated Ventana Staining ............................................................................. 36
  2.4.3 Hematoxylin and Eosin Staining ......................................................................... 38
2.5 Western Blotting .......................................................................................................... 39
  2.5.1 Preparation of Tissue Homogenates ................................................................... 39
  2.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) ....................................... 39
  2.5.3 Immunoblotting .................................................................................................... 40
  2.5.4 Densitometric Analysis ......................................................................................... 40
2.6 Human Tissue Microarray (TMA) .............................................................................. 41
  2.6.1 Selection Criteria, Strategy and Construction ..................................................... 41
  2.6.2 Preparation of Cell Culture Blocks ...................................................................... 42
2.7 Scoring and Statistical Analysis .................................................................................. 44
  2.7.1 Mouse .................................................................................................................... 44
  2.7.2 Human Tissue Microarray .................................................................................... 45
2.8 In vivo Protocols ......................................................................................................... 45
  2.8.1 Mice used for intramammary engraftment of tumour cell lines ......................... 45
  2.8.2 Tumour engraftment into the mammary fat pad ................................................ 46
  2.8.3 Measurement of primary tumour growth and assessment of metastasis .......... 46
2.9 Light Microscopy and Image acquisition ................................................................... 47

CHAPTER 3—RESULTS ............................................................................................................. 48

3.1 Evaluation of Src/ezrin expression and activation as a marker of tumour grade in human breast cancer ............................................................................................................................. 48
  3.1.1 Validation of antibodies for Immunohistochemistry .......................................... 48
  3.1.2 Differential localization of Ezrin and Src in nulliparous, lactating mammary glands and PyMT-induced tumours ................................................................. 49
  3.1.3 Increased expression and activation of Ezrin and Src in PyMT-induced mammary tumours compared to normal or lactating mammary glands .......................... 50
  3.1.4 Increased expression of HGF and Met in PyMT-induced mammary tumours compared to nulliparous breast tissue ................................................................. 51
3.2 Differential localization of ezrin expression in normal and neoplastic human breast tissues .................................................................................................................................................. 62
  3.2.1 Tests of Replicability ............................................................................................ 63
3.3 Characterization of the Y477F ezrin phenotype using a mouse tumour xenograft model . 67
  3.3.1 Expression of Y477F ezrin mutant in AC2M2 cells ............................................. 67
  3.3.2 The AC2M2 Mouse tumour engraftment model ............................................... 69
  3.3.3 Y477F ezrin inhibits local invasion of primary tumour outgrowths ..................... 69
  3.3.4 Y477F ezrin inhibits lymphovascular invasion (LVI) in primary AC2M2 breast tumours .................................................................................................................. 74
  3.3.5 Y477F ezrin display more membranous ezrin in primary AC2M2 breast tumours . 76

CHAPTER 4- DISCUSSION......................................................................................................... 80

4.1 Evaluation of Src/ezrin expression and activation in a PyMT-induced breast tumour mouse model ........................................................................................................................................ 81
  4.1.1 Differential localization of Ezrin in mouse nulliparous, lactating mammary glands and PyMT-induced tumours ................................................................. 81
  4.1.2 Increased expression and activation of Ezrin and Src in PyMT-induced mammary tumours compared to normal or lactating mammary glands .......................... 82
LIST OF FIGURES

Figure 1. Domain structure of the ERM proteins................................................................. 9
Figure 2. Mechanism of activation of ezrin................................................................. 13
Figure 3. Structural diagram and mechanism of activation of Src................................. 18
Figure 4. Structural diagram of the Met receptor tyrosine kinase............................... 22
Figure 5. Model of the Ezrin/Src signalling network...................................................... 28
Figure 6. pCB6 expression vector and VSVG sequence.................................................... 31
Figure 7. Polyomavirus Middle T transgene structure................................................... 33
Figure 8. Immunohistochemistry.................................................................................. 37
Figure 9. Tissue Microarray Strategy Flow Chart......................................................... 43
Figure 10. Validation of nonspecific binding through isotype matched negative control antibodies in normal mouse breast and Polyoma virus Middle T-induced tumour tissue. 52
Figure 11. Validation of pT567 ezrin antibody specificity by pT567 ezrin peptide block ....... 53
Figure 12. Validation of pan Src and pY419 Src antibodies by pan Src and pY419 Src peptide block.................................................................................................................. 54
Figure 13. Immunohistochemical staining of ezrin in nulliparous and lactating mammary glands; and PyMT-induced tumour tissues. ................................................................. 55
Figure 14. Immunohistochemical staining of pT567 ezrin in nulliparous and lactating mammary glands; and PyMT-induced mammary tumour tissues........................................ 56
Figure 15. Immunohistochemical staining of pan Src in nulliparous and lactating breast and PyMT-induced mammary tumour tissues................................................................. 57
Figure 16. Immunohistochemical staining of pY419 Src in nulliparous and lactating breast; and PyMT-induced mammary tumour tissues................................................................. 58
Figure 17. Expression and activation levels of ezrin in tumourigenic compared to normal and lactating mammary glands................................................................. 59
Figure 18. Expression and activation levels of Src in tumourigenic compared to normal and lactating mammary glands................................................................. 60
Figure 19. Expression levels of pY1234/1235 Met, pY705 Stat3, and of pro- and α− HGF in PyMT induced tumours compared to nulliparous and lactating breast tissue......... 61
Figure 20. Expression and localization of ezrin and Src in invasive breast carcinoma compared to normal human mammoplasty.......................................................... 64

Figure 21. Tests of association for cytoplasmic pan ezrin or pY419 Src staining with neoplastic versus normal human breast tissue.................................................................65

Figure 22. Western blotting analysis of VSVG ezrin mutant expression compared to endogenous ezrin in AC2M2 clones........................................................................... 68

Figure 23. Detection of Y477F ezrin by western blotting in xenograft tumours ....................... 70

Figure 24. Effect of Y477F mutant on primary tumour growth................................................. 71

Figure 25. Effect of Y477F ezrin mutant on local invasion of AC2M2 breast carcinoma cells 72

Figure 26. Characterization of invasion and metastasis of pCB6 tumour xenografts................. 73

Figure 27. Effect of Y477F ezrin on lymphovascular invasion (LVI) in primary AC2M2 breast tumours................................................................. 75

Figure 28. Comparison of cytoplasmic and membranous ezrin localization in pCB6 control and Y477F ezrin mutant tumours................................................................. 77

Figure 29. Comparison of pT567 ezrin in pCB6 control and Y477F ezrin mutant tumours ...... 78

Figure 30. Localization of pan and pY419 Src localization in pCB6 control and Y477F ezrin mutant tumours................................................................. 79
LIST OF TABLES

Table 1. Reagents and sources.................................................................29
Table 2. Blocking peptides for immunohistochemistry...............................35
Table 3. Primary antibodies used for immunohistochemistry.........................38
Table 4. Primary antibodies used for Western blotting analysis......................40
Table 5. HRP-linked secondary antibodies used for Western blotting...........41
Table 6. Cytoplasmic H-scores of pan ezrin and pY419 Src .........................65
Table 7. Test of association between pan ezrin or pY Src with SBR score........66
Table 8. Test of association between pan ezrin or pY Src with Histologic Grade..66
Table 9. Proportion of mice with local invasion of 1° tumour xenografts...........72
Table 10. Lymphovascular involvement in pCB6 and Y477F expressing tumours...75
Table 11. Pan ezrin expression in pCB6 and Y477F expressing tumours ..........77
Table 12. pT567 ezrin expression in pCB6 and Y477F expressing tumours .......78
Table 13. Pan and pY419 Src expression in pCB6 and Y477F expressing tumours...79

LIST OF APPENDICES

Appendix 1. Effect of Y477F ezrin on wound healing in AC2M2 cells.................96
Appendix 2. Effect of Y477F ezrin on growth and morphology of AC2M2 cells in 3D
Matrigel cultures.................................................................97
Appendix 3. Immunohistochemical staining of γ-tubulin in pCB6 control and Y477F ezrin
mutant tumours........................................................................98
Appendix 4. Comparison of cytoplasmic and membranous ezrin localization in pCB6 control
and Y477F ezrin mutant tumours..................................................99
## LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2D</td>
<td>two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>three dimensional</td>
</tr>
<tr>
<td>a.a</td>
<td>amino acid</td>
</tr>
<tr>
<td>BRCA1</td>
<td>breast cancer 1</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CDK5</td>
<td>cyclin dependent kinase 5</td>
</tr>
<tr>
<td>C-ERMAD</td>
<td>carboxy ezrin radixin moesin associated domain</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CSF1R</td>
<td>colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CSK</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>DCIS</td>
<td>ductal carcinoma in situ</td>
</tr>
<tr>
<td>E3KARP</td>
<td>sodium hydrogen exchanger type 3 kinase A regulatory protein</td>
</tr>
<tr>
<td>EBP50</td>
<td>ezrin radixin moesin binding protein 50</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix protein</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase-1</td>
</tr>
<tr>
<td>ERM</td>
<td>ezrin radixin moesin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FERM</td>
<td>four point one ezrin radixin moesin</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>Gab1</td>
<td>Grb2 associated binding protein 1</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GRK2</td>
<td>G protein-coupled receptor kinase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>HER-2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF1α</td>
<td>hypoxia induced factor-1-alpha</td>
</tr>
<tr>
<td>ICAM</td>
<td>inter-cellular adhesion molecule</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>mammary tumour virus</td>
</tr>
<tr>
<td>N-ERMAD</td>
<td>amino ezrin radixin moesin associated domain</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium hydrogen exchanger</td>
</tr>
<tr>
<td>PAI1</td>
<td>plasminogen activator inhibitor 1</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>PDGFR</td>
<td>platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3 kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 2,4-bisphosphate</td>
</tr>
<tr>
<td>PKCα</td>
<td>protein kinase Cα</td>
</tr>
<tr>
<td>PKCθ</td>
<td>protein kinase Cθ</td>
</tr>
<tr>
<td>PLC-γ</td>
<td>phospholipase C-γ</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>PTP1B</td>
<td>protein tyrosine phosphatase 1B</td>
</tr>
<tr>
<td>PTPα</td>
<td>protein tyrosine phosphatase α</td>
</tr>
<tr>
<td>Rho</td>
<td>Ras homolog gene family</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>ROCK</td>
<td>rho kinase</td>
</tr>
<tr>
<td>SF</td>
<td>scatter factor</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family of kinases</td>
</tr>
<tr>
<td>SH</td>
<td>src homology</td>
</tr>
<tr>
<td>SHIP-2</td>
<td>Sh2 containing 5' inositol phosphatase</td>
</tr>
<tr>
<td>SHP</td>
<td>SH2 containing phosphatase</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>SOS1</td>
<td>Son of sevenless homolog 1</td>
</tr>
<tr>
<td>Src</td>
<td>Rous sarcoma non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>Stat3</td>
<td>Signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>TEB</td>
<td>terminal end buds</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Tpr</td>
<td>translocated promoter region</td>
</tr>
<tr>
<td>uPA</td>
<td>urinary plasminogen activator</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>vascular endothelial growth factor C</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>vascular endothelial growth factor D</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>β2A</td>
<td>beta 2 adrenergic receptor</td>
</tr>
</tbody>
</table>
CHAPTER 1—INTRODUCTION

1.1 The Breast

1.1.1 Breast Cancer

Breast cancer continues to be the most frequently diagnosed cancer worldwide and in Canada. Every year, 1 in 9 women is expected to develop breast cancer in her lifetime and 1 in 28 women will die from it. In 2009-2010, an estimated 22,700 Canadian women will be diagnosed with breast cancer and 5,400 will die from it. The majority of breast cancers occur primarily in women between ages 50-69, although 75-79 year olds have the highest rate of incidence and at least 20% of breast cancers have been diagnosed in women under the age of 50 (1). Although breast cancer mortality rates are declining, due to better adjuvant therapy and screening programs, it remains the most common cause of cancer death in females aged 20-59 (2). Metastasis, the process by which tumour cells, from the primary tumour site, spread and travel to distant regions of the body, is the major cause of death due to breast cancer (3). While currently lymph node positivity is a major predictive factor for the development of distant metastases, 20-30% of patients with lymph node negative breast cancer will also develop distant metastases (4-6). Thus, predicting which patients are at high-risk of developing metastases remains a challenge.

There are a number of established risk factors that have been associated with the development of breast cancer, of these, the greatest being gender and age. Some established non-modifiable risk factors include personal or familial history of breast cancer, inherited genetic mutations or alterations (eg. BRCA1), high breast tissue density, early menarche, late menopause, and ethnicity (7). Other potentially modifiable risk factors include previous radiation exposure; late, few or no pregnancies; no breastfeeding; use of hormone replacement therapy, especially those with progestins; high dose oral contraceptives; greater alcohol consumption; and tobacco smoke (8).
Breast cancer is a clinically heterogeneous disease where origin and outcome can vary significantly. Traditionally, classification has been based on histopathological presentation of the tumours. Breast neoplasms are broadly divided into invasive and non-invasive subgroups (9). Non-invasive neoplasms are confined within the basement membrane and have not yet invaded into the surrounding stroma. The most common type of non-invasive breast cancer is ductal carcinoma in situ (DCIS) (10). Invasive tumours, on the other hand, have invaded through the basement membrane into the surrounding stroma and can metastasize to distant organs. The most common types of invasive carcinomas are ductal and lobular carcinomas, which account for 50-80% of all invasive breast cancers (reviewed in 9). Less common types of breast cancer include medullary carcinoma (characterized by a distinct boundary between tumour and normal tissue), mucinous/colloid carcinoma (arising from mucus produced by cancer cells) and tubular carcinoma (characterized by the tubular phenotype exhibited by the cancer cells) (10). The most common sites for metastatic spread are to the lung, liver and bone.

While histological typing gives a broad classification of the different types of breast cancers, there are very few reliable prognostic markers of metastasis, which is one of the most important factors governing overall survival and outcome. Currently, prognostic classification of breast cancer is based on a pathological staging of the disease (TNM score). TNM staging is a numerical and categorical system based on tumour size and the degree of local invasion (T), lymph node involvement (N) and presence of metastasis (M), where a lower tumour stage is associated with better prognosis (9). Histological grade is also used and provides prognostic information. One of the most common grading systems is the Scarff-Bloom Richardson grading system which is based on tubule formation (% carcinoma composed of identifiable tubules), nuclear grade and mitotic activity. Other established prognostic/predictive markers include lymphovascular invasion, hormone-receptor expression (eg. estrogen and progesterone receptor status), HER-2 amplification and uPA/PAI1 protein levels (reviewed in 11).
With the advent of gene and protein microarray profiling, breast tumours were grouped into five previously unrecognized subtypes based on their gene-expression patterns (12). Three distinct estrogen receptor negative subgroups: normal-breast like, HER-2 positive and basal-like, and two estrogen receptor positive subgroups: luminal A and luminal B were identified. In addition, molecular profiling of stromal tissue has yielded complementary information that further defines these cancer subtypes (13). These combined approaches have yielded biomarker sets with promising prognostic potential (eg. Mammaprint)(14).

Conventional treatment options for malignant breast cancer are typically surgery, radiation, especially following lumpectomy, endocrine therapy and/or chemotherapy (15). Unfortunately, many of the current procedures lack tumour specificity and pose great toxicity to normal, rapidly proliferating cells, such as hair follicles and gastrointestinal cells. By applying the growing understanding of oncogenic pathways that drive metastasis, targeted therapies such as trastuzumab, against the HER-2 receptor, are slowly being developed in the hopes of decreasing exposure of cancer patients to the toxic effects of broad-range chemotherapeutics. However, the small number of predictive and prognostic markers remains an ongoing challenge. Current prognostic and predictive factors are still unrefined and often only serve as coarse indicators. As a result, current methods are unable to identify who will relapse and who will or will not benefit from treatment. Without a high degree of certainty, many patients are overtreated and are at risk of being exposed to the toxic side effects of treatment such as menopausal symptoms, cardiotoxicity, neurotoxicity, thromboembolic complications, cognitive impairment, and development of secondary cancers from treatment (16). On the other hand, a subset of patients may be under-treated as favourable test results could give a false sense of security and a reduction of vigilance in detection of metastases. Lack of refined factors also poses a huge economic burden to the healthcare system as treatments are quite expensive. Therefore, increased understanding of tumour biology may lead to improved prediction and novel pharmacological treatments of breast cancer.
1.1.2 Mammary Gland Development

In both rodents and humans, mammary gland development occurs during three main stages: embryonic, pubertal, and adult reproductive (reviewed in 17). The mammary gland is a secretory organ which is composed of two main compartments, the epithelium and the surrounding stroma (18). The epithelium component consists of two main types, luminal and basal myoepithelia (17). The luminal epithelium forms the ducts and the secretory alveoli while the basal epithelium consists of myoepithelial cells responsible for the contraction and secretion of mammary products. The stroma is comprised of adipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cell types (17). During embryogenesis, placodes (areas of thickening) form at the site of each future nipple and invaginate into the underlying mesenchyme to become bulb-shaped buds (primary mammary rudiment/anlage) (reviewed in 19-20). The buds elongate to form a mammary sprout which then branches to form a rudimentary structure containing five ductules embedded in the subdermal fat pad (17). At this point, mammary gland development pauses at birth and remains inactive until puberty (19).

At puberty, terminal end buds (TEBs) appear at the tips of ducts and begin to invade the fat pad. As systemic levels of estrogen rise, proliferation within the TEBs results in ductal elongation while clefting of the TEBs leads to ductal bifurcation (17). A wide range of epithelial and stromal factors, including hormones, growth factors, extracellular matrix molecules, matrix metalloproteases (MMPs) and immune cells, works together to regulate ductal development. For instance, HGF induces site-specific branching; TGF-β maintains proper ductal spacing and lumen formation, while MMPs play a role during branching morphogenesis (reviewed in 20). When the limits of the fat pad are reached, growth ceases.

During pregnancy, the mammary gland epithelium proliferates in preparation for lactation. Progesterone induces extensive side-branching and alveologenesis. Along with prolactin, progesterone also promotes the conversion of ductal epithelium into secretory cells
In addition, estrogen and progesterone stimulate mammary development, but inhibit milk secretion. Myoepithelial cells become discontinuous, allowing luminal epithelial cells to contact the basement membrane which is critical for full differentiation and proper milk secretion. During lactation, estrogen and progesterone decrease. Oxytocin is released causing myoepithelial contraction, propelling milk from the alveoli into the ducts. Once suckling ceases, the mammary gland undergoes involution where drastic tissue remodelling and apoptosis occurs, reverting back to its pre-pregnant state. Due to the continual remodelling that occurs within the mammary gland, the tissue is particularly susceptible to tumourigenesis.

1.1.3 Epithelial-Mesenchymal Transition and Metastasis

Metastasis, as mentioned above, is the underlying cause of morbidity and mortality in breast cancer, and is a process whereby malignant cells spread from the primary tumour site to distant organ sites. The process, also known as the metastatic cascade, is divided into sequential stages including epithelial-mesenchymal transition (EMT), inhibition of anoikis, invasion, angiogenesis, intravasation, extravasation and outgrowth.

Epithelial-Mesenchymal transition (EMT) is a process which normally occurs during embryogenesis. For example, during gastrulation or migration of neural crest cells, epithelial cells acquire mesenchymal-like attributes. This allows cells to migrate away from the neural tube through the extracellular environment and differentiate into bone, smooth muscle, peripheral neurons, and melanocytes. The process is tightly regulated during development, but may be deregulated in tumourigenesis, leading to a prolonged EMT phenotype. The EMT phenotype is characterized by a loss of cell polarity, cell scattering and downregulation of epithelial proteins, in particular, E-cadherin. Loss of E-cadherin expression leads to a decrease in cell-cell contacts, increasing its motility, and allowing for anchorage independent growth. Other proteins are upregulated, such as N-cadherin and members of the Snail and Twist families, which have been found to induce cell migration, invasion, metastasis, and inhibit apoptosis. Some
epithelial cells that have undergone EMT also have the ability to secrete matrix degrading proteases, such as MMPs, which are able to remodel the tumour microenvironment and release mitogenic or angiogenic factors (25).

Invasion is a crucial initial step in the metastatic cascade as cells must migrate through the extracellular matrix and cross several basement membranes in order to metastasize from within the duct to surrounding tissues. Typically, metastases occur through two distinct routes: directly via the vasculature or indirectly, via lymphatics (29-31). Typically during vascular invasion, tumour cells will intravasate and undergo transendothelial migration into the vasculature. From there, they will travel and extravasate at a distant site where they may stay dormant or begin to proliferate. Tumours can also interact with lymphatics in a number of ways, including vessel incorporation into the tumour, chemotactic migration towards lymphatics via chemokine gradients or induction of lymphangiogenesis through secretion of VEGF-C and VEGF-D by the tumour (32). Due to the structure of lymphatics, such as loose overlapping cell-cell junctions, lack of a complete basement membrane, lymphatic vessels are intrinsically more amenable to tumour invasion compared to blood vessels (33). Tumour-induced lymphangiogenesis has been associated with metastasis and poor prognosis (33). In addition, changes in the adhesive properties of the lymphatic endothelium promote lymphatic spread. During lymphatic invasion, tumour cells disseminate and travel to lymph nodes where they proliferate to form solid metastases. Once established, the tumour cells can then disseminate to distant sites, through the vasculature, where they form secondary metastases.

Interaction with the extracellular matrix, in particular via integrins, activates FAK/Src complexes that modulate the activity of RHO family GTPases which regulate cytoskeletal rearrangement (34). Through 2D and 3D studies, it has been shown that there are two main mechanisms by which cells can invade through tissue: amoeboid and fibroblastic movement, also known as, mesenchymal movement (35). Amoeboid movement, the faster of the two movements, is characterized by a rounded morphology, high actomyosin contractility, membrane blebbing and
independence from matrix proteolysis. The process is RhoA/ROCK-dependent and involves weak integrin adhesion (36). On the other hand, fibroblastic movement is characterized by an elongated morphology and is dependent on extracellular proteolysis. The process is Rac dependent and requires integrin-dependent adhesion (36). These two mechanisms are interconvertible, where Rho or ROCK inhibition causes cells to switch from amoeboid invasion to fibroblastic invasion. Tumours expressing podoplanin, a small transmembrane glycoprotein, have been found to mediate collective tumour cell migration in the absence of EMT by reorganizing the actin cytoskeleton via RhoA/ROCK and ezrin (37). Furthermore, podoplanin functions as a specific marker for lymphatic endothelium, as it is not expressed in vascular endothelium, and plays an important role in lymphangiogenesis. Loss of podoplanin expression disrupts normal lymphatic vasculature formation that is needed for lymphangiogenesis (38).

In order for tumours to grow beyond a limiting size (~500 µm in diameter), vascularisation of the tumour through a process known as angiogenesis, is required (39). In addition to angiogenesis, vasculogenic mimicry can also occur in which tumour cells arrange themselves into vessel-like structures (40). As a tumour grows to a certain size, it outgrows its blood supply and regions of hypoxia develop. The hypoxic environment, in turn, induces further angiogenesis through the downstream effects of the transcription factor hypoxia induced factor-1-alpha (HIF1α). In some instances, hypoxia can also promote invasion through the activation of Met signalling (41).

Understanding the mechanisms of the metastatic cascade can offer potential prognostic markers and novel targeted treatment options that may predict relapse and decrease the mortality of breast cancer due to metastasis.

1.2 Ezrin

Ezrin is a member of the ezrin-radixin-moesin (ERM) family of plasma membrane-cytoskeleton linkers that play roles in cortical structure, cell adhesion, motility, endocytic
trafficking, signal transduction and regulation of growth (reviewed in 42). Ezrin is an 81 kDa polypeptide, first described as a substrate of receptor protein tyrosine kinases in a human carcinoid cell line (A431) in response to EGF stimulation (43). It was subsequently purified from the microvillus cytoskeleton of chicken intestinal epithelial cell brush borders (44). Although most cells in culture express all three ERM proteins, in vivo, ezrin is primarily found in the apical regions of epithelial cells, while moesin is concentrated in endothelial and hematopoietic cells (45-48). Other regions of ezrin expression are along basolateral surfaces of ductal epithelial cells, renal podocytes, and retinal epithelial cells (45, 49).

1.2.1 Biochemical Features and Ezrin Activation

The ezrin gene, villin-2, resides on human chromosome 6. It has 13 exons which encodes a protein of 586 residues (50, 51). Among vertebrates, the ERM proteins are highly conserved, where they share a ~75% sequence homology (42). Ezrin contains two functionally important domains, the N-terminal ERM associated domain (N-ERMAD) also known as the FERM (4.1 ERM) domain and the C-terminal ERM associated domain (C-ERMAD) (52, 53) (Figure 1). The N-ERMAD consists of 297 residues, and is composed of three structural modules, F1, F2, and F3, which are arranged into a tight clover-shaped structure (54). The N-ERMAD domain is responsible for binding to type 1 transmembrane proteins, such as CD43, CD44, ICAM-1, ICAM-2, NHE-1 and Syndecan-2 (55, 56). The N-ERMAD domain can also indirectly bind via EBP50 (ERM binding protein 50) or E3KARP (NHE type 3 kinase A regulatory protein) to multipass transmembrane proteins, such as NHE-3, CFTR, β2A, PDGF-4 and podocalyxin (57, 58). Between the N-ERMAD and C-ERMAD is a central α domain that forms an α helical coiled-coil structure. The C-ERMAD is an 80 residue domain that is arranged into one β strand and six helical regions (54). Within the C-ERMAD is an F-actin binding domain, located on the last 34 charged amino acids, which binds to the actin cytoskeleton and completes the membrane-cytoskeleton link (59-62).
Figure 1. Domain structure of the ERM proteins.

A) A comparison of the domain organization among members of the 4.1 (FERM-domain containing) superfamily of proteins, ezrin, radixin and moesin as well as merlin. The ERM proteins show high sequence identity with one another, but diverge from merlin. Adapted from (42).

B) Ezrin consists of two major domains, the amino ERM associated domain (N-ERMAD), also known as the FERM (4.1 ERM) domain and a carboxy ERM associated domain (C-ERMAD) that are linked by an α-helical domain. The N-ERMAD domain is 297 residues long and is composed of three structural modules, F1, F2, and F3, which are arranged into a tight clover-shaped structure (53). The C-ERMAD is an 80 residue domain that is arranged into one β strand and six helical regions. Within the C-ERMAD is an F-actin binding domain, located on the last 34 charged amino acids. The ribbon diagram shows ezrin in its closed conformation, taken from (62). While in its closed conformation, the N-ERMAD masks the F-actin binding domain of the C-ERMAD. The N-ERMAD is shown in light blue, the α-helical domain is shown in yellow and the C-ERMAD is shown in red.
ERM proteins are negatively regulated by an intramolecular interaction between the N-ERMAD and the C-ERMAD. In this conformation, the F-actin binding site is masked (52). Thus, disruption of the intramolecular interactions between domains is necessary to activate the molecule. However, the mechanism behind ERM protein activation remains to be fully elucidated. Evidence for the interaction between the N- and C-ERMAD was discovered when ERM proteins were found to homo- and heterodimerize with one another in cultured cells (64, 110). It was subsequently found that the N-ERMAD binds with high affinity to approximately 90 residues on the C-ERMAD (54). In several *in vitro* studies, high-level expression of the C-terminal domain of ezrin induced membrane spreading, formation of lamellipodia and microspike protrusions, possibly due to excess of exposed F-actin binding sites (65). However, overexpression of N-terminal ezrin fragments can suppress this phenotype (65, 66). It is thought that multiple interactions involving hydrophobic and hydrogen-bonding interactions as well as bridging through water molecules contribute to the high affinity between the N-ERMAD and C-ERMAD domains (42).

Therefore, in its inactive state, C-ERMAD associates with N-ERMAD to mask positively charged residues in the C-terminal domain that are believed to be responsible for F-actin binding (52). It is thought that binding of phosphatidylinositol 2,4-bisphosphate (PIP$_2$) to the N-ERMAD, and subsequent phosphorylation of, a conserved C-terminal threonine are involved in the activation or unmasking of ezrin (67). Based on crystallization studies and site-directed mutagenesis, it has been found that PIP$_2$ binds to four clusters of lysine, located within a basic groove between subdomains F1 and F3 on the N-ERMAD (68). Binding of PIP$_2$ may induce a conformational strain in the F3 domain which is then transmitted through the α helix to the C-ERMAD (69). Mutation of the binding sites interfered with proper ezrin localization to the plasma membrane in conjunction with loss of cell extensions. Thus, PIP$_2$ plays an important role in recruiting ezrin to the plasma membrane and to induce a conformational shift, thereby
decreasing the intramolecular interaction between the amino and carboxy terminal domains (67, 68).

Disruption of the tight interaction between the two domains allows for the phosphorylation of a conserved threonine residue in the ERM proteins. The conserved threonine residue has been mapped to T567 in ezrin, T558 in moesin, and T564 in radixin (70-73). Initial evidence for serine/threonine phosphorylation as a mechanism of ERM activation came when treatment with a serine/threonine protein phosphatase inhibitor was observed to block cytoplasmic translocation of ERM proteins from microvilli in cells undergoing apoptosis (75). Structurally, the threonine residue is buried at the interface between the N- and C-ERMAD. Addition of a negatively charged phosphate group would introduce repulsive forces that could weaken the interaction between the two domains (54). Furthermore, transfection of cells with T567D ezrin in vitro induced the formation of abundant cell-surface structures, such as lamellipodia, membrane ruffles, and microvilli tufts (76). T567D ezrin is a constitutively activated ezrin form, where the threonine residue is replaced by an aspartic acid residue through site specific mutagenesis, mimicking the phosphorylated protein. Phosphorylation at T567 interferes with the intramolecular associations between N-ERMAD and C-ERMAD, due to electrostatic and steric effects (54). Intramolecular interference result in an ‘open’ conformation, exposing the N- and C-ERMAD domains for further phosphorylation and protein interactions with other molecules, including phosphatidylinositol 3-kinase (PI3K) and Src (77-79) (Figure 2). The serine/threonine kinase responsible for the phosphorylation in vivo has yet to be identified. However, potential candidates include serine/threonine kinases such as Rho-kinase (ROCK) (59, 71, 74, 80) protein kinase Cα (PKCα), and PKCθ, (59, 81, 82), Cdc 42 (83), and G protein-coupled receptor kinase 2 (GRK2) (84).
1.2.2 Other Mechanisms of Ezrin Regulation

Ezrin can be phosphorylated on other serine/threonine and tyrosine residues, where these phosphorylation events are linked to specific functions. Phosphorylation of ezrin S66 by protein kinase A mediates gastric hydrochloric acid secretion (85), while phosphorylation of ezrin T235 by cyclin dependent kinase 5 (cdk5) was coincident with pRb-induced cell senescence (86). Ezrin can also be phosphorylated on specific tyrosine residues, namely on Y145 and Y353 (87), upon stimulation by growth factors, including EGF and HGF (88, 89). Phosphorylation of Y353 has been associated with cell survival during epithelial cell differentiation through the PI3K/Akt pathway (90). As a tyrosine kinase, Src plays an important role in ezrin phosphorylation where it phosphorylates and binds to pY190 of ezrin via its SH2 domain. Following phosphorylation and binding to pY190, Src phosphorylates Y145 (91). pY145 ezrin has been observed to promote and stabilize Src activity perhaps through conformational changes in the ezrin molecule. Expression of a loss-of-function ezrin mutant, Y145F, caused delayed cell spreading, as well as inhibition of stress fibre formation and focal adhesion assembly in LLC-PK1 cells (91). More recently, Src has been found to phosphorylate Y477 ezrin, independent of binding to pY190 (92). Fes kinase has been found to interact with pY477 through its SH2 domain and this interaction is required for the recruitment and activation of Fes. The inability to do so renders the cells defective in spreading and scattering following HGF stimulation (93). However, the functional significance of the Src/ezrin interaction in cancer remains to be fully elucidated.

In addition, ERM proteins can be differentially regulated via degradation pathways within the cell. In leukocytes, ezrin, but not moesin, is cleaved to form 55 kDa and 30 kDa fragments by calpain following stimulation with phorbol 12-myristate 13-acetate (PMA) (48). The functional significance of this differential degradation remains to be elucidated. Insights can be drawn from talin, a related FERM superfamily member. Upon cleavage by caplain, the head domain of talin acquires an increased affinity for the cytoplasmic tail of β3 integrin, facilitating clustering and activation of integrins (94).
Figure 2. Mechanism of activation of ezrin.

A) Ezrin is typically found in its closed, inactive conformation. Binding of PIP$_2$ to the FERM domain recruits ezrin to the plasma membrane and induces a conformational shift decreasing the interaction between the N- and C-terminal domains. Subsequent phosphorylation at T567 results in an open conformation, exposing the N- and C-ERMAD domains for further phosphorylation and interactions with other proteins (67).

B) Ezrin can also be phosphorylated on other sites, which can mediate more specific functions. Ezrin can be phosphorylated at other serine/threonine sites. Phosphorylation of Y353 has been associated with cell survival signalling during epithelial cell differentiation through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (90). Src can phosphorylate and bind to pY190 of ezrin. Following phosphorylation and binding of the SH2 domain of Src to Y190, Src can phosphorylate Y145. pY145 ezrin has been observed to sustain and positively influence Src activity. Inactivating mutant, Y145F, demonstrates delayed cell spreading, inhibition of stress fibre formation and focal adhesion assembly in LLC-PK1 cells (91). More recently, Src has been found to phosphorylate Y477. The inability to do so renders the cells defective in spreading and scattering under HGF stimulation (93). However, its function and significance remains to be fully elucidated. Figure adapted from (91).
A) Inactive ezrin and Active ezrin are depicted. EZRIN interacts with EBP50 and F-actin. PIP2 binds to active ezrin.

B) Src phosphorylates ezrin at Y145, Y190, Y353, Y477, and T567. Ezrin is divided into N-ERMAD (1-296) and C-ERMAD (296-585) domains.
If ezrin cleavage functions in a similar manner, cleavage could cause the dissociation of ezrin from its binding partners or F-actin, altering ezrin role in cell adhesion and cell shape.

1.2.3 Ezrin in Normal Development

Ezrin is expressed in a wide variety of tissues, where its expression is especially high in the small intestine, kidney, thymus, lung and organs which possess epithelial microvilli. Thus, it is mainly expressed in the apical surface of epithelial cells and plays an important role in maintaining epithelial polarity during normal development. Ezrin is phosphorylated and translocated to the microvilli after stimulation of gastric acid secretion (95, 96). In rabbit renal proximal tubules during anoxia, dissociation of ezrin from the cytoskeleton correlates with microvillar breakdown (97). Ezrin is the only ERM protein expressed in the gut. Systemic knockout of ezrin causes death soon after birth, due to abnormal aggregated intestinal microvilli resulting from the incomplete transition from pluristratified to columnar epithelium during development. Neonates are unable to survive past weaning as they are unable to absorb sufficient nutrients. Ezrin has also been implicated in development of gastric parietal cells, responsible for hydrochloric acid production, and retinal pigment epithelial cells (98).

Mouse knockouts for the other ERM proteins have also been generated and all have been found to play a major role in membrane organization. Due to their structural similarities, it is possible that functional redundancy exists among the ERM proteins. Inactivation of radixin in mice yields viable animals that only show subtle liver defects, but mice moesin knockout show no apparent defects. The few phenotypical defects seen in the mice suggest that other ERMs can compensate for the loss of individual ERMs in many tissues. However, the proteins show some tissue specificity. Mice defective in radixin, which is mainly expressed in the liver and cochlear stereocilia, are normal at birth but develop liver injury, bilirubin deficiency and deafness but (99-101).
1.2.4 Ezrin in Cancer

Over-expression of ezrin occurs in a number of cancers, including breast, soft tissue sarcomas, gliomas, ovarian carcinomas, endometrioid carcinomas, lung adenocarcinomas, and primary cutaneous melanomas (102-106). It has also been demonstrated that ezrin is required for metastasis in osteosarcomas and rhabdomyosarcomas and increased ezrin expression is associated with advancing histological grade in sarcomas (105). More recently, increased membranous expression of ezrin is associated with poor overall survival in pancreatic cancer and with lymph node positivity in breast cancer (107, 108). In invasive breast cancer, over-expression of ezrin is frequently observed (109). In several invasive breast carcinoma cell lines, ezrin localized to motile structures but had more diffuse cytoplasmic staining relative to non-invasive and non-tumourigenic cell lines in which ezrin tended to localize to the apical surface (109). Similarly, ezrin expression in normal breast epithelium is localized to the apical surface, while breast tumours showed increased cytoplasmic ezrin localization. Cytoplasmic ezrin expression is associated with adverse tumour characteristics such as high grade, high Ki-67 expression, hormonal receptor negativity and lymph node metastases, while membranous ezrin staining is associated with high grade, strong HER-2 and p-Akt expression (109). However, the functional significance of cytoplasmic ezrin localization in cancer still remains unknown; possibilities include ezrin forms that are inactive (closed configuration), degraded (48) or head-to-tail oligomers (110). In contrast, pT567 ezrin, an active ezrin form, remains localized to the membrane. Ezrin acts co-operatively with Src to induce the disruption of cell-cell contacts and cell scattering, which increase cell motility and the potential for invasion and metastasis. Suppression of ezrin expression through shRNA reverses the invasive and motile behaviour of breast carcinoma cells (108). Similarly, over-expression of a dominant negative truncated ezrin mutant (a.a. 1-309) blocks cell motility, invasion and metastasis of breast carcinoma cells in a mouse tumour transplantation model (111). ERM proteins, ezrin and more recently moesin, have been shown to be independent prognostic factors for overall survival in breast cancer patients.
Furthermore, increased expression of ezrin binding protein 50 (EBP50) has been found to correlate with advanced tumour stage and lymph node positivity (113). Together, these findings demonstrate that the ezrin signalling network is a possible prognostic indicator of invasiveness in human breast cancer.

1.3 Src

1.3.1 Biochemical Features and Src Activation

Src is a 60 kDa non-receptor tyrosine kinase belonging to the Src family of kinases (SFK), which includes members such as Yes, Fyn, Fgr, Lck, Lyn, Blk and Yrk. Src was first discovered in 1911 by Peyton Rous who described a viral v-src gene, encoded by the Rous sarcoma virus, which was responsible for transmissible growth of sarcomas in chickens (114). It was subsequently found that the v-src gene was derived from the cellular homologue, c-Src (115). Unlike v-src, cellular c-src is poorly transforming, but has the potential to become an oncogene when activated. v-Src differs from c-Src in that it lacks the C-terminal negative regulatory domain and contains 12 substituted C-terminal amino acids along with numerous point mutations throughout the molecule allowing for higher levels of activity and greater transforming ability (116,117).

c-Src is composed of five highly conserved domains: the C-terminal tail, four Src homology (SH) domains and a unique amino-terminal domain of unknown function. While the function of the amino terminal domain is unknown, mutation to this domain has been observed to reduce the transforming potential of v-src (116). The SH1 domain contains the kinase domain and a conserved autophosphorylation site (Y419 in humans, Y418 in mice, and Y416 in chicken) (115). The SH2 domain recognizes and binds to tyrosine-phosphorylated peptide sequences which determine the substrate specificity of the protein (118). The SH3 domain binds to proline-rich regions while the SH4 domain contains a myristoylation site which is important for membrane localization (119). Normally, inactive Src is localized in the perinuclear region of the
cell. It has been found that targeting Src to the membrane is essential for its transforming ability as it localizes Src into close proximity with both upstream and downstream signalling molecules (120, 121). Lastly, the C-terminal tail contains a negative-regulatory tyrosine residue (Y530 in humans, Y529 in mouse, Y527 in chicken). When phosphorylated by kinases, such as C-terminal Src kinase (CSK), Y530 binds to the SH2 domain, simultaneously forming intramolecular bonds between SH2 and SH3 domains, causing Src to assume a closed configuration (122) (Figure 3). The closed conformation blocks substrate binding sites in both SH2 and SH3, rendering Src inactive. All the intramolecular bonds are relatively weak, so a single activating step can destabilize and expose other residues for further activation. Dephosphorylation of Y530 by phosphatases including SH2-containing phosphatase (SHP) 1 and 2, protein tyrosine phosphatase α (PTPα), and protein tyrosine phosphatase 1B (PTP1B), disrupt these intramolecular bonds, resulting in an open, active configuration (124-126). In order to fully activate Src, membrane localization-dependent autophosphorylation at Y419 is required and provides a binding site for downstream SH2 domain-containing proteins. Another level of regulation occurs through ubiquitination by CBL ubiquitin ligase and subsequent degradation by the proteasome (127).

1.3.2 Src in Normal Development

Src is expressed ubiquitously in many adult tissues with greater levels of expression in platelets, neurons and osteoclasts (128). It is involved in numerous cellular processes such as transcription, cell cycle progression, adhesion, angiogenesis, migration, apoptosis and differentiation (129, 130). In vivo studies have observed that mice possessing a null mutation of the Src gene, introduced through homologous recombination in mouse embryonic stem cells, survive through embryogenesis, possibly due to the functional overlap between members of the Src family of kinases (131). The mice, however, develop osteopetrosis; a disease resulting from deficiency in bone resorption by osteoclasts, suggesting a direct role of Src in osteoblast/osteoclast regulation (131-134).
Figure 3. Structural diagram and mechanism of activation of Src.

A) The structural domains of Src are shown. The Src protein is composed of a myristoylation sequence (M) that targets Src to the membrane, a unique region (U), SH2 and SH3 protein-interaction domains, a kinase domain that contains the autophosphorylation site, Y419 and a carboxy-terminal negative regulatory domain (R) that contains Y530 which controls the conformational state of Src (116). Adapted from (115).

B) When phosphorylated by kinases, such as C-terminal Src kinase (CSK), Y530 binds to the SH2 domain, simultaneously forming intramolecular bonds between SH2 and SH2 domain, causing Src to assume a closed configuration. The closed confirmation blocks substrate binding sites in both SH2 and SH3, rendering Src inactive. Deposphorylation of Y530 disrupts these intramolecular bonds, resulting in an open, active configuration. After localizing to the membrane, through its myristoylation site, subsequent autophosphorylation, at Y419, fully activates Src and provides a binding site for downstream SH2 domain-containing proteins. Structural and ribbon diagrams show Src in both its active (right) and inactive (left) conformations. The SH3 domain is shown in navy blue, the SH2 domain is in light blue and green, the activation loop is yellow and the C-terminal tail is red. Ribbon diagrams were adapted from (115, 121)
Since Src, Yes and Fyn are the most ubiquitously expressed proteins of the Src family of kinases, double knockouts of Src, Yes and Fyn show that most Src/Fyn and Src/Yes mice die perinatally while Fyn/Yes deletions are generally viable (135). Furthermore, mouse embryos with homozygous null mutations in Src, Yes and Fyn genes die in utero by day 9.5 and display severe developmental defects (136). In addition, germ line deletion of Src cause defects in the initial stages of mammary ductal outgrowth as well as uterine and ovarian development (137). When fibroblasts were isolated from these embryos and plated on to ECM proteins, they displayed severe cell spreading and migration deficiencies (136). Similarly, Src-deficient mammary epithelial cells were unable to respond to exogenous estrogen stimulation and displayed severe cell spreading and migration deficiencies when plated on ECM proteins (137). This suggests that developmental deficiencies are due to impaired transmission of ECM signals. It is still unclear as to where activated Src localizes in the cell. It is postulated that cytosolic Src plays a role in protein trafficking, perinuclear Src is associated with the microtubule organizing centre, while nuclear Src may function as a cell cycle regulator (138,139). However, the regulation of the subcellular distribution of Src still remains largely unknown. Findings reveal that inactive Src tends to localize in the perinuclear region until stimulation, at which point, Src translocates to the plasma membrane through endosome-mediated recycling (139).

1.3.3 Src in Cancer

Src was the first oncogene to be discovered and has been strongly implicated in the development, growth, progression and metastasis of many human cancers. Studies indicate that Src expression and/or activity is upregulated in a number of human cancers and cancer cell lines including breast, colon, pancreas, esophageal, gastric, ovarian as well as head and neck cancer (140-147). When expressed under the control of a mammary tumour virus promoter (MMTV), polyomavirus middle T antigen transgenic mice develop mammary tumours with high penetrance. However, in the absence of functional Src, these mice rarely develop mammary tumours (149).
contrast, transgenic mice expressing constitutively activated Src under the control of the MMTV promoter frequently develop mammary epithelial hyperplasias (148). This suggests that Src is required for mammary tumourigenesis but is insufficient to induce mammary tumour formation. Therefore, it is likely that Src interacts with other signalling molecules to bring about the tumourigenic phenotype (147). It has been demonstrated that Src is activated by a plethora of RTKs including EGFR, ErbB2, PDGFR, FGFR, CSF1R, VEGFR and Met (150-157). In the case of Met, Src is often regarded as a downstream effector. Src has been found to play a role in HGF-induced disruption of cell-cell contacts, cell spreading and membrane ruffling, and cell migration, all of which contribute to the metastatic potential of the cell (158, 159). Src destabilizes focal adhesion contacts, resulting in more rapid focal adhesion turnover and increased cell migration. In addition, Src acts to decrease cell-cell contacts through binding and phosphorylating β-catenin and p120 catenin, leading to disassembly of adherens junctions (160). Src also stimulates ubiquitination of E-cadherin leading to its endocytosis and further loss of cell-cell contacts (161). There is recent evidence demonstrating an upstream role in ligand-independent and cell matrix adhesion-dependent Met regulation (162). It has been found that constitutively active Src increases Met phosphorylation within the activation loop and when expressed with constitutively active ezrin, further increases Met phosphorylation levels (163). Immunohistochemical staining of a breast tumour cohort found that high cytoplasmic total Src levels and high membranous activated pY419 Src levels were associated with decreased overall survival. In addition, membrane pY419Src expression was positively correlated with grade, size, and HER-2 status and inversely correlated with ER status while cytoplasmic pY419 Src expression positively correlated with HER2 status (164). Interestingly, over-expression of pY215 Src was associated with improved survival within a cohort of ER, PR and HER-2 negative breast cancer patients (164). In contrast, expression of Src within the nucleus of ER positive breast tumours is associated with improved patient outcome. This demonstrates the importance of intracellular localization and activation status in determining potential predictive markers.
1.4 Met

1.4.1 Biochemical Features and Met Activation

Met was originally identified as the product of a proto-oncogene, Tpr-Met, in a human osteosarcoma cell line treated with a potent carcinogen, N-methyl-N’-nitro-N-nitrosoguanidine (165). The gene encodes for a receptor tyrosine kinase and is located on 7q21-q31 (165). The nascent 150 kDa protein product is partially glycosylated to form the 170 kDa Met precursor, which is further glycosylated. The 170 kDa precursor is cleaved to form a heterodimer consisting of a 50 kDa α chain and a 145 kDa β chain (166). The β chain is subdivided into five domains. The extracellular domain binds the only known ligand of Met, referred to as hepatocyte growth factor (HGF), but is also known as scatter factor (SF) (167, 168). The transmembrane domain contains hydrophobic residues necessary to transverse the cell membrane. The juxtamembrane domain contains negative regulatory sites at S985 and Y1003, where pS985 inhibits Met kinase activity while phosphorylated Y1003 is required for Met receptor ubiquitination by Cbl ubiquitin ligase (169). The kinase domain possesses intrinsic kinase activity. When Met is activated, transphosphorylation occurs at Y1234 and Y1235. Lastly, the carboxy-terminal region, which includes Y1349 and Y1356, provides a docking site for numerous proteins following Met activation (170).

Normally, binding of HGF, the only known ligand of Met, to its receptor induces dimerization of Met. Dimerization triggers transphosphorylation of tyrosine residues, Y1234 and Y1235, in the activation loop of the kinase domain (171). Autophosphorylation further activates the intrinsic kinase activity of Met which results in the phosphorylation of two tyrosine residues, including Y1349 and Y1356, in carboxy terminus, forming a multistubstrate docking site (reviewed in 172) (Figure 4). Phosphorylation of the docking site allows for the recruitment and binding of numerous adaptor proteins via their SH2 domains, including Src, Grb2 (Growth factor receptor-bound protein 2), Gab1 (Grb2 associated binding protein 1),
Figure 4. Structural diagram of the Met receptor tyrosine kinase.

The Met receptor tyrosine kinase is a heterodimer which consists of a 50 kDa α chain and a 145 kDa β chain joined through a disulfide bond (166). The β-chain is divided into five domains. The extracellular domain, along with the α chain, binds to HGF (167,168). The transmembrane domain contains hydrophobic residues necessary to span the plasma membrane. The juxtamembrane domain contains a number of regulatory sites, including S985 (176) and Y1003, phosphorylation of which targets Met for ubiquitination by Cbl ubiquitin ligase followed by proteosomal degradation (169). The kinase domain contains the activation loop which contains residues Y1230, Y1234 and Y1235 that are transphosphorylated upon ligand binding. The last domain is the carboxy terminal docking site containing residues Y1349 and Y1356 which mediate the majority of downstream signalling by serving as binding sites for adaptor and effector proteins (170).

The ribbon diagram shows the extracellular and kinase domain. The extracellular domain is composed of 2 sema domains and 4 immunoglobin domains. Ribbon diagram was adapted from (172).
HGF

α chain

β chain

Extracellular domain

S985
Y1003

Transmembrane domain

Y1230
Y1234
Y1235

Juxtamembrane domain

Y1349
Y1356

Kinase domain

C-terminal docking site

Gab1, Grb2, Src, Shp2, Shc, PI3K, PLCγ, Grb10
SHIP-2 (SH2-containing 5’inositol phosphatase), PLC-\(\gamma\) (phospholipase C-\(\gamma\)), and PI3K that will then lead to downstream signalling cascades (172-174). Through its downstream effectors, Met is involved in numerous biological functions. Grb2 interacts with SOS1 which activates Ras, triggering ERK/MAPK signalling and is involved in HGF induced cell migration, invasion and branching morphogenesis, possibly through the disassembly of adherens junctions (175). Gab1 is considered one of the most crucial substrates for Met. Embryos nullizygous for Gab1 display all of the defects observed in Met or HGF null embryos (177). Activated Met phosphorylates Gab1 at several tyrosine residues which allows binding of downstream molecules, such as PI3K, Shc, Shp2 (SH2-containing protein tyrosine phosphatase 2) and CRK (retroviral protein CT10 regulator of kinase-like) (178). Src is implicated in the involvement of HGF-induced cell transformation (170) while Stat3 induces transcription of genes required for HGF-induced branching morphogenesis and anchorage-independent cell growth (179, 180). Apart from directly mediating signal transduction, Met can interact with other cell surface proteins, including \(\beta_4\) integrin, ezrin, the hyaluronan receptor CD44, the Fas receptor, semaphorin receptors or E-cadherin (88, 181-184). These interactions may allow localization of Met to specialized regions of the cell where it can interact with specific substrates.

1.4.2 Met in Normal Development

Met is mainly expressed on the membrane of epithelial cells in many tissues (185). Normally, Met is activated in a paracrine manner, where mesenchymal cells produce and secrete HGF, which diffuse and bind to Met, expressed in epithelial and endothelial cells. In some instances, mesenchymal stem cells can express both Met and HGF which promotes proliferation and migration of these cells during tissue repair and wound healing (186, 187). Migration of these cells involves the disruption of cadherin-based cell-cell contacts (188, 189).

During embryogenesis, HGF/SF and Met are essential. Met is known to be expressed in a many stages of development, and mediates differential signalling pathways or biological
responses dependent upon downstream effectors that are being activated. Without either Met or HGF, mice die in utero (reviewed in 189). The presence of Met or HGF is essential to cell proliferation and cell survival of placental trophoblasts and hepatocytes. Lack of Met also decreases survival of sensory or sympathetic neurons as well as impairs outgrowth of certain motor nerves (190). HGF treated mouse mammary epithelial cells embedded in collagen form tubules with well-defined lumens, supporting its role in ductal development (191). In rat mammary gland development, analysis of HGF and Met transcripts showed a decrease during pregnancy until lactation and increased during involution, suggesting a role in mammary ductal morphogenesis (192). In addition, overexpression of HGF was found to enhance the size, increase the number and branching activity of end buds (193). Due to the role of Met and HGF in regulating cell motility, Met is also involved in the migration of cells to their target organs, including long-range migration of skeletal muscle progenitor cells. Ablation of the Met or HGF gene results in a complete absence of all muscle groups derived from these cells (194). Under normal physiological development, Met and its downstream pathways controlling survival, growth and motility are tightly regulated. However, during tumourigenesis, Met expression can be deregulated resulting in abnormal cell proliferation, migration and survival (195).

1.4.3 Met in Cancer

Deregulation of the Met-HGF signalling network can manifest itself through various mechanisms. In the original discovery of Met, chromosomal rearrangement within a human osteogenic sarcoma cell line induced by a potent carcinogen, resulted in a fusion of two genetic loci, a translocated promoter region and Met, generating Tpr-Met (196). Through its Tpr region, Tpr-Met dimerizes and is constitutively activated in the absence of a ligand (197). Constitutive activation through the expression of Tpr-Met in transgenic mice induced development of multiple epithelial-derived tumours, including breast (198). Similarly, overexpression of Met, through mechanisms such as transcriptional activation or an enhanced population of progenitor cells
expressing Met, can lead to ligand-independent spontaneous dimerization of Met receptors leading to unregulated downstream signalling. Overexpression of Met is correlated with poor prognosis and has been found in a number of cancers, including breast, ovarian, pancreatic and thyroid cancers (199-204). In a cohort of axillary lymph node negative breast carcinoma patients, it was found that tumours with high Met expression levels were associated with death due to metastatic disease (205). Further studies show that Met overexpression and activation is prominent in the highly aggressive basal-like breast cancer subtype (206, 207). Met tends to show a membranous-cytoplasmic localization, but there have been reports observing nuclear staining (208). Mutations within the domains of Met also have been found to be associated with certain cancers. Activating point mutations within the kinase domain have been found in sporadic and hereditary forms of human papillary renal cancer while mutations in the juxtamembrane domain are found in human gastric & lung cancers (189). Autocrine activation of Met by HGF has been proposed as another mechanism mediating breast tumourigenesis (209-215). The Elliott lab has identified a Src/Stat3 pathway that activates HGF transcription and autocrine activation of Met in breast carcinoma cells (214, 215). Multiple downstream signalling cascades mediate numerous biological functions of Met, including cell proliferation, cell survival, cell scattering, motility, induction of cell polarity, angiogenesis, tissue regeneration, invasion and tumour metastasis (216). Therefore, Met likely plays an important role in several stages of tumour progression.

1.5 Rationale, Hypothesis and Objectives

In collaboration with Dr. Monique Arpin (Curie Institute, Paris, FR), our group has shown that Src acts co-operatively with ezrin in the transformation of epithelial cells, through phosphorylation at specific tyrosine residues (Y145 and Y477F) (91, 93). Co-operation of Src and ezrin results in the disruption of cadherin-based cell-cell contacts and increased cell scattering and motility (91, 217). Src and ezrin also cooperate in the formation of stress fibres and cell
spreading in mammary epithelial cells (91). More recently, our group has shown that co-expression of activated Src and activated ezrin strongly increased Met phosphorylation relative to phosphorylation levels induced with activated Src or ezrin alone, and induces the formation of invadopodia, extracellular matrix-degrading actin-rich protrusions that are characteristic of invasive tumour cells (162). However, expression of a dominant negative truncated ezrin mutant (a.a. 1-309) abolishes Src-induced Met phosphorylation and invadopodia formation (91, 162). Two groups have studied the interaction between Src and ezrin (79, 91) which involve two key tyrosines on ezrin, Y145 and Y477 which are phosphorylated by Src. Inactivating mutations at these two tyrosine sites have been shown to block the cooperative effects of Src and ezrin in the transformation of epithelial cells, such as cell spreading, disruption of cell-cell contacts and cell motility (91). It has also been recently shown that recruitment of Fps/Fes kinase to Y477 of ezrin promotes HGF-induced cell scattering (93). This novel Src/ezrin interaction may play an important role in metastatic tumour progression. Based on the cumulative findings, we propose the following hypothesis (summarized in Figure 5):

**In this study, I have examined the hypothesis that: Src/ezrin activation is required at specific steps of the metastatic process, and is a potential predictor for local invasion and distant metastasis in human breast cancer.**

My experimental approaches to test this hypothesis included:

1. **Evaluating Src and ezrin as markers of tumour stage and metastasis in human breast cancer**
   
   i) Determining localization and expression levels in normal mammary glands and PyMT induced tumours
   
   ii) Assessing the pathological association of Src/ezrin expression in a breast tumour TMA
2. *Assessing the role of Src/ezrin activation at specific stages of the metastatic cascade in breast cancer.*

The combined pre-clinical and clinical aspects of this study have provided new insight into the role of the Src/ezrin signalling network during specific stages of the metastatic cascade in malignant breast cancer, and may lead to new strategies for the treatment of metastatic disease. Furthermore, associations of Src/ezrin activation with clinical parameters may lead to potential prognostic/predictive markers of metastasis in lymph node negative breast cancer.
Met
pY
Disruption of
cell-cell contacts
Invasion
Metastasis
Cell scattering
Cell motility
Carcinoma
Src
pY145 pY477 pY190 pT567
Ezrin
Actin filaments
Disruption of

Figure 5. Model of the Ezrin/Src signalling network.

In normal epithelial cells, Src and Met activation is tightly regulated and the majority of ezrin is in its inactive state. During tumourigenesis, sustained activation and overexpression of Met can cause the loss of cadherin-based cell-cell contacts, the mechanism of which remains unclear. Loss of cadherin is one of the primary hallmarks of EMT, which can lead to invasion and subsequently metastasis. A possible mechanism of sustained Met activation is mediated through integrins which requires both Src and an intact cytoskeleton (218). Ezrin also acts downstream of Met during cell migration (219) and is required for metastasis of breast carcinoma cells (111). More recently, Src and ezrin have been found to act cooperatively in the formation of invadopodia, stress fibres and cell spreading in mammary epithelial cells (91). Interactions between Src and ezrin suggest a positive feedback loop in which binding of Src to Y190 of ezrin increases Src kinase activity. In return, Src phosphorylates ezrin at Y145 and Y477, resulting in a fully activated molecule of ezrin, which is required for cooperative effects of Src and ezrin in the transformation of epithelial cells and allows the initiation of several downstream signalling pathways which may potentiate the effects of Met.
### CHAPTER 2—MATERIALS AND METHODS

#### 2.1 Reagents

The sources of the reagents used in the study are listed in Table 1.

**Table 1. Reagent sources.**

<table>
<thead>
<tr>
<th>Reagent Name</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-dithiothreitol (DTT)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>2,2,2-tribromoethanol</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>20x TBS, pH 7.4</td>
<td>TEKnova Inc., Hollister, CA</td>
</tr>
<tr>
<td>Absolute Ethanol</td>
<td>Queen’s University, Stores</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>Fisher scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Ammonia Solution</td>
<td>BDH Inc., Toronto, ON</td>
</tr>
<tr>
<td>Ammonium Persulfate (APS)</td>
<td>ICN Biomedicals Inc, Irvine, CA</td>
</tr>
<tr>
<td>Benzamidine</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Bio-Rad DC Protein Assay Kit</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Bio-Rad Kaleidoscope Protein Marker</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Bis-Acrylamide</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Bovine Serum Albumin (BSA)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Buprenorphine</td>
<td>Merck &amp; Co., Inc., Whitehouse Station, NJ</td>
</tr>
<tr>
<td>DAKO Cytomation LSAB + System-HRP</td>
<td>Dako Inc., Carpinteria, CA</td>
</tr>
<tr>
<td>Deoxycholate</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Disodium Hydrogen Orthophosphate (Na₂HPO₄·H₂O)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagles Medium (DMEM)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Enhanced Chemi-Luminescence Kit</td>
<td>Perkin Elmer, Wellesley, MA</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic Acid EDTA</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Fetal Bovine Serum</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Formaldehyde solution 37%</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Glycine</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Harris’ Modified Hematoxylin Reagent</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Horse Serum</td>
<td>Invitrogen Corp., Camarillo, CA</td>
</tr>
<tr>
<td>Hydrochloric acid (HCl)</td>
<td>BDH Inc., Toronto, ON</td>
</tr>
<tr>
<td>Leupeptin/Pepstatin</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Low melting point agarose</td>
<td>BRL Research Lab</td>
</tr>
<tr>
<td>Methanol</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>N,N,N’,N’-Tetramethylethylenediamine (TEMED)</td>
<td>BioRad, Mississauga, ON</td>
</tr>
<tr>
<td>Nonidet P-40 (NP-40)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>PAP pen</td>
<td>Daido Sangyo Co. Ltd., Tokyo, Japan</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Permoun</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Phenyl-methyl-sulfonyl-fluoride (PMSF)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Potassium Chloride (KCl)</td>
<td>Merck Frost Canada Ltd., Kirkland, QC</td>
</tr>
<tr>
<td>Potassium Dihydrogen Orthophosphate (KH₂PO₄)</td>
<td>ICN Biomedicals Inc, Irvine, CA</td>
</tr>
</tbody>
</table>
Table 1. (cont’d)

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Chloride (NaCl)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Sodium citrate trisodium salt dihydrate (C₆N₅Na₃O₇·2H₂O)</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulphate (SDS)</td>
<td>ICN Biomedicals Inc., Irvine, CA</td>
</tr>
<tr>
<td>Sodium Fluoride (NaF)</td>
<td>ICN Biomedicals Inc., Irvine, CA</td>
</tr>
<tr>
<td>Sodium Orthovanadate (Na₃VO₄)</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Streptavidin/Biotin Blocking Kit</td>
<td>Vector Laboratories, Burlingame, CA</td>
</tr>
<tr>
<td>Tertiary amyl alcohol</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Toluene</td>
<td>Fisher Scientific, Ottawa, ON</td>
</tr>
<tr>
<td>Trasylol</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Tris Base</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>Triton-X-100</td>
<td>MP Biomedicals Inc., Solon, OH</td>
</tr>
<tr>
<td>Tween-20</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>β-Glycerophosphate</td>
<td>Sigma Aldrich, Oakville, ON</td>
</tr>
<tr>
<td>β-mercaptopethanol</td>
<td>Calbiochem, Darmstadt, Germany</td>
</tr>
</tbody>
</table>

2.2 Cell Lines and Tissue Culture

2.2.1 Cell lines and tissue culture

The predominant cell line used in this study was the AC2M2 cell line. The AC2M2 cell line is a lung metastatic variant which was previously selected from the SP1 cell line following three times serial intramammary injections of lung metastatic nodules into syngeneic mice (221). The SP1 cell line was derived from culturing tissue explants of a spontaneous, poorly metastatic murine mammary intraductal adenocarcinoma that arose from an 18th month old female CBA/J breeder (111, 220, 221). AC2M2 cells exhibit constitutive activation of Src and a scattered invasive phenotype, which is characteristic of malignant breast carcinoma cells. All AC2M2 and derived cell lines were maintained in DMEM with 10% FBS at 37°C, and were harvested using EDTA/trypsin (Gibco).

2.2.2 DNA Constructs and Transfection of AC2M2 cells with pCB6 expression vector

cDNA coding for wildtype ezrin or mutant ezrin forms were fused to oligonucleotides, encoding the 11 amino acid carboxy-terminus of vesicular stomatitis viral glycoprotein (VSVG) for independent detection of the mutant from endogenous ezrin (222). The sequence of the VSVG tag is shown in Figure 6. Fusion of the ezrin cDNA to the VSVG fragment was
Figure 6. pCB6 expression vector and VSVG sequence

A) pCB6 expression vector is shown (Courtesy of Monique Arpin, Curie Institut, Paris, FR).

B) Sequence of VSVG tag is shown flanked with restriction sites. Below are the 11 amino acids that encode for the carboxy terminus of the VSVG protein (222).

C) Y477F ezrin construct. In the ezrin construct, a point mutation at 477 is made where the tyrosine residue is substituted with phenylalanine. The phenylalanine substitution creates a loss of function mutant where phosphorylation cannot occur. Thus downstream signalling from this residue is inhibited. The construct was tagged with VSVG for independent detection from endogenous ezrin.
A pCB6 vector 8.2 kb

B

CMV Promoter

Hind III

DNA insert

XbaI

Lac Z

AmpR

SmaI SfII BglII KpnI XbaI

cccggagggcccaccgcccctaaccgatcagtagtgacgctgagctgagtaaatagctaatctaga

Y T D I E M N R L G K

VSVG Tag

C

N-ERMAD

Y477F

C-ERMAD

VSVG

1 296 585
performed using the Bluescript-KS plasmid. The fused ezrin-VSVG cDNA was then excised through digestion by HindIII-XbaI and inserted into the eukaryotic expression vector, pCB6, downstream of the cytomegalovirus promoter. Point mutation for Y477F ezrin was obtained using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). pCB6 vector only, with WT or mutant ezrin constructs were kindly obtained from Dr. Monique Arpin (Curie Institute, Paris, FR). Initial transfections to produce pCB6 control and Y477F ezrin-expressing clones were performed by the Arpin lab.

2.3 Mouse Strains

2.3.1 Polyomavirus Middle T (PyMT) transgenic mouse

The PyMT antigen is an oncogene which has been known to constitutively activate c-Yes and c-Src, the latter being one of the key molecules in our signalling network, through the formation of stable protein-protein interactions with the C-terminus of PyMT antigen (149). c-Src and c-Yes-deficient PyMT transgenic mice are unable to develop tumours, indicating the importance of these kinases in mediating PyMT-induced tumourigenesis and progression (149). Other cytoplasmic proteins, such as Shc and Grb2, also form complexes with activated PyMT that are important for the induction of the Ras signalling pathway and are required for tumour progression (223). Furthermore, PyMT-induced mammary tumours exhibit a tumour progression cascade morphologically similar to that of human breast cancer. In addition, biomarkers expressed in PyMT-induced tumours were consistent with those associated with poor outcome in humans, including loss of ER and PR, overexpression of Erb-B2 and cyclin D1, as well as an aberrant expression of β1 integrins (224). Tissue sections from transgenic PyMT-induced mouse breast tumours were provided by Dr. W. Muller (McGill University, Montreal, Canada). The transgene was developed by inserting the PyMT antigen cDNA under the transcriptional control of the mouse mammary tumour virus (MMTV) promoter (described in 226) (Figure 7). As a result, PyMT antigen expression is primarily directed to the mammary epithelium at puberty.
Figure 7. Polyomavirus Middle T transgene structure.

The blue region represents sequences within the Bluescript vector backbone, followed by the mammary tumour virus long terminal repeat derived from the plasmid pA9 and directs expression primarily to the mammary epithelium. Adjacent to the promoter is an inert region derived from the original pA9 vector. The red region is the insertion site of the cDNA encoding the PyV middle T antigen followed by transcriptional processing sequences derived from the SV40 early transcription unit (226). Transcription start site, indicated by the arrow is shown. Modified from (226).
### 2.3.2 Nulliparous and Lactating Mice

Nulliparous and lactating mammary gland blocks were a kind gift from Dr. Peter Truesdell at the Queen’s Cancer Research Institute, Kingston, Canada. Females with a SVJ/129-CD1 hybrid background were mated at 8-9 weeks of age. The # 4 mammary gland was isolated and used in all experiments. Mammary gland tissues were used in order to provide a non-neoplastic reference point to assess differences in protein expression and localization in our tumour models. Nulliparous mammary glands taken from FVB female mice were used as a control for experiments involving transgenic PyMT mice.

### 2.4 Staining

#### 2.4.1 Antibody Control and Validation

Controls for immunostaining served as an important indicator for any false positive or false negative staining that arose. Antibodies were tested on select tissues and cells to validate their specificity. To validate specificity of ezrin, tissue sections taken from mouse intestine were used as intestinal brush border cells are known to express ezrin (45). To validate the specificity of Src, tonsil was used as a positive control based on correspondence with Dr. M. Frame (Edinburgh Cancer Research Centre, Edinburgh, UK) and obtained from Lee Boudreau (QLMP). Cell line blocks, known to express ezrin and Src were placed into the training TMA to provide another level of positive controls. Three types of negative controls were used. At the most minimal level, a no primary antibody control was used. An immunoglobulin control was used in the PyMT studies to eliminate staining caused from non specific binding via the Fc portion of the primary antibody. Due to the instability of phosphorylated epitopes on fixed tissue, a blocking peptide specific to the phospho-specific antibody was used (Table 2).
Table 2. Blocking peptides for Immunohistochemistry.

<table>
<thead>
<tr>
<th>Blocking peptide</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pT567 ezrin</td>
<td>Cell Signaling, Beverly, MA (1047)</td>
</tr>
<tr>
<td>Pan Src (36D10)</td>
<td>Cell Signaling, Beverly, MA (1235B)</td>
</tr>
<tr>
<td>pY418 Src</td>
<td>Cell Signaling, Beverly, MA (1940B)</td>
</tr>
</tbody>
</table>

2.4.2 Immunohistochemistry

2.4.2.1 Manual

For formalin-fixed paraffin embedded (FFPE) tissue, tissue sections of 6µm thickness were deparaffinised and rehydrated prior to staining. Sections were submerged in three toluene baths for 4 minutes then washed 7 times in graded ethanol baths of 100%, 85% and 70% sequentially. Tissues were then placed in a water wash for 4 minutes. Following deparaffinization, tissues were placed in a sodium citrate antigen retrieval solution, pH 6.0 at 95°C for 30 minutes. Tissues were removed after retrieval, cooled for 20 minutes then washed twice with 1x TBS buffer. 3% hydrogen peroxide block was applied and tissues were incubated for 5 minutes. After blocking, the tissues were rinsed with TBS and placed in a buffer bath of 0.025% Triton X-100 in TBS. Excess buffer was tapped off and 5% horse serum was applied and tissues were incubated for 30 minutes. If background staining was high, a streptavidin/biotin block would be applied after incubation with serum for 15 minutes each. After a brief wash with TBS, the primary antibody, diluted in 5% horse serum was applied and incubated overnight at 4°C in a humidity chamber.

Depending on whether the tissue was mouse or human, slightly different methods were utilized to ultimately attach the horseradish peroxidase (HRP) to the antigen site. For human tissue, a biotinylated universal secondary antibody cocktail, consisting of antibodies against mouse, rabbit and goat was applied and tissues were incubated for 15 minutes. Following a TBS
wash, HRP conjugated streptavidin was applied and tissues were incubated for 15 minutes. For murine tissue, a HRP conjugated rabbit secondary antibody was incubated on the tissue for 30 minutes. Next, 3,3'-Diaminobenzidine (DAB) was allowed to react with HRP for up to 10 minutes to form a brown precipitate (Figure 8). Tissues were rinsed with water to stop the reaction. Tissues were then counterstained with hematoxylin for 17 seconds and placed in a running water bath for 5 minutes. After the wash, sections were dipped 10 times in ammonia water, the bluing step, and placed back in the running water bath for 2 minutes. Following the wash, tissues were dehydrated by dipping the slides 10 times through graded ethanol baths of 70%, 85% and 100%, respectively, and three toluene baths. Tissues were then coverslipped using Permount.

2.4.2.2 Automated Ventana Staining

Human tissue microarray slides were stained using the Ventana Discovery XT staining module. The Ventana was chosen as an alternative to manual staining for a number of reasons. Due to the automated nature of the Ventana, it introduces less variability as the staining environment is carefully controlled. Since the tissues are taken from human as opposed to mouse, there is limited cross-reactivity from the universal secondary antibody cocktail. It also provides a secondary validation technique apart from the manual method. Furthermore, conditions and dilutions that are optimized with the Ventana can be easily applied to clinical settings where automated staining for large numbers of samples will be advantageous. Slides were warmed to 75°C and deparaffinization solution was applied and incubated for 8 minutes. Slides were subject to an antigen retrieval process, pH 8.0 at 100°C. Following antigen retrieval, slides were allowed to cool for 8 minutes. Inhibitor D, similar to the hydrogen peroxide (HPO) block from the manual method, was applied and incubated for 4 minutes.
Figure 8. Immunohistochemistry.

Primary antibody for detection of antigen of interest is applied, represented by the orange antibody. For human tissue, a biotinylated universal secondary antibody cocktail is applied. Depending on which animal the antibody is raised in, the appropriate secondary antibody binds to the Fc portion of the primary antibody. The secondary antibody is represented by the yellow antibody and the biotin by the green symbol. HRP conjugated streptavidin, symbolized by the red drop, binds to the biotin. For murine tissue, a HRP conjugated rabbit secondary antibody is applied instead of a biotinylated secondary. Application of 3,3'-Diaminobenzidine (DAB) reacts with HRP forming a brown precipitate.
Following HPO inhibition, diluted primary antibody was applied manually and incubated for 60 minutes. Antibody binding sites were amplified and followed by incubation of a universal secondary antibody which contains a cocktail of antibodies against rabbit, mouse and goat. Stain was visualized through precipitation of DAB by horseradish peroxidase. A copper wash was applied for 4 minutes which increases the brown DAB colour. Tissues were then counterstained with hematoxylin for 4 minutes, and rinsed twice. Bluing reagent was then applied and incubated for 4 minutes. Slides were rinsed 3 times. Following the wash, dehydration and coverslipping was done manually as described above.

Table 3. Primary antibodies used for Immunohistochemistry.

<table>
<thead>
<tr>
<th>1° Antibody</th>
<th>Clonality</th>
<th>Source</th>
<th>Concentration</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan ezrin</td>
<td>Polyclonal</td>
<td>Cell Signaling, Beverly, MA (3145)</td>
<td>IHC: 1:200</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pT567 ezrin</td>
<td>Monoclonal</td>
<td>Cell Signaling, Beverly, MA (3149)</td>
<td>IHC: 1:200</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Pan Src (36D10)</td>
<td>Monoclonal</td>
<td>Cell Signaling, Beverly, MA (2109)</td>
<td>IHC: 1:100</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pY418 Src</td>
<td>Polyclonal</td>
<td>Biosource of Invitrogen Corp., Camarillo, CA (44660G)</td>
<td>IHC: 1:300</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Pan Met</td>
<td>Monoclonal</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-8057)</td>
<td>IHC: 1:500</td>
<td>Mouse</td>
</tr>
<tr>
<td>pY1234/1235 Met</td>
<td>Polyclonal</td>
<td>Cell Signaling, Beverly, MA (3126)</td>
<td>IHC: 1:100</td>
<td>Rabbit</td>
</tr>
<tr>
<td>HGF</td>
<td>Polyclonal</td>
<td>R&amp;D Systems Inc. Minneapolis, MN (AF2207)</td>
<td>IHC: 1.5 µg/µL</td>
<td>Goat</td>
</tr>
<tr>
<td>VSVG</td>
<td>Polyclonal</td>
<td>Monique Arpin</td>
<td>IHC: 1:100</td>
<td>Rabbit</td>
</tr>
</tbody>
</table>

2.4.3 Hematoxylin and Eosin Staining

To visualize tissue morphology, tissue sections were stained with hematoxylin and eosin. Hematoxylin stains basophilic structures, such as the nucleus and ribosomes, while eosin stains eosinophilic structures such as intracellular or extracellular protein. Tissues were deparaffinized by submerging the sections in 3 toluene baths for 4 minutes, followed by 7 dips in three graded...
ethanol baths of 100%, 85% and 70%, then a water bath for 4 minutes. Tissues were stained for 4 minutes in hematoxylin and placed in a running water bath for 5 minutes. Next, the sections were dipped 6-7 times in an acid-alcohol wash and placed back in the running water bath for 8 minutes. The tissues were then transferred into ammonia water for 2 minutes and then to eosin for 1 minute. Following a quick water wash, tissue sections were dipped 10 times through the graded ethanol baths of 70%, 85% and 100% followed by 10 dips in 3 consecutive toluene baths. Tissues were mounted with Permount and coverslipped.

2.5 Western Blotting

2.5.1 Preparation of Tissue Homogenates

Tissues were homogenized using a homogenizer in RIPA buffer containing a cocktail of protease and phosphatase inhibitors, as listed in the Appendix. Following homogenization, homogenates were sonicated and nutated for 15-20 minutes at 4°C. Afterwards, homogenates were centrifuged at 13000x rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was discarded. 2x SDS was then added to the supernatant and the homogenates were stored at -20°C for future biochemical assays.

2.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

β-mercaptoethanol (3%) was added to the homogenates, from herein referred to as samples, and boiled at 100°C for 5 minutes. Samples were loaded and resolved on 8% polyacrylamide gels for SDS-PAGE along with BioRad Kaleidescope (pre-stained protein) Marker for size reference. Samples were then transferred to PVDF (polyvinyl difluoride) membranes (Millipore, Villerica, MA), which were pre-treated in 100% methanol for 10 minutes. The semi-dry transfer method was used at 100mA/gel for 1 hour.
2.5.3 Immunoblotting

Following transfer, membranes were blocked in either 5% milk or BSA in TBST as suitable for 1 hour before probing overnight with diluted primary antibodies of interest as indicated in Table 4. After overnight incubation, membranes were washed 3 times, 10 minutes each, with TBST and appropriate secondary antibodies (Table 5), diluted to 1:2500 in TBST were applied to the membranes and incubated for 1 hour at room temperature. Membranes were washed 3 times, 10 minutes each, with TBST. Proteins were visualized using enhanced chemiluminescence reagent (Perkin Elmer, Wellesley, MA), followed by exposure to autogrophic film (Fuji Medical x-ray film, Christie Group Ltd., Mississauga, ON).

2.5.4 Densitometric Analysis

Densitometric analysis for Western blotting was performed on Adobe Photoshop CS2. Relative band intensity was derived from the product of mean intensity and pixel area. Each measurement was taken in triplicate and averaged. Values were normalized to the background intensity of the gel as well as to γ-tubulin, the loading control. A ratio of phospho-protein to pan protein was also calculated and expressed as a percentage relative to the nulliparous control.

<table>
<thead>
<tr>
<th>1° Antibody</th>
<th>Type</th>
<th>Source</th>
<th>Concentration</th>
<th>Blocking</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan ezrin</td>
<td>Polyclonal</td>
<td>Cell Signaling, Beverly, MA (3145)</td>
<td>WB: 1:1000</td>
<td>5% Milk</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pT567 ezrin</td>
<td>Monoclonal</td>
<td>Cell Signaling, Beverly, MA (3141)</td>
<td>WB: 1:1000</td>
<td>5% BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Pan Src</td>
<td>Monoclonal</td>
<td>Cell Signaling, Beverly, MA (2109)</td>
<td>WB: 1:1000</td>
<td>5% BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>pY418 Src</td>
<td>Polyclonal</td>
<td>Biosource of Invitrogen Corp., Camarillo, CA (44660G)</td>
<td>WB: 1:1000</td>
<td>5% BSA</td>
<td>Rabbit</td>
</tr>
<tr>
<td>Pan Met</td>
<td>Monoclonal</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-8057)</td>
<td>WB: 1:500</td>
<td>5% Milk</td>
<td>Mouse</td>
</tr>
</tbody>
</table>
Table 5. HRP-linked secondary antibodies used for Western blotting.

<table>
<thead>
<tr>
<th>2° Antibody</th>
<th>Source</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-goat HRP-linked</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA (sc-2020)</td>
<td>1:2500</td>
</tr>
<tr>
<td>Donkey anti-rabbit HRP-linked</td>
<td>GE Healthcare Ltd., Buckinghamshire, UK (NA934V)</td>
<td>1:2500</td>
</tr>
<tr>
<td>Sheep anti-mouse HRP-linked</td>
<td>GE Healthcare Ltd., Buckinghamshire, UK (NA931V)</td>
<td>1:2500</td>
</tr>
</tbody>
</table>

2.6 Human Tissue Microarray (TMA)

2.6.1 Selection Criteria, Strategy and Construction

Paraffin embedded formalin fixed breast cancer tissue, between 2005 and 2007, were retrieved by Dr. Jerry Chen from the Kingston General Hospital archives. Tissue accrued was based on a novel methodological practice where selection was based on several patient criteria as opposed to random tissue selection without defining an investigative patient subpopulation.

Tissues were taken from premenopausal women that were 49 years of age or younger at the time of diagnosis, presenting with primary infiltrating ductal or lobular carcinomas and staged within T1-3a, N0-1 and M0, indicators of tumour grade, nodal status and metastases. Patients were excluded if they had any cancer history, except for non-melanoma skin or in situ cervix, excluding the BRCA genotypes, bilateral breast cancer or had histological breast cancer types other than ductal or lobular carcinomas. Patients were also excluded if they were treated with neoadjuvant chemotherapy.

Since tissue specimens are extremely valuable, we chose to construct several smaller TMAs that will serve as pilots for a larger TMA (Figure 9). Triplicate cores of 0.6 cm diameter
were extracted from the tissue and were used to construct two TMAs in the Queen’s laboratory of Molecular Pathology (QLMP). The first TMA is the Technical TMA which was used to optimize technical parameters, including staining conditions, optimal antibody dilutions and replicability between staining runs. The first TMA, the Technical TMA, consists of 8 breast tumours, 4 normal breast mammoplasties and 7 cell lines: SP1, T47D, SK-BR-3, MCF-7, MDA-MB 231 and its lung and bone seeking metastatic variants (see Appendix for sources and culture conditions). Cell lines were obtained from our lab. The second TMA is from the Patient Cohort Test TMA, which consists of 59 breast tumours and 20 breast mammoplasties, where the cohort of mammoplasties provided a reference for staining of tumour tissues. This TMA was used as a pilot to establish initial correlations between potential novel markers of interest and prognostic parameters. Within the cohort, histological parameters available included tumour type, size, histological grade, lymphovascular invasion status (26% positive), axillary lymph node status (34% positive), ER/PR (18% positive), HER-2/neu (18% positive) and p53 (17% positive). The QLMP is in the process of constructing a third TMA, the Clinical Outcome Cohort TMA which consists of 450 tumours that contain at least 5 year clinical data. The clinical outcome data provided will aid in examining established associations in the Patient Cohort Test TMA with patient outcome. This project was approved by the Queen’s University Health Sciences and Affiliated Teaching Hospital’s Research Ethics Board.

2.6.2 Preparation of Cell Culture Blocks

Cell culture blocks were made as controls for the test TMA. Cells, mentioned in 2.6.1, were harvested and centrifuged for 10 minutes at 500x g. The supernatant was removed and the pellet was resuspended in PBS. The resuspension was centrifuged again and the pellet was suspended in 10% Neutral buffered formalin. Cells were incubated at 4°C for 2-3 hrs and centrifuged.
Since tissue specimens are extremely valuable, we chose to construct several smaller TMAs that will serve as pilots for a larger TMA. The first TMA is the Technical TMA which was used to optimize technical parameters, including staining conditions, optimal antibody dilutions and replicability between staining runs. The first TMA, the Technical TMA, consists of 8 breast tumours, 4 breast mammoplasties and 7 cell lines: SP1, T47D, SK-BR-3, MCF-7, MDA-MB 231 and the lung and bone seeking metastatic variants of MDA-MB-231. The second TMA is the Patient Cohort Test TMA, which consists of 59 breast tumours and 20 breast mammoplasties, where the cohort of mammoplasties provided a reference for staining of tumour tissues. This TMA was used as a pilot to establish initial correlations between potential novel markers of interest and prognostic parameters. QLMP is in the process of constructing a third TMA, the Clinical Outcome Cohort TMA which consists of 450 tumours that contain at least 5 year clinical data. The clinical outcome data provided will aid in examining established associations in the Patient Cohort Test TMA with patient outcome.
**Patient Criteria**

**Inclusion criteria:**
- Premenopausal (<49 of age at diagnosis)
- Primary infiltrating ductal or lobular carcinomas
- Stage: T1-3a, N0-1, M0

**Exclusion criteria:**
- Any other cancer history as well as mutated BRCA1 genotypes
- Bilateral breast cancers
- Histologic types other than ductal or lobular
- Treatment with neoadjuvant chemotherapy

**Tumour profiles:**
- Lymphatic vascular invasion (26%)
- Axillary lymph node status (34% +ve)
- ER/PR (18% +ve)
- HER-2/neu (18% +ve)
- p53 (17% +ve)
After removing the supernatant and resuspending the pellet, cells were transferred into a 1mL eppendorf tube and centrifuged for 10 minutes at 500x g. After removing the supernatant, 1% low melting point (LMP) agarose in PBS was added to the cell pellet. The agarose-cell suspension was allowed to harden for 15 minutes and incubated with 10% neutral buffered formalin overnight at 4°C. Cell plugs were then paraffin-embedded in the Queen’s Laboratory for Molecular Pathology.

2.7 Scoring and Statistical Analysis

2.7.1 Mouse

Hematoxylin and eosin staining was used for visual assessment of histologic grade. Tumour cells typically exhibited a spindle-like elongated morphology, with an increased number of mitotic figures for a given area. Tumour nuclei tended to have large, prominent nucleoli, thus resulting in a darker appearance.

IHC staining was analyzed based on protein localization of at the cellular level; whether staining was membranous (diffuse or apical), cytoplasmic or nuclear. In addition, the distribution of the staining in the regions of the tumour and/or surrounding tissues (peripheral, perivascular etc.) was observed. The percentage of positive cells and the intensity of the stain categorized as negative, weak, moderate, and strong, were also recorded. Regions of necrosis were assessed visually and omitted from the analysis. Local invasion was detected at the gross pathological level through adhesion or invasion primarily into the adjacent abdominal muscle wall. At the histological level, tumours were classified as non-invasive if a clear circumscribed border, between the tumour and the stroma, was observed. Tumours that are invasive will often display invasive wisps that have migrated into the surrounding stroma, abdominal wall or draining lymph node. Metastases were detected grossly as discrete hard, white nodules within the tissue, typically from the lung and visceral organs via coelomic spread. Lymphovascular invasion (LVI) was considered positive if tumour cells were observed within a blood vessel that possessed a
complete endothelial lining. Vessels located at the periphery of the tumour were assessed since intratumoral vessels often have abnormal drainage systems that have an unlikely chance of leading to metastases. LVI was assessed as a categorical factor and values were compared between groups using a non-parametric Fisher’s Exact test (224).

2.7.2 Human Tissue Microarray

Scoring was carried out by two Pathology residents, Dr. Sonal Varma and Dr. Ashish Rajput. Discrepant cases were resolved by senior Staff Pathologist, Dr. Sandip SenGupta. Scoring criteria was based on the percentage of positive cells (0-100%), staining intensity on a scale of 0 to 3 and the distribution of staining; whether staining was nuclear, cytoplasmic, membranous or apical. Based on the product of percent positive cells and staining intensity, an average H-score, ranging from 0 to 300 was obtained. Pearson/Spearman correlation coefficients were used to establish associations between continuous markers of interest and continuous clinical factors (tumour size, Scarff-Bloom-Richardson score) while ANOVA was used for continuous marker and categorical clinical factors (TNM stage, histologic grade) and Fisher’s Exact tests were used for 2 categorical factors. In previous correlative work with the NCIC CTG MA.22 study, 50 patients were sufficient to indicate significant associations between investigative factors (personal communication with Dr. Judy-Anne Chapman). Thus, the 59 tumour TMA should have sufficient power for exploratory investigations between new and established factors through Pearson/Spearman correlation tests. Statistical analysis was carried out by Dr. Judy-Anne Chapman and Alex Perry (NCIC Clinical Trials Group).

2.8 In vivo Protocols

2.8.1 Mice used for intramammary engraftment of tumour cell lines

Athymic female nude mice (Tac: NCRNU-M, Taconic Farms) were used for all studies involving intramammary engraftment of tumour cell lines. This mouse strain originated from the
cross between a BALB/c inbred nude and a NIH(S) outbred nude mice. Deficiency in T cell function, due to abnormal thymus development, allows for the xenografting of a broad range of normal and malignant tissues and cell lines. This engraftment method allows easy evaluation of tumour growth, local invasion and metastasis.

2.8.2 Tumour engraftment into the mammary fat pad

Mice to be injected were given 0.6 mL of Avertin intraperitoneally. To ensure mice reached the surgical plane of anesthesia, stimulation of foot pads and rate of breathing were observed before surgery. Surgery proceeded when foot pads ceased twitching upon stimulation and when breathing was slow and deep. Tear gel was applied to the eyes to prevent drying and blindness. A small incision above the right abdominal mammary gland was made. 7.5 x 10^3 AC2M2 cells, suspended in 10µL of PBS, were injected into the mammary fat pad of each mouse with a 100µL Hamilton syringe. The wound was closed with two surgical clips which were removed 9 days later. 0.1 mL of Buprenorphine and 0.5 mL of Lactated Ringer’s solution was administered subcutaneously for pain management and fluid resuscitation, respectively. Buprenorphine was administered every 8-10 hours over a period of two days to alleviate pain. The protocol for all animal experimentation was approved by the Queen’s University Animal Care Committee.

2.8.3 Measurement of primary tumour growth and assessment of metastasis

Primary tumour growth was monitored and measured every 2-3 days. Two perpendicular dimensions of the tumour were measured using callipers and tumour volume was calculated using the following formula: (a)(b^2)/2; where ‘a’ was the larger of the two measurements. Primary tumour volumes, up to 21 days, were averaged in each group and plotted. Tumour growth rate was approximated by the slope of the growth curve. Statistical significance between groups was determined using Student's t-test in Microsoft Excel.
For metastasis studies, primary tumours were resected at 21 days, and mice were allowed to survive post-surgery. It has been reported that resection of tumours alleviates factors that suppress metastases and has been shown to promote metastatic growth (227). This process is also representative of the management of clinical breast cancers in which the primary tumour is excised before treatment. Thus, primary tumours were resected after 21 days. Animals were sacrificed and autopsies were performed after 40 days. During the autopsy, local invasion and metastasis into viscera, liver and draining and axillary lymph nodes, diaphragm, visible vertebrae, rib cage and lung were assessed. Samples of primary tumour including surrounding margins of normal tissue as well as various organs were fixed in 4% paraformaldehyde, followed by paraffin embedding. Parallel fresh samples were snap frozen in liquid nitrogen for biochemical studies. Hematoxylin and eosin staining was performed to assess primary tumour morphology and the presence of local invasion and metastases. Invasion was assessed as a categorical factor and a non-parametric Fisher’s Exact test was used to determine statistical significance between groups.

2.9 Light Microscopy and Image acquisition

All histological slides were viewed under the Nikon Eclipse E600 and pictures were acquired with the Nikon Digital camera DXM1200 using Nikon ACT-1 software. Images were brightened and contrasted using Adobe Photoshop CS2 software, using regions of normal tissue as a reference point. Similar fields of serial sections were imaged in order to compare staining patterns of different antibodies within the same tissue region and to minimize the effects of tissue heterogeneity.
CHAPTER 3—RESULTS

3.1 Evaluation of Src/ezrin expression and activation as a marker of tumour grade in human breast cancer

As a first step to evaluate the Src/ezrin signalling network in human breast cancer progression, the expression and localization patterns of Src/ezrin were validated in normal and neoplastic murine mammary glands. A murine Polyoma middle T (PyMT) viral model which exhibits a clear morphological progression from premalignant to malignant state was used. The essential involvement of Src in the induction of PyMT mammary tumours, as well as the similarity of PyMT tumours with receptor tyrosine kinase-driven tumourigenesis, provides a model that simulates our proposed Src/ezrin signalling network (cf. Material and Methods Section 2.3.1). Expression of biomarkers, such as loss of ER, PR and constitutive expression of HER-2, in PyMT tumours has also been found to be consistent with those associated with poor outcome in human breast cancer, thereby providing a practical model for linking our findings to human cancer (224) (cf. Material and Methods Section 2.3.1). Preliminary evaluation of Src/ezrin at different stages of normal mammary development, including tissues from nulliparous (non-pregnant), parous and lactating mice, was performed to validate biomarker specificity and expression profiles, as well as to assess differences in protein expression in a controlled model of breast tumourigenesis. Validated biomarkers were then used to assess Src/ezrin in a cohort of human breast cancer tissues.

3.1.1 Validation of antibodies for Immunohistochemistry

Since statistical associations of biomarkers with clinical-pathological parameters are heavily dependent on the reproducibility and specificity of the immunohistochemical staining, the need for well-validated probes to provide confidence in the observations is essential. Several levels of controls were utilized. First, ‘no-primary antibody’ control was used to control for non-specific secondary antibody binding. Second, non-immune rabbit immunoglobulin was used to
control for non-specific staining via the Fc portion of the primary antibody (Figure 10). Third, a blocking peptide consisting of the peptide used for immunization was utilized to confirm the epitope specificity of the primary antibody (cf. Materials and Methods Section 2.4.1). The specificities of the antibodies used in the present study are illustrated in Figures 10-12. Normal tissues or cell lines with known expression patterns of the proteins of interest were chosen as positive controls. For ezrin staining, mouse intestinal mucosa was used as a positive control, whereas human tonsil was used for Src. Apical staining against pT567 ezrin was inhibited with its respective blocking peptide, but not with the corresponding non-phosphorylated ezrin peptide (Figure 11). Likewise, staining of human tonsil with pan Src and pY419 Src antibodies was inhibited with Src and pY419 Src blocking peptides, respectively (Figure 12).

3.1.2 Differential localization of Ezrin and Src in nulliparous, lactating mammary glands and PyMT-induced tumours

As a cytoskeletal plasma membrane cross-linker, ezrin is typically localized to the apical membrane of differentiated glandular epithelial cells (45). On the other hand, several groups have shown using panels of various carcinoma cell lines and breast tumour tissues, that ezrin is localized predominantly in the cytoplasm in malignant cells. Furthermore, previous work in our lab showed that activated Src disrupted ezrin localization to the apical membrane (162). In order to validate this observation in the context of our cancer progression model, we examined the localization patterns of ezrin and Src in nulliparous, lactating mammary glands and PyMT-induced tumours by immunohistochemistry. No-primary antibody and non-immune rabbit immunoglobulin (IgG) were used as negative controls while mouse small intestine was used as a positive control (Figure 10). In nulliparous and lactating tissue, total/pan ezrin was localized to the apical region of the mammary luminal epithelium (Figure 13). In contrast, pan ezrin staining of PyMT-induced tumours exhibited intense, diffuse atypical cytoplasmic staining (Figure 13). The localization pattern of pT567 ezrin, which represents the open, active form of ezrin, an
epitope shared by the homologous phosphorylated threonine residue on moesin and radixin, was also determined. In both nulliparous and lactating mammary glands, epithelial apical localization of pT567 ezrin was similar to that of pan ezrin. However, in the PyMT tumour model, pTezrin exhibited heterogeneous staining, but remained localized to the plasma membrane in PyMT cells at the periphery of the tumour (Figure 14).

Src is ubiquitously expressed, and plays multiple roles in normal and malignant development as mentioned in the Introduction (Section 1.3.3). Src is predominantly associated with the plasma membrane through its myristoylation site (119). During transformation, active (pY419) Src migrates to focal adhesions and filopodia extensions (228). A possible nuclear role of Src as a cell cycle regulator has also been described (120). Active Src recycles via vesicular transport through endosomes and functions in protein trafficking (139). In the present study, both pan Src and active Src were found to localize in the cytoplasm of nulliparous mammary glands, while in lactating glands both pan and active Src localized predominantly in the apical epithelia. In PyMT tumours, both pan and active Src exhibited an intense diffuse cytoplasmic staining pattern throughout the tumour. Nuclear staining was also evident in both pan and pY419 Src (Figure 15 & 16).

3.1.3 Increased expression and activation of Ezrin and Src in PyMT-induced mammary tumours compared to normal or lactating mammary glands

In addition to exhibiting changes in cellular localization, Src and ezrin are frequently over-expressed or over-activated in invasive breast cancer. To determine the level of expression and activation of Src and ezrin within the PyMT model, western blotting of snap frozen tumour lysates was used. There was a marked increase in expression of both pan Src and pan ezrin in lactating compared to nulliparous breast tissues. During pregnancy, extensive proliferation and side branching of the mammary ductal tree occurs. Mammary gland growth continues from early stage to the peak of lactation (229). Thus, it is possible that the elevated levels of Src and ezrin
seen within the lactating stage reflect the proliferative and differentiated nature of the lactating tissue. Interestingly, PyMT tumours exhibited elevated expression of both pan ezrin and pan Src compared to normal tissue counterparts. Concomitant elevated phosphorylated forms of Src (pY419) and ezrin (pT567) were detected in three PyMT tumours compared to basal levels in nulliparous (control) and lactating tissues (Figure 17 & 18).

3.1.4 Increased expression of HGF and Met in PyMT-induced mammary tumours compared to nulliparous breast tissue

The Arpin and Elliott labs have previously shown that active Src phosphorylates ezrin at specific tyrosine residues, thereby regulating various Src-induced EMT functions (91, 93). Furthermore, the Elliott lab observed that Src and ezrin act co-operatively to increase phosphorylation of specific tyrosine residues in both the common docking site (Y1356) and the auto-activation loop of the kinase domain (Y1234, Y1235) of Met (162). The Elliott lab has also shown that sustained Src-dependent activation of Stat3, which occurs in invasive breast cancer, can stimulate HGF transcription, hence protein expression in several carcinoma cell lines (230). Normally HGF is secreted by mesenchymal cells and activates Met in a paracrine manner. However during tumourigenesis, Src may stimulate Stat3-dependent HGF expression and establishment of an HGF/Met autocrine loop (215, 230) (*cf.* Introduction Section 1.4).

As a first step to assess the above model, we determined whether increased HGF expression and Stat3/Met activation occurs during PyMT-induced tumourigenesis. Tumour samples, along with control breast tissue of the same mouse strain, were homogenized and expression was determined by western blotting. Antibodies against pan Met, the phosphorylated tyrosines in the activation loop of the Met kinase domain (Y1234 and Y1235, hereafter referred to as pY Met), pan Stat3, and pY705 Stat3, the active form of Stat, were used. γ-tubulin was used as a normalization control. An increase in total HGF, Met and Stat3 proteins was observed in the tumour compared to the control breast tissues (Figure 19).
Figure 10. Validation of nonspecific binding through isotype matched negative control antibodies in normal mouse breast and Polyoma virus Middle T-induced tumour tissue.

Nulliparous (A-B), lactating (C-D) and Polyoma viral-induced mammary glands (E-F) were stained by immunohistochemistry (IHC) as described in Materials and Methods. An immunoglobulin class matched negative control antibody was used to evaluate nonspecific binding. Photographs were taken at 40x and 100x objective, and scale bars are 50 and 20 µm respectively. Results are representative of at least 2 experiments.
IgG control

Nulliparous

A

40x

B

100x

Lactating

C

D

PyMT

E

F
Figure 11. Validation of pT567 ezrin antibody specificity by pT567 ezrin peptide block.

IHC against pT567 ezrin was performed on intestinal mucosa (A), human mammary glands (D), and a human mammary carcinoma cell line, MDA-MB-231 (F). Apical membranous staining was observed (arrows). On serial sections, IHC against pT567 ezrin with the respective blocking peptide was performed, shown in B, E and G. In addition, IHC was performed against pT567 ezrin with a control (non-phosphorylated) peptide as described in Materials and Methods, shown in C. A proportion of cells exhibited membranous pT567 ezrin staining in the control (F) but was absent following treatment with blocking peptide (G) Photographs were taken at 40x objective and scale bars indicate 50 µm. Results are representative of at least 2 experiments.
pT567 ezrin

A

No blocking peptide

B

Blocking peptide

C

Control peptide

D

No blocking peptide

E

Blocking peptide

F

No blocking peptide

G

Blocking peptide
Figure 12. Validation of pan Src and pY419 Src antibodies by pan Src and pY419 Src peptide block.

IHC against pan Src was performed in human tonsil (A), human mammary glands (B), and human invasive ductal carcinoma tissue (C), as described in Materials and Methods. On serial sections, IHC against pan Src with corresponding blocking peptide was performed (E-G). Likewise, IHC against pY419 Src was performed on human tonsil, along with the corresponding blocking peptide, shown in D and H, respectively. Photographs were taken at 40x objective. Scale bars indicate 50 µm. Results are representative of at least 2 experiments.
Pan Src

Pan Src + peptide block

A
Tonsil

B
Normal Breast

C
Breast Carcinoma

D
Tonsil

E

F

G

H

Figure 13. Immunohistochemical staining of ezrin in nulliparous and lactating mammary glands; and PyMT-induced tumour tissues.

IHC against pan ezrin was performed on nulliparous (A-B) and lactating mammary glands (C-D); and PyMT-induced mammary tumours (E-F). Photographs were taken at 40x and 100x objective, and scale bars are 50 and 20 μm respectively. Staining is described as membranous, cytoplasmic or atypical cytoplasmic (See Materials and Methods) (arrows). Results are representative of at least 2 experiments.
Pan ezrin

**Nulliparous**

40x

**Lactating**

Membranous

Apical

**PyMT**

Intense, diffuse atypical cytoplasm
Figure 14. Immunohistochemical staining of pT567 ezrin in nulliparous and lactating mammary glands; and PyMT-induced mammary tumour tissues.

IHC against pT567 ezrin was performed on nulliparous (A-B) and lactating mammary glands (C-D); and PyMT-induced mammary tumours (E-F). Photographs were taken at 40x and 100x objective, and scale bars are 50 and 20 µm respectively. Staining is described as in Fig. 4 (see arrows). Results are representative of at least 2 experiments.
pT567 ezrin

A  40x  Nulliparous

B  100x  Membranous

C  Lactating

D  Apical

E  PyMT

F  Heterogenous membranous
Figure 15. Immunohistochemical staining of pan Src in nulliparous and lactating breast and PyMT-induced mammary tumour tissues.

IHC against pan Src was performed on nulliparous (A-B) and lactating mammary glands (C-D); and PyMT-induced mammary tumours (E-F). Photographs were taken at 40x and 100x objective, and scale bars are 50 and 20 μm respectively. Staining is described as in Fig. 4 (see arrows). Results are representative of at least 2 experiments.
Pan Src

A 40x

Nulliparous

C

Lactating

E PyMT

Membranous

Cytoplasmic

Diffuse cytoplasmic
Figure 16. Immunohistochemical staining of pY419 Src in nulliparous and lactating breast; and PyMT-induced mammary tumour tissues.

IHC against pY419 Src was performed on nulliparous (A-B) and lactating mammary glands; (C-D) and PyMT-induced mammary tumours (E-F). Photographs were taken at 40x and 100x objective, and scale bars are 50 and 20 µm respectively. Staining is described as in Figure 13. Results are representative of at least 2 experiments.
pY419 Src

Nulliparous

A

B

C

D

Lactating

Cytoplasmic

Cytoplasmic

PyMT

Nuclear; Diffuse cytoplasmic
Figure 17. Expression and activation levels of ezrin in neoplastic compared to normal and lactating mammary glands.

Fresh frozen nulliparous, lactating mammary glands and PyMT-induced tumours were homogenized and lysed in RIPA buffer. Equal protein concentrations were then subjected to SDS-PAGE under reducing conditions on an 8% polyacrylamide gel. Western blotting was performed with antibodies against pan ezrin and pT567 ezrin. γ-tubulin served as a loading control (Panel A). Densitometric analysis for western blotting, as described in Material and Methods, was performed using Adobe Photoshop and represents the product of band intensity (mean) and area (pixels). All values were normalized to the background intensity of the gel. Results are representative of 2 experiments.
Nulliparous control

PyMT tumour #1

PyMT tumour #2

PyMT tumour #3

kDa

-81

-81

-48

pT567 ezrin

pan Ezrin

γ-tubulin

Relative Band intensity

pan Ezrin/tubulin

Control

PyMT #1

PyMT #2

PyMT #3
Figure 18. Expression and activation levels of Src in neoplastic compared to normal and lactating mammary glands.

Fresh frozen nulliparous, lactating mammary glands and PyMT-induced tumours were homogenized and lysed in RIPA buffer. Equal protein concentrations were then subjected to SDS-PAGE under reducing conditions on an 8% polyacrylamide gel. Western blotting was performed with antibodies against pan Src and pY419 Src. γ-tubulin served as a loading control (Panel A). Densitometric analysis was performed as described in Fig. 8 and Material and Methods. Results are representative of 2 experiments.
Nulliparous  Lactating  Nulliparous control  PyMT tumour #1  PyMT tumour #2  PyMT tumour #3

**pY419 Src**

**pan Src**

**γ-tubulin**

---

**Nulliparous control**

**PyMT tumour #1**

**PyMT tumour #2**

**PyMT tumour #3**

---

**Relative Band Intensity**

- Control
- PyMT #1
- PyMT #2
- PyMT #3

---

**pan Src/tubulin**

**Relative Band Intensity**

- 0
- 0.5
- 1
- 1.5
- 2
- 2.5
- 3
- 3.5
- 4
Figure 19. Expression levels of pan and pY1234/1235 Met; pan and pY705 Stat3; pro- and α− HGF in PyMT induced tumours compared to nulliparous and lactating breast tissue.

Fresh frozen nulliparous mammary gland and PyMT-induced tumours were homogenized and lysed in RIPA buffer. Equal protein concentrations were then subjected to SDS-PAGE under reducing conditions on an 8% polyacrylamide gel. Western blotting was performed with antibodies against pan and pY1234/1235 Met; pan and pY705 Stat3; pro- and α-HGF. γ-tubulin served as a loading control (Panel A). The 145 kDa band corresponds to the β chain of Met. The upper band (~190 kDa) most likely corresponds to the uncleaved precursor Met protein. Densitometric analysis for western blotting was performed as described in Fig. 8 and Material and Methods. Results are representative of 2 experiments.
A marked increase in pY705 Stat3 correlated with increased expression of pro-HGF (92 kDa precursor chain), both of which were absent in control tissues. A band corresponding to the 69 kDa α chain of HGF (proteolytic cleavage product) was present in control breast tissue, and increased in PyMT tumour lysates (Figure 19). Increased pY705 Stat3 also correlated with increased pYMет expression. However, due to the low level of corresponding pan proteins in control tissues, the ratio of phosphorylated/total Stat3 and Met proteins could not be evaluated. Taken together, these results are consistent with the activation of an autocrine HGF/Met loop during PyMT-induced mammary tumourigenesis.

3.2 Differential localization of ezrin expression in normal and neoplastic human breast tissues

The next phase of this study was to relate our findings from the PyMT mouse tumour model to a clinical context through assessing Src/ezrin localization or activation status in human normal and neoplastic breast tissues. Previous reports regarding ezrin localization in tumour progression have been controversial. One study showed that basal lateral membranous expression of ezrin within invasive cellular processes is associated with shorter disease-free survival in pancreatic cancer (231). Another study showed that membranous ezrin expression in breast cancer is associated with lymph node positivity (109). However, cytoplasmic ezrin expression has also been correlated with invasive breast cancer (108, 109). To address this controversy, we assessed Src/ezrin expression and localization in a human breast TMA consisting of a cohort of primary invasive breast cancers from premenopausal women (n=59), and 20 normal breast mammoplasties. The process of optimizing the TMA, both technically and analytically, is still ongoing and is critical for the success in subsequent studies of the larger TMAs.

Immunohistochemical staining for Src/ezrin was performed on a human breast TMA, and H-scoring by two pathologists was carried out as described in Materials and Methods. Results revealed Src and ezrin expression patterns similar to that of the PyMT transgenic mouse model
Apical expression of pan ezrin, pT567 ezrin and pan Src was observed in breast lobule units from normal mammoplasties (Figure 20). Statistical analysis of ezrin expression in the breast TMA showed a significantly greater cytoplasmic localization of pan ezrin within invasive breast tumour cases compared to the normal mammoplasties. The majority (89%) of breast carcinomas showed strong cytoplasmic ezrin staining, compared to normal breast lobule units which did not (Pearson/Spearman correlation coefficients, p = 0.003) (Figure 21). In addition, a subset of breast cancer cases (10%) showed strong membranous staining of pT567 ezrin. pY419 Src showed both membranous and cytoplasmic staining in both normal and neoplastic breast tissues, but no significant difference in cytoplasmic pY419 Src was observed (Figure 21, Table 6). Correlations of pan Src and pT567 ezrin in normal versus tumour tissues are in progress.

Histologic grade and Scarff-Bloom Richardson (SBR) scores were obtained from patient charts for each tumour tissue sample. Pearson and Spearman correlations were performed and showed no significant association of pan ezrin or pYSrc with SBR score, nor did ANOVA with histologic grade (Table 7 & 8).

### 3.2.1 Tests of Replicability

In view of the known mechanism of Src/ezrin interaction, we tested the association of cytoplasmic pan ezrin with pY419Src in our human breast cancer cohort between two TMAs made from the same set of tissue blocks. When compared, TMA1 showed a significant association, while TMA2 did not (Pearson/Spearman correlation coefficients, p = 0.003 and 0.65, respectively) (Table 7). This prompted an assessment on the concordance and replicability between the two TMAs for pan ezrin and pYSrc: There were significant differences in pY419Src and pan ezrin scores between the two TMAs (Pearson/Spearman correlation coefficients, p=0.0002 and p=0.04, respectively). In contrast, no evidence of difference in p53 staining between the two test TMAs (Pearson/Spearman correlation coefficients, p=0.79) was observed.
Figure 20. Expression and localization of ezrin and Src in invasive breast carcinoma compared to normal human mammoplasty.

Normal human mammoplasties and invasive ductal carcinoma were taken from a patient cohort, the criteria for which are listed in Materials and Methods. Normal mammoplasties (A-D) and invasive breast carcinoma (E-H) tissue sections were stained using immunohistochemistry for pan ezrin (A, E); pT567 ezrin (B, F); pan Src (C, G) and pY419Src (D, H). Photographs were taken at 100x objective, and scale bars indicate 20 µm. Results are representative of at least 2 experiments.
Normal human mammoplasty

A. pan ezrin
B. pT567 ezrin
C. pan Src
D. pY418 Src

Invasive breast carcinoma

E. Cytoplasmic (weak membranous)
F. Membranous
G. Membranous (weak cytoplasmic)
H. Cytoplasmic (weak membranous)
Figure 21. Tests of association for cytoplasmic pan ezrin or pY419 Src staining with neoplastic versus normal human breast tissue.

Human mammoplasty (n=20) and invasive breast tumour (n=59) cores were stained for pan ezrin and pY419 Src with immunohistochemistry. Average H-scores are derived from the product of percent positive cells and staining intensity. Pearson/Spearman correlation coefficients were used to determine statistical significance of cytoplasmic pan ezrin (A) or cytoplasmic pY419 Src (B) between neoplastic versus normal breast tissues.

Table 6. Cytoplasmic H-scores of pan ezrin and pY419 Src

Table indicates number of tissue sections scored, mean H-scores along with the standard error of mean (s.e.m). * indicates statistical significance by Pearson/Spearman correlation coefficients.
Table 6. Cytoplasmic H-scores of pan ezrin and pY419 Src

<table>
<thead>
<tr>
<th>Marker</th>
<th>Tissue type</th>
<th>N</th>
<th>Mean H-score (s.e.m)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan Ezrin</td>
<td>Tumour</td>
<td>54</td>
<td>110.68 (10.19)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mammoplasty</td>
<td>13</td>
<td>56.54 (13.17)</td>
<td>0.003*</td>
</tr>
<tr>
<td>pY419 Src</td>
<td>Tumour</td>
<td>53</td>
<td>74.69 (8.85)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mammoplasty</td>
<td>11</td>
<td>63.64 (20.33)</td>
<td>0.61</td>
</tr>
</tbody>
</table>

* Significant by Pearson/Spearman coefficients
Table 7. Test of association between pan ezrin or pYSrc with SBR score.

Statistical analysis using Pearson and Spearman correlation coefficients was performed to evaluate association of pan ezrin or pY419Src with Scarff-Bloom Richardson (SBR) score. N indicates number of tissue samples.

Table 8. Test of association between pan ezrin or pYSrc with Histologic Grade
Likewise, Pearson and Spearman correlation coefficients were used to evaluate association between pan ezrin or pYSrc with histologic grade. N indicates number of tissue samples.
### Table 7. Test of association between PanEzrin or pYSrc with SBR score

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Pearson Correlation</th>
<th>P-value</th>
<th>Spearman Correlation</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan Ezrin with pYSrc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMA 1</td>
<td>53</td>
<td>0.44</td>
<td>0.001</td>
<td>0.43</td>
<td>0.001*</td>
</tr>
<tr>
<td>TMA 2</td>
<td>52</td>
<td>0.04</td>
<td>0.76</td>
<td>0.06</td>
<td>0.65</td>
</tr>
<tr>
<td>Pan Ezrin with SBR score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMA 1</td>
<td>54</td>
<td>-0.15</td>
<td>0.29</td>
<td>-0.08</td>
<td>0.55</td>
</tr>
<tr>
<td>TMA 2</td>
<td>53</td>
<td>0.10</td>
<td>0.50</td>
<td>0.06</td>
<td>0.65</td>
</tr>
<tr>
<td>pYSrc with SBR score</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMA 1</td>
<td>53</td>
<td>-0.07</td>
<td>0.62</td>
<td>-0.03</td>
<td>0.85</td>
</tr>
<tr>
<td>TMA 2</td>
<td>52</td>
<td>-0.09</td>
<td>0.52</td>
<td>-0.14</td>
<td>0.31</td>
</tr>
</tbody>
</table>

* significant by Pearson or Spearman correlation coefficients

### Table 8. Test of association between PanEzrin or pYSrc with Histologic Grade

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Pan Ezrin H-score (s.e.m)</th>
<th>P-value</th>
<th>N</th>
<th>pYSrc H-score (s.e.m)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologic Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>6</td>
<td>88.89 (18.59)</td>
<td>0.62</td>
<td>5</td>
<td>83.33 (35.75)</td>
<td>0.76</td>
</tr>
<tr>
<td>Grade 2</td>
<td>20</td>
<td>102.33 (15.78)</td>
<td></td>
<td>19</td>
<td>83.33 (14.42)</td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>27</td>
<td>117.78 (15.81)</td>
<td>0.62</td>
<td>28</td>
<td>69.94 (12.14)</td>
<td>0.76</td>
</tr>
</tbody>
</table>

* significant by Pearson or Spearman correlation coefficients
3.3 Characterization of the Y477F ezrin phenotype using a mouse tumour xenograft model

In Results Section 3.1, both Src and ezrin showed increased expression and activation during PyMT-induced breast tumourigenesis. In addition, Y477 ezrin is a key phosphorylation site for Src, and is required for HGF-induced cell spreading and scattering (93). Functions associated with Y477 are unique to ezrin as this residue is absent in both radixin and moesin. We therefore investigated the role of Y477 ezrin in tumourigenesis and metastasis in a mouse breast carcinoma engraftment model.

3.3.1 Expression of Y477F ezrin mutant in AC2M2 cells

To assess the role of phosphorylation at Y477 ezrin in breast tumours, a VSVG-tagged Y477F ezrin mutant, which cannot be phosphorylated by Src, was expressed in AC2M2 breast carcinoma cells using a pCB6 bacterial-based vector system. Polyclonal cells were selected for G418 resistance, subjected to single cell cloning and individual clones were screened for VSVG-ezrin. Two independent clones expressing VSVG-Y477F ezrin, A43 and C13, were derived as shown in Figure 22. Quantitative analysis by 2-fold serial dilutions of corresponding cell lysates by Western blotting against total ezrin revealed an approximately 2- and 4-fold increase of ezrin levels in the A43 and C13 clones, respectively (Figure 22). In addition, wound healing assays showed a delayed scattering and spreading of the Y477F (A43 and C13) compared to the pCB6 control clones (Appendix 1). Furthermore when AC2M2 cells were cultured on 20% Matrigel, cells expressing Y477F ezrin (A43 and C13) formed cohesive aggregates compared to pCB6 cells which formed loose aggregates with branching extensions invading into surrounding Matrigel (Appendix 2).
Figure 22. Western blotting analysis of VSVG ezrin mutant expression compared to endogenous ezrin in AC2M2 clones.

AC2M2 cells were previously transfected with an empty pCB6 vector, with a Y477F ezrin mutant. A pCB6 clone (#2) and two Y477F clones (A43 and C13) were lysed in 2x SDS sample buffer, and equal protein amounts were subjected to SDS-PAGE and Western blotting with antibodies against VSVG (A) and pan ezrin (B). In panel A, the band at ~81 kDa represents Y477F ezrin tagged with VSVG (A43 and C13). In panel B, the band at ~81 kDa in pCB6 represents endogenous ezrin, while the bands in A43 and C13 represent Y477F mutant + endogenous ezrin. γ-tubulin served as a loading control.

(C) Semiquantitation of exogenous versus endogenous ezrin expression was determined by western blotting as a 2-fold serial dilution of total cell lysates (1.25–20 μg), as described in Materials and Methods. Western blotting was performed with an antibody against pan ezrin. A near absence of the pan ezrin band in the pCB6 lane in the 2.5μg samples loaded compared to the A43 and C13 ezrin bands indicates a 2-4 fold increase in ezrin mutant compared to endogenous ezrin expression.
3.3.2 The AC2M2 Mouse tumour engraftment model

The Elliott lab has previously developed the mouse carcinoma cell line, AC2M2, which is highly metastatic upon engraftment into the mammary glands of immunodeficient (Tac: NCRNU-M) athymic nu-/- mice (221). As previously reported, overt lung metastases were seen in >90% of mice injected with parental tumour cells alone, or expressing pCB6 control vector and sacrificed at least 30 days after engraftment (111). In the present study to investigate the Y477F ezrin phenotype in an \textit{in vivo} tumour engraftment model, AC2M2 clones expressing the pCB6 empty vector (control) or the Y477F ezrin mutant (Figure 23) were engrafted into the mammary gland of athymic nude mice (7.5x10^3 cells/mouse), and tumour outgrowth was monitored. Interestingly, no difference in the primary tumour growth rates between pCB6 control or Y477F ezrin expressing tumours were observed (Figure 24). After 21 days, tumours and surrounding stroma were surgically removed and animals were sacrificed 30-40 days post engraftment.

3.3.3 Y477F ezrin inhibits local invasion of primary tumour outgrowths

Local invasion of pCB6 control and Y477F ezrin AC2M2 tumour cells were characterized using gross and histo-pathological assessments of tumours excised at 21 days post injection. On gross pathological examination, the majority (11/12) of pCB6 control tumours were found to invade through the abdominal wall and underlying muscle, with tumour nodules occasionally visible in visceral organs (Figure 25, 26 & Table 9). Histopathological analysis of excised primary tumour tissues confirmed that the majority (>90%) of pCB6 tumours had invaded into underlying abdominal muscle. Lymphovascular invasion and seeding of tumour cells in the viscera, disseminating into various abdominal organs via peritoneal spread, was observed; such organs included the intestine, pancreas and spleen (Figure 26). In a rare instance, perineural invasion was seen in one of the pCB6 tumours.
Figure 23. Detection of Y477F ezrin by western blotting in xenograft tumours.

Fresh tumour tissues were removed at 21 days post engraftment with AC2M2 tumour cells either expressing mutated Y477F ezrin (A43 or C13) or an empty control vector (pCB6) (see Fig. 23). Tumour tissues were snap frozen in liquid nitrogen, and subsequently homogenized and lysed in RIPA buffer with protease inhibitors as described in Materials and Methods. Equal protein amounts were then subjected to SDS-PAGE under reducing conditions on an 8% polyacrylamide gel. Western blotting was performed against VSVG (A) or pan ezrin (B). A non-specific band migrating faster than the VSVG-ezrin band was present in all samples, and served as an internal loading control. M denotes mouse number. Results are representative of 2 separate SDS-PAGE analyses of the same cell lysates.
Figure 24. Effect of Y477F mutant on primary tumour growth.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude (Tac: NCRNU-M) mice (7.5 x 10³ cells per mouse). Tumours were monitored every two days, and palpable tumours were measured using Vernier callipers. Volume estimates were determined and plotted as a function of days post engraftment. The slopes, derived from the tumour growth curves of each mouse, were averaged and statistical significance was assessed using Student’s t-test. Tumours were surgically removed at 21 days post engraftment and cut into three pieces for histopathology and biochemistry as follows: i) formalin fixed paraffin embedded, ii) formalin fixed frozen, and iii) snap frozen fresh tissue. See Materials and Methods for details. Results are representative of two experiments.
Average tumor growth of AC2M2 Y477F ezrin clones

Mean Slope Values per Mouse Group

Student’s t-test:
(pCB6, A43): p=0.299
(pCB6, C13): p=0.799
Figure 25. Effect of Y477F ezrin mutant on local invasion of AC2M2 breast carcinoma cells.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice. Primary tumours were excised after 21 days and photographs of gross pathology (A-B) were taken. Tumour tissues were processed as described in Fig. 24, and 5 µm sections of FFPE tissues were stained with hematoxylin and eosin for histopathological analysis. Arrows indicate either invasion into underlying abdominal muscle wall (A, C) or circumscribed tumour margin (D). Invasion was assessed based on combined observations of gross and histopathology. Labels with “T” indicates tumour, “Mus” indicates muscle and “LN” indicates lymph node.

Table 9. Proportion of mice with local invasion of 1° tumour xenografts

Proportion of mice with local invasion relative to total number of mice per group is shown. P-values of two pooled experiments (Fisher’s Exact test) are shown.
Table 9. Proportion of mice with local invasion of 1° tumour xenografts

<table>
<thead>
<tr>
<th>Group</th>
<th>pCB6</th>
<th>A43</th>
<th>C13</th>
<th>A43+C13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. #1</td>
<td>6/6</td>
<td>2/6</td>
<td>1/6</td>
<td>3/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p=0.03*)</td>
<td>(p=0.008*)</td>
<td>(p=0.004*)</td>
</tr>
<tr>
<td>Exp. #2</td>
<td>5/6</td>
<td>1/6</td>
<td>2/6</td>
<td>3/12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p=0.04*)</td>
<td>(p=0.1)</td>
<td>(p=0.01*)</td>
</tr>
<tr>
<td>Combined</td>
<td>11/12</td>
<td></td>
<td></td>
<td>6/24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(p=0.0002*)</td>
</tr>
</tbody>
</table>

* Significant by Exact Fisher Test
Figure 26. Characterization of invasion and metastasis of pCB6 tumour xenografts.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice. Primary tumours were surgically excised after 21 days, and animals were allowed to survive for 40 days. Primary tumours, excised previously, and additional organs, which were removed at autopsy, were processed as described in Fig. 24. Paraffin embedded tissues were sectioned (5 μm), and stained with hematoxylin and eosin. Images show the diverse invasive and metastatic characteristics of the pCB6 control tumours, including: abdominal wall invasion (A), perineural invasion (B), intestinal invasion (C), lymphovascular invasion (D), pancreatic invasion (E), and peritoneal seeding on the splenic capsule (F) and lung (G). Labels with “T” indicates primary tumour, “Mus” indicates muscle and “V” indicates vessel wall.
pCB6-Abdominal wall invasion

pCB6-Splenic mesentery invasion

pCB6-Intestinal invasion

pCB6-Perineural invasion

pCB6-Lymphovascular invasion

pCB6-Pancreatic invasion

pCB6-Lung metastasis

Pancreas
Thus, extensive local invasion and seeding of visceral organs as well as distant metastasis to lungs was observed in the pCB6 AC2M2 tumour model. In contrast, only a few (6/24) of Y477F expressing tumours (total of two independent clones) showed invasive characteristics (Fisher’s Exact test, p=0.0002) (Figure 25). The majority of Y477F ezrin tumours remained circumscribed, with very little invasion into surrounding stroma and abdominal wall. These results indicate that Y477F ezrin inhibits local invasion and spreading into visceral organs of primary breast tumour outgrowths.

As previously reported in the AC2M2 carcinoma model, overt lung metastases were seen in >90% of mice with pCB6 control tumours at least 30 days after engraftment (111). However, differences in lung metastases were not observed in mice with Y477F ezrin tumours under similar conditions (data not shown). Since overt lung metastases are rarely detected within 21 days, the focus has largely been on local invasion during early tumour growth period. Further experiments are in progress to elucidate a possible role of Y477F ezrin in lung metastasis.

3.3.4 Y477F ezrin inhibits lymphovascular invasion (LVI) in primary AC2M2 breast tumours

One of the important prognostic indicators of relapse in clinically advanced human breast cancers is lymphovascular invasion (LVI), which involves dissemination of tumour cells via the lymphatics or the vasculature (232). LVI was therefore assessed in hematoxylin and eosin stained sections of pCB6 and Y477F tumours. LVI was evaluated based on the presence of tumour cells within the thin lymphatic channels surrounding the lymph node while vascular spread was based on the presence of tumour cells within a complete endothelial lined blood vessel (Figure 27, Table 10). Since intratumoural vessels are often structurally abnormal and leaky, they were not included in the analysis. Thus, only vessels that were located at the periphery of the tumour were evaluated.
Figure 27. Effect of Y477F ezrin on lymphovascular invasion (LVI) in primary AC2M2 breast tumours.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice (see Fig. 25). Primary tumours were excised after 21 days and histopathological analysis of hematoxylin and eosin stained sections was performed. LVI was defined as the presence of tumour cells in a well defined, endothelial-lined blood vessel. Confirmation was carried out by Dr. S. Varma and Dr. S. SenGupta. Arrows with “E” indicate endothelial cells while arrows with “T” indicate tumour cells within the vessel.

Table 10. Lymphovascular involvement in pCB6 and Y477F expressing tumours

Proportion of mice with detectable LVI is shown for each group. Combined numbers for two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) are also shown. Fisher’s Exact test was performed to determine statistical significance for the presence of lymphovascular involvement in pCB6 versus Y477F ezrin expressing tumours. * indicate statistical significance by Fisher’s Exact test.
Lymphovascular involvement (LVI)

Table 10. Lymphovascular involvement in pCB6 and Y477F expressing tumours

<table>
<thead>
<tr>
<th>Group</th>
<th># +ve mice</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCB6</td>
<td>5/6</td>
<td>-</td>
</tr>
<tr>
<td>A43</td>
<td>0/6</td>
<td>0/6 (p= 0.008)*</td>
</tr>
<tr>
<td>C13</td>
<td>0/6</td>
<td>0/6 (p= 0.008)*</td>
</tr>
<tr>
<td>A43 + C13</td>
<td>0/12</td>
<td>0/12 (p=.0007)*</td>
</tr>
</tbody>
</table>

* Significant by Exact Fisher Test
Evaluations were validated by a pathology resident, Dr. S. Varma and questionable cases reviewed by the senior pathologist, Dr. S. SenGupta. Using sections taken from pCB6 or Y477F tumours, peripheral vessels were assessed. Presence of at least one example of LVI constituted a positive tumour. LVI was detected in the majority (5/6) of pCB6 control tumours examined. No LVI was detected in tumours from two Y477F ezrin clones (Table 10). These findings indicate that Y477F ezrin expression inhibits LVI in primary tumour outgrowths (Fisher’s Exact test, p= 0.0007).

3.3.5 Y477F ezrin display more membranous ezrin in primary AC2M2 breast tumours

As we have observed, ezrin expression in normal breast epithelium is localized to the apical surface, while invasive breast tumours showed increased cytoplasmic ezrin localization (cf. Results Section 3.1.2). These findings prompted us to examine cytoplasmic versus membranous expression of ezrin in pCB6 control versus Y477F ezrin tumours. Sections of pCB6 and Y477F ezrin tumours were stained for pan ezrin (Figure 28), pT567 ezrin (Figure 29), pan Src and pY419 Src (Figure 30). Both pCB6 control and Y477F expressing tumours displayed moderate/strong cytoplasmic ezrin staining. However, Y477F tumours showed multiple foci of incomplete membranous staining of pan ezrin that were rarely present in the pCB6 tumours (Table 11). Staining with pT567 ezrin of both pCB6 control and Y477F ezrin tumours displayed complete membranous staining pattern that was predominantly localized at the tumour periphery (Table 12). Expression patterns of pan and pY419 Src were similar in both pCB6 and Y477F tumours, which exhibited both membranous and cytoplasmic staining (Figure 30, Table 13). Staining for γ-tubulin showed a broad heterogeneous distribution in both pCB6 and Y477F tumours except in regions of necrotic tissue damage (Appendix 3, in progress).
Figure 28. Comparison of cytoplasmic and membranous ezrin localization in pCB6 control and Y477F ezrin mutant tumours.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice (see Fig. 25). Primary tumours were excised after 21 days, sectioned, and stained by immunohistochemistry for pan ezrin. A red box indicates area photographed at higher magnification. Photographs were taken at 4x, 40x, and 100x objective. Scale bars indicate 500, 50, 20 µm, respectively. Results are representative of at least 4 tumours per group. Strong foci of incomplete membranous pan ezrin staining were visible in the Y477F tumours, whereas cytoplasmic ezrin staining was also evident in both groups. Enlargement of cells with membranous staining is shown in insert.

Table 11. Pan ezrin expression in pCB6 and Y477F expressing tumours

Number of tumours in each group, intensity of stain and cellular localization (membranous or cytoplasmic) of pan ezrin are indicated. P-values of two pooled experiments (Fisher’s Exact test) are shown.
Table 11. Pan ezrin expression in pCB6 and Y477F expressing tumours

<table>
<thead>
<tr>
<th>Group</th>
<th>pCB6</th>
<th>C13</th>
<th>A43</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mem</td>
<td>0/4</td>
<td>-ve</td>
<td>4/6 (p=0.07)</td>
</tr>
<tr>
<td>Pan ezrin</td>
<td></td>
<td></td>
<td>Combined: 9/12 (p=0.02)*</td>
</tr>
<tr>
<td>Cyt</td>
<td>4/4</td>
<td>Moderate-strong</td>
<td>5/6</td>
</tr>
</tbody>
</table>

* Significant by Exact Fisher Test
Figure 29. Comparison of pT567 ezrin in pCB6 control and Y477F ezrin mutant tumours.

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice. Primary tumours were excised after 21 days, sectioned, and stained by immunohistochemistry for pT567 ezrin. A red box indicates area photographed at higher magnification. Photographs were taken at 4x, 40x, and 100x objective. Scale bars indicate 500, 50, 20 µm respectively. Results are representative of at least 4 tumours per group.

Table 12. pT567 ezrin expression in pCB6 and Y477F expressing tumours
Number of tumours in each group, intensity of stain and cellular localization (membranous or cytoplasmic) of pT567 ezrin are indicated.
Table 12. pT567 ezrin expression in pCB6 and Y477F expressing tumours

<table>
<thead>
<tr>
<th>Group</th>
<th>pCB6</th>
<th>C13</th>
<th>A43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># mice</td>
<td>Intensity</td>
<td># mice</td>
</tr>
<tr>
<td>pT567 ezrin Mem</td>
<td>4/4</td>
<td>Strong</td>
<td>6/6</td>
</tr>
<tr>
<td>pT567 ezrin Cyt</td>
<td>0/4</td>
<td></td>
<td>0/6</td>
</tr>
</tbody>
</table>
Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fat pad of nude mice. Primary tumours were excised after 21 days, sectioned, and stained by immunohistochemistry against pan and pY419Src. A red box indicates area photographed at higher magnification. Due to similar staining patterns, only pCB6 tumours are shown. Photographs were taken at 4x, 40x, and 100x objective. Scale bars indicate 500, 50, 20 µm respectively. Results are representative of at least 4 tumours per group.

Table 13. Pan and pY419 Src expression in pCB6 and Y477F expressing tumours

Number of mice possessing tumour phenotype, intensity of stain and cellular localization (membranous or cytoplasmic) of pan or pY419 Src are indicated.
Table 13. Pan and pY419 Src expression in pCB6 and Y477F expressing tumours

<table>
<thead>
<tr>
<th>Group</th>
<th>pCB6</th>
<th>C13</th>
<th>A43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># mice</td>
<td>Intensity</td>
<td># mice</td>
</tr>
<tr>
<td>Pan Src</td>
<td>Mem</td>
<td>2/2 Strong</td>
<td>5/6    Strong</td>
</tr>
<tr>
<td></td>
<td>Cyt</td>
<td>2/2 Weak</td>
<td>6/6    Weak</td>
</tr>
<tr>
<td>pY419 Src</td>
<td>Mem</td>
<td>2/2 Strong</td>
<td>5/6    Strong</td>
</tr>
<tr>
<td></td>
<td>Cyt</td>
<td>2/2 Weak</td>
<td>5/6    Weak</td>
</tr>
</tbody>
</table>
CHAPTER 4- DISCUSSION

This thesis represents a transdisciplinary approach to study a novel Src/ezrin pathway in breast cancer metastasis. Overall, we have demonstrated differential localization and activation of ezrin and Src between normal and malignant mammary tissues, which suggests that the Src/ezrin pathway may have the potential to be used as a predictive marker in breast cancer. Furthermore, the results support the role of a precise phosphorylation state of ezrin in local invasion within an in vivo breast tumour engraftment model. Thus, the study demonstrates the following:

1. In a murine system, total ezrin is differentially localized in nulliparous, lactating mammary glands and PyMT tumours, with pronounced apical expression of these proteins in nulliparous mammary glands but strong cytoplasmic expression in PyMT-induced tumours.

2. There is increased expression and activation of ezrin, Src and Met in PyMT-induced tumours compared to normal breast tissues. A concomitant increased expression of activated Stat3 and HGF was also observed in PyMT-induced tumours, consistent with the establishment of an HGF/Met autocrine loop.

3. In invasive human breast tumours from a premenopausal patient cohort, ezrin showed significantly greater cytoplasmic localization compared to non-neoplastic epithelial ducts in normal mammoplasties.

4. In a mouse breast carcinoma engraftment model, the Y477F ezrin mutant, which is not phosphorylatable by Src, significantly reduced local invasion of primary tumours and spreading into visceral organs. Furthermore, Y477F ezrin had no effect on primary tumour growth rate.

5. Y477F ezrin-expressing tumours exhibited multiple focal areas of incomplete membranous ezrin staining which were absent in pCB6 control tumours. Moderate/strong cytoplasmic staining was evident in both tumour groups.

These studies correlate our signalling network to PyMT-induced breast tumourigenesis, and suggest an association of cytoplasmic ezrin expression with invasive human breast cancers.
Furthermore, Src/ezrin interaction via phosphorylation at a specific tyrosine residue (Y477) plays a rate limiting role in local invasion and spreading of breast cancer.

4.1 Evaluation of Src/ezrin expression and activation in a PyMT-induced breast tumour mouse model

4.1.1 Differential localization of Ezrin in mouse nulliparous, lactating mammary glands and PyMT-induced tumours

As a test of principle to evaluate the expression of the Src/ezrin signalling network in breast cancer progression, the expression and localization patterns of Src/ezrin were first determined in normal murine mammary glands and PyMT-induced tumours through immunohistochemistry. In nulliparous and lactating tissue, pan ezrin was localized to the apical region of the mammary luminal epithelium (Figure 13). In contrast, pan ezrin staining exhibited an intense, diffuse atypical cytoplasmic staining in PyMT tumours. In both nulliparous and lactating mammary glands, apical epithelial localization of pT567 ezrin was similar to that of pan ezrin. However in the PyMT tumour model, pTezrin exhibited heterogeneous staining, but remained localized to membranous regions at the periphery of the tumour (Figure 14). Both pan Src and active Src were found to localize in the cytoplasm of nulliparous mammary glands, while in lactating glands both pan and active Src localized predominantly in the apical epithelia. In PyMT tumours, both pan and active Src exhibited an intense, diffuse cytoplasmic staining pattern throughout the tumour (Figure 15 &16). In this study, semi-quantitative comparisons of PyMT tumours and nulliparous tissues were made using the same mouse background (FVB). Due to tissue availability of FFPE tissues, IHC for nulliparous and lactating were done on the SVJ strain. However, immunohistochemical staining patterns were representative to corresponding non-neoplastic and neoplastic human breast tumours.

In a normal physiological state, activation of ezrin targets the molecule to the plasma membrane. However, in a breast carcinoma cell line as well as a cohort of breast tumours,
cytoplasmic localization of ezrin was associated with adverse tumour characteristics (109). Cytosolic ezrin is thought to exist in a folded, inactive monomeric configuration, although homo- or hetero- oligomerization, with other ERM proteins could also occur (110). Loss of ezrin in the apical region may lead to deregulation of ezrin-mediated processes, such as cell-cell contacts and cell-matrix adhesion. Thus, loss of apical ezrin localization may disturb the maintenance of epithelial polarity, a characteristic of EMT progression, into a more aggressive and metastatic phenotype. Alternatively, loss of polarity may disturb ezrin localization.

During Src activation, Src is also targeted to the membrane. However, immunohistochemical staining of breast tumours revealed that tumours with high cytoplasmic Src expression were significantly associated with shorter disease specific survival (164). Interestingly, nuclear localization of active Src was also observed in the above study (164) as well as our PyMT tumour model, though its function is not yet known. Thus, it is possible that increased proliferative or tumourigenic events, as in the case of lactating mammary glands and PyMT tumours, could be responsible for the observed aberrant localization of Src. It is also possible that Src could complex with nuclear localizing molecules, such as Rac, to localize to the nucleus (245).

4.1.2 Increased expression and activation of Ezrin and Src in PyMT-induced mammary tumours compared to normal or lactating mammary glands

In addition to examining the differences in localization of Src and ezrin in PyMT tumours and non-tumourigenic breast tissue, we also examined the levels of total and activated ezrin and Src. There was a marked increase in expression of both pan Src and pan ezrin in lactating compared to nulliparous breast tissues. When compared to nulliparous tissue, PyMT tumours also exhibited elevated expression of both pan ezrin and pan Src. During pregnancy, extensive proliferation and side branching of the mammary ductal tree occurs. Mammary ductal growth and alveoli development continue from early stage to the peak of lactation (233). Thus, it is
possible that the elevated levels of Src and ezrin seen within the lactating stage are due to the proliferative and differentiated nature of the lactating tissue. Cells with ezrin siRNA showed cell cycle downregulation. Tumourigenesis is also often accompanied by increased proliferation through various mechanisms, including the activation of tyrosine receptor kinases such as Met. Activation of receptor kinases can lead to the activation of Src, which, in turn, becomes capable of phosphorylating multiple downstream molecules. Activated Src may also phosphorylate ezrin directly at specific tyrosine residues or indirectly at threonine-567, via Rho GTPase regulated kinases, such as ROCK, resulting in ezrin activation. One group found that expression levels of ezrin were significantly higher in primary breast cancer than in lymphatic metastases, suggesting that ezrin may play a role in invasion and the initial stages of metastasis (108). While there is a prominent difference in the pan protein levels of Src and ezrin between nulliparous and malignant tissue, the corresponding increases in the phosphorylated levels of ezrin (pT567) and Src (pY419) are notably smaller. This may suggest that while there is an increase in Src and ezrin expression overall, only a small proportion of these molecules remain in an active state.

4.1.3 Increased expression of HGF and Met in PyMT-induced tumours compared to nulliparous breast tissue

In addition to increase in total and phosphorylated forms of Src and ezrin, we observed increased HGF, Met and Stat3 expression levels as well as increased Stat3/Met activation in PyMT induced tumours relative to the control tissues. Interestingly, a non-cleaved pro-HGF protein band was seen in the PyMT tumours that was not present in the control tissue. Under normal physiological conditions, pro-HGF is secreted from the surrounding mesenchymal cells and is cleaved into α- and β-chains that are non-covalently linked to form active HGF (172). Active HGF then binds to and activates Met, which is expressed on epithelial cells, in a paracrine manner (186, 187). In contrast, HGF and Met are frequently over-expressed in many cancers, including invasive human breast carcinomas (199-204). The Elliott lab has recently shown a
novel activating role of Src and Stat3 on HGF transcription resulting in increased HGF expression and the establishment of an autocrine HGF/Met loop in breast epithelial cells (215, 234). Over-expression of pro-HGF and Met in the PyMT tumours indicates that the tumour tissue may in fact regulate production of these proteins in an autocrine fashion distinct from stromal sources of HGF. This could be due to the increased expression of activated Stat3 and Src, which is required for HGF transcription. Autocrine HGF, along with stromal-derived HGF, can then lead to sustained activation of Met, as evidenced by the increase in Met phosphorylation in PyMT-induced tumours. Taken together, these results are consistent with the activation of an autocrine HGF/Met loop during PyMT-induced mammary tumourigenesis.

4.2 Assessing the correlation of Src/ezrin expression in a breast tumour TMA

4.2.1 Validation of antibody specificity for immunohistochemistry

Validation of antibody specificity is critical for ensuring accuracy of immunohistochemical analysis. As an example, it was shown that four out of five commercially available antibodies, against the carboxy terminal domain of Met, were not reproducible at a quantitative level although able to identify the protein on a Western blot (235). Therefore, great caution must be taken when selecting antibodies, as variability may also exist between lots.

To control for antibodies, the first step was to assess non-specific secondary antibody binding by omitting the primary antibody: these staining results were all negative. To take this further, non-specific primary antibody binding due to the Fc region was controlled by using a non-immune rabbit immunoglobulin control. Since the antibodies used for immunohistochemistry were derived from rabbit, but the tissues probed were of murine origin, any staining present would be attributed to rabbit immunoglobulin binding. As shown in Figure 10, rabbit IgG staining in murine tissue was generally negative. Other methods to validate antibody specificity would be to use knockout or knockdown cells such as SYF-/- or ezrin -/- cell lines and compare the differences in protein expression to the wild-type.
Validating phospho-specific epitopes for immunohistochemistry has been an ongoing challenge in the field. While phospho-specific antibodies provide the possibility of looking at the dynamic changes in cell signalling networks, there has been much scepticism with the use of phospho-epitopes in immunohistochemistry. The most common problem encountered is a false-negative result which can be due to antigen masking (236). Another problem is the instability or lability of phospho-epitopes which may be dependent on how quickly tissues are fixed as well as the method of storage. While phospho-epitopes, once fixed, are usually stable at room temperature, the optimal handling, extent of stability or the dephosphorylation kinetics have yet to be objectively examined. To gather an idea of the quality of fixation, tissue sections can be stained for general house-keeping proteins, such as vimentin or gamma tubulin (Appendix 3). It is expected that well-fixed tissue should have an even, diffuse distribution throughout the section.

While testing for phospho-directed antibodies is similar to that for total protein, additional controls specific to phospho-epitopes are needed. As a first approach, a peptide blocking system was used on positive control tissues as well as our tissues of interest. It is expected that the specific peptide binds to the primary antibody competing with its binding to the endogenous epitope. The level of blockage is quite dramatic with peptides specific for pT567 ezrin, pan Src and pY419 Src as indicated in Figure 11 & 12. While these controls demonstrate the specificity of the antibody to the blocking epitope, this epitope may be similar to unrelated endogenous. As an illustration, the phospho-specific antibody against ezrin is known to recognize pT567 of ezrin, pT564 of radixin and pT558 of moesin. Since the specificity of the antibody is targeted towards these three epitopes, further controls such as ezrin KO cells are needed to determine whether staining is representative of true pTezrin, or whether localization patterns are confounded by staining against similar epitopes.

A more general control would be treatment of tissue sections with alkaline phosphatase where it is expected that immunoreactivity should be absent (237). Other possibilities of antibody control would be to perform site-directed mutagenesis at T567 ezrin or Y419 Src, and
express these mutants in corresponding KO cell lines. This approach should reveal a loss of immunoreactivity as compared to the wild-type protein.

4.2.2 Developing the Tissue Microarray

The development of a breast tumour tissue microarray posed various technical challenges apart from antibody validation. Initially, one test TMA (TMA1) consisting of 60 tumours was made. However, difficulties in paraffin constitution made sectioning difficult and eventually, TMA1 cracked. Learning from this, a second test TMA (TMA2) was made taking different cores from the same blocks from the same patient cohort that were seemingly equivalent to those from TMA1. In addition to the neoplastic patient cohort, 20 normal mammoplasties were included in the TMA. TMA2 was stained and scored in the same way as TMA1. When performing tests of association through Pearson and Spearman correlation coefficients, a significant association between pan ezrin and pY419 Src was found in TMA1 but significance was lost in TMA2 (Table 7). Further analysis revealed that p53 and HER-2 profiles were concordant between the two TMAs while ezrin and pYSrc were not. This finding prompted a study on the extent of reproducibility and potential tumour heterogeneity between regions or even different sections of the same core.

While this aspect of the study is still ongoing, there are possible steps to address this issue. The aspect of reproducibility can be addressed through automated staining using the Ventana processor in the Queen's Laboratory of Molecular Pathology on recently cut serial sections in two separate runs. Automated staining should eliminate variables introduced when staining manually from one run to the next, including temperature, tissue drying and mixing. While tissue blocks are usually quite stable at room temperature, epitopes on tissue sections have been known to degrade over time once cut. Thus, cutting sections and immediately staining them would decrease the possibility of epitope loss. Breast carcinoma heterogeneity has long been a source of imprecision in histological grading and of reproducibility issues. Cores will tend to
shift as sections are cut and slight thickness variations may be present for each section. Staining serial sections should help control for any possible tissue heterogeneity between different depths of the tumour. It is possible that issues of tumour heterogeneity may need to be evaluated based on whole tissue sections rather than cores (238). Even then, there may be heterogeneity that is highly dependent on the biomarker to be analyzed. Furthermore, including internal positive and negative controls, such as cell lines or normal tissues, can provide baseline and maximum staining intensities relative to which test tissues, on the same slide, can be scored. While there will always be a certain level of variation from one section to the next, there needs to be a defined level of replicability; localization should remain the same and intensities ought to be within an accepted range.

Technical variability is also an issue that needs to be well controlled. A preliminary run showed that there were different staining intensities between sections stained against Stat5A and pan ezrin. In contrast, HER-2 and p53 showed similar staining intensities. This finding suggests that there may be some technical issues which may affect antibody function such as antigen retrieval, pH and temperature.

4.2.3 Differential localization of ezrin expression in normal and neoplastic human breast tissues

Statistical analysis of the differential localization of ezrin expression showed a significantly greater cytoplasmic localization of pan ezrin within the invasive breast tumour cases compared to the normal mammooplasties. The majority (89%) of breast carcinomas showed strong cytoplasmic ezrin staining, compared to normal breast lobule units which did not (Pearson/Spearman correlation coefficients, p = 0.003). Results showed an expression pattern of ezrin similar to that of the PyMT transgenic model. Previous studies with breast cancer cell lines and breast tumours have shown that apical localization of ezrin is associated with well-differentiated, hormonal receptor positive, low Ki-67 (low proliferative) and node negative
tumours. In contrast, cytoplasmic localization of ezrin is associated with higher grade, high Ki-67, hormonal receptor negative, and lymph node positive tumours (108, 109). As mentioned above, current understanding is that ezrin activation occurs when localized to the plasma membrane where it plays a role in cortical organization, cell motility and cell division. Loss of ezrin in the apical region may lead to a deregulation of ezrin-mediated processes, such as the loss of normal anchorage of adhesion molecules or growth factor receptors to the membrane. Thus, loss of apical and increased cytoplasmic ezrin localization may disturb the maintenance of epithelial polarity, a characteristic of EMT progression, leading to a more aggressive and metastatic phenotype. Thus, aberrant localization patterns of ezrin expression may provide important clues to its role as a prognostic marker. There has also been evidence that proteases such as calpain can cause ezrin cleavage leading to a 55 kDa ezrin fragment which may have some physiological relevance (47). For example, it was found that the ezrin cleavage product localized to the nucleolus (239). One can speculate that cleaved ezrin products may lack the regulation of the closed conformation, which in turn, can mediate downstream signalling in the cytoplasm or nucleolus.

Staining of tumour cells for pY419 Src showed both membranous and cytoplasmic staining in normal and neoplastic breast tissues (Figure 21). Statistical analysis showed that there was no significant difference in cytoplasmic pY419 Src between normal and neoplastic breast tissue (Table 6). Since Src is ubiquitously expressed, it is possible that there may be similar levels of Src in both membranous and cytoplasmic compartments. However, differences in localization, and in some cases combinations of localization patterns, may be highly important in predicting patient outcome. For example, it has been shown that high cytoplasmic Src and high membranous Y419 Src is associated with decreased disease-specific survival. In contrast, nuclear and cytoplasmic Y215 Src was significantly associated with improved disease-specific survival (164). Thus, further statistical analysis is needed to look at possible differences in staining localization patterns, and their association with disease outcome in our patient cohort.
More recently, many groups have started to compare a more objective approach with visual scoring through the use of automated image analysis technologies, such as ARIOL and AQUA. In contrast to ordinal scales associated with manual scoring, the automated platforms can produce a continuous scale of intensities, which gives greater information, better differentiation and less overlap or subjectivity. In the ARIOL platform, the system calculates mean staining intensity and % of positive tumour area selected based on nuclear diameter. Further studies to assess the correlation between paired visual and ARIOL derived H-scores are needed.

4.3 To assess the role of the Src/ezrin activation at specific stages of breast cancer metastasis.

4.3.1 Y477F ezrin inhibits local invasion of primary tumour outgrowths and lymphovascular invasion in primary AC2M2 breast tumours

There is increasing evidence that ezrin is linked to malignant progression in a variety of human cancers, including osteosarcoma, rhabdomyosarcoma and breast (107, 111, 240, 241). Overexpression and/or aberrant activation of ezrin can lead to increased proliferation, cell survival, motility and increased metastatic potential. Ezrin has been shown to be required for metastasis in breast carcinoma cells (111). Using the Y477F ezrin mutant that cannot be phosphorylated by Src, Arpin et al. have shown that Src/ezrin interaction via pY477 ezrin is required for HGF-induced scattering and tubule formation in porcine kidney epithelial cells grown in 3-D collagen gels (93). In our AC2M2 breast carcinoma model, wound healing assays showed a delayed healing rate of tumour cells expressing Y477F ezrin compared to the pCB6 control (Appendix 1). Furthermore when AC2M2 cells were grown on Matrigel, tumour cells expressing Y477F ezrin formed cohesive aggregates, while pCB6 control cells formed loose aggregates with branching extensions (Appendix 2, courtesy of Colleen Schick). These findings support a role of Src/ezrin interaction via phosphorylatedY477 in regulating migration and invasion in Matrigel cultures.
The AC2M2 breast carcinoma mouse engraftment model developed in Dr. Elliott’s laboratory was previously used to study the role of ezrin in breast cancer metastasis. Published findings from the Elliott lab show that over-expression of an N-terminal truncated ezrin domain (N-term, a.a. 1-309) in AC2M2 cells blocks cell migration and invasion in vitro (111). Furthermore, lung metastasis is greatly attenuated in N-term AC2M2 tumour cells engrafted into the mammary fat pad compared to tumour cells expressing wild type ezrin or pCB6 control vector. Interestingly, no difference in primary tumour growth rate was detected. In this thesis, local invasion and spreading of AC2M2 primary tumours were examined and the effect of Y447F ezrin on this process. Gross and histopathological analysis showed that the majority (>90%) of pCB6 tumours invaded into underlying abdominal muscle (Figure 25). In addition, marked seeding and dissemination into visceral organs including intestine, spleen, and pancreas were evident. Thus, the pCB6 control tumours displayed a phenotype reminiscent of clinically advanced human breast cancers, where locally advanced primary tumours tend to anchor to the chest wall and invade into underlying muscle tissue (242).

In contrast to the pCB6 tumours, few (<25%) of the Y477F-tumours (two independent clones examined) showed locally invasive characteristics (Fisher’s Exact test, p=0.0002) (Figure 25). Instead, the majority of Y477F tumours remained circumscribed, with little invasion into surrounding stroma and the abdominal wall. This novel finding suggests that phosphorylation at a single tyrosine residue (Y477) on ezrin is required for local invasiveness of tumour cells.

Lymphovascular invasion, a known prognostic marker for high risk human breast cancers (232), was also detected in the majority (5/6) of pCB6 control tumours examined. No LVI was detected in tumours from two Y477F ezrin clones examined (Table 10). These findings indicate that Y477F ezrin expression inhibits lymphovascular invasion in primary tumour outgrowths. Since the major route of initial local spread in breast cancer is often through the lymphatic system (243), the above findings suggest that Y477 may play a role in mediating this process. The mechanism by which pY477 ezrin regulates invasion is not known. However, recent evidence
shows that pY477 ezrin interacts with Fps kinase causing its activation and localization to cell-cell contacts. Further studies are required to establish a role of Fps or other ezrin partners in the invasion process.

Additional studies are also needed to determine the effect of Y477F on distant metastasis of AC2M2 tumour cells. Thus, next steps can be to repeat the experiments with a larger mouse cohort over a longer period of time. Since overt metastatic nodules in our model are rarely detected within 21 days, lengthening the duration after primary tumour resection may give us insight into the metastatic phenotype of the tumours. However, there are potential limitations and confounders. We have found it difficult to detect Y477F ezrin via the VSVG tag in metastatic outgrowths, suggesting that Y477F ezrin may be unstable or the VSVG tag is lost during tumour progression. This may suggest loss of Y477F ezrin expression in tumour cells and thus, loss in phenotype, especially following extended periods of time required for outgrowth of distant metastases.

Another useful addition to the study would be to express GFP in the Y477F ezrin cells so that metastatic nodules could be imaged through bioluminescence imaging. Immunofluorescent staining for CD31 to detect vasculature (244), or for podoplanin to detect lymphatics (38) can also assist in interpreting the effects of Y477F ezrin on lymphovascular invasion of GFP-tagged tumour cells. With the use of spinning disk microscopy, there is also the possibility of doing live imaging studies where invasion and extravasation mechanisms can be studied.

4.3.2 Y477F ezrin promotes increased membranous ezrin localization in primary AC2M2 breast tumours

Both pCB6 control and Y477F ezrin expressing tumours displayed moderate/strong cytoplasmic ezrin staining. However, Y477F ezrin tumours also exhibited multiple foci of incomplete membranous staining of pan ezrin that was absent in the pCB6 tumours. In contrast, pT567 ezrin showed strong membranous staining in both tumour groups, as expected (cf.
Introduction Section 1.2.3). A possible interpretation of these differential ezrin localization patterns could be that cytoplasmic staining represents endogenous ezrin which promotes a tumourigenic phenotype. On the other hand, Y477F ezrin may tend to localize to the plasma membrane and would promote a less malignant (non-invasive) phenotype. Staining for VSVG would validate the membranous staining seen in the Y477F ezrin tumours. Automated imaging tools such as ARIOL or AQUA can be used to further quantify and determine whether membranous staining patterns are significantly different between the two groups.

Interestingly, Y477 is located between a stretch of proline residues and the C-ERMAD which houses the F-actin binding site. It has been shown that phosphorylation of Y477 and regulation between the active and inactive conformations of ezrin are independent of each other (79). Therefore due to its unique location, it is possible that phosphorylation at Y477 would occur even when ezrin is in its closed conformation. This characteristic suggests that the effects of Y477 phosphorylation could be mediated without ezrin localization to the membrane. Further studies are required to elucidate ezrin dynamics in the model.

4.4 Significance

There is a great need for better predictive and prognostic markers for node negative breast cancer. Ezrin and Src have been strongly implicated in metastasis where they can act cooperatively to induce the disruption of cell-cell contacts and cell scattering, thereby increasing the invasive and metastatic potential of these cells (217). Ezrin and Src have also been found to mediate a positive feedback loop in which phosphorylation of ezrin stabilizes Src activity (91). Furthermore, ezrin has been found to be required for metastasis in breast carcinoma cells (111). Expression of an inactivating ezrin mutant (T567A) or ezrin shRNA knockdown have also been shown to block metastasis of both osteosarcoma and rhabdomyosarcoma cell lines (240, 241). Our findings suggest that ezrin, Src and their related signalling network may be a potential
metastasis marker in breast cancer. The combined pre-clinical and clinical aspects of the study observe this potential association between marker and invasiveness on many levels.

In the xenograft model, we have found that, within ezrin, phosphorylation of the Y477 amino acid residue controls regional local invasiveness of the primary tumour as well as metastasis through lymphovascular invasion. Y477F ezrin tumours displayed less abdominal wall invasion compared to their pCB6 control counterpart. Furthermore, there was an absence of LVI in the Y477F ezrin tumours compared to pCB6 control tumours. In the PyMT transgenic mouse model and in the human breast cancer TMA studies, differential membranous versus cytoplasmic staining of ezrin between nulliparous mammary glands and breast tumour tissue indicate that precise localization of the markers are important in understanding the outcome of the disease. Furthermore, the differences in staining localization between endogenous ezrin and an ezrin phosphorylation mutant (Y477F ezrin) suggest that not only is intracellular localization important, but that the precise state of phosphorylation may be crucial in understanding tumour invasiveness and metastatic potential.

In conclusion, this study has provided insight into potential mechanism surrounding the Src/ezrin pathway from an in vivo perspective and preliminary differences in ezrin expression and localization between non-tumourigenic and malignant breast tumours holds promise as a possible prognostic or predictive marker for treatment response in breast cancers.
APPENDIX

5.1 General Reagents

**Phosphate Buffered Saline (PBS)**
- 137 mM NaCl
- 2.68 mM KCl
- 21.7 mM Na₂HPO₄·H₂O
- 1.47 mM KH₂PO₄
- Adjust to volume with ddH₂O and pH 7.3

**Avertin**
- 2.5 g 2,2,2-Tribromoethanol
- 5 mL tertiary amyl alcohol
- 200 mL distilled water-neutral pH

**Tissue Homogenization**

**RIPA buffer**
- 150 mM NaCl
- 0.5% Deoxycholate
- 0.1% SDS
- 50 mM Tris
- 1% Nonidet P-40 (NP-40)
- 10 mM EDTA
- Adjust to volume with ddH₂O and pH 7.4

**Protease and phosphatase inhibitor cocktail**
- 1% trasylol (Sigma)
- 1µg/mL leupeptin (Sigma)
- 1 mM 1, 4-dithiothreitol (DTT) (Sigma)
- 0.1 mM phenylmethanesulphonylfluoride (PMSF) (Sigma)
- 1 mM benzamidine (Sigma)
- 1 mM sodium fluoride
- 1 mM sodium orthovanadate (Sigma)
- 20 mM β-Glycerophosphate (Sigma)

**2xSDS**
- 20% glycerol
- 4.6% SDS
- 1% Bromophenol Blue
- 0.15% Tris
- Adjust to volume with ddH₂O and pH 6.8

**Immunohistochemistry and Tissue Staining**

**Sodium citrate buffer (10mM)**
- Sodium citrate trisodium salt dehydrate (C₆N₅Na₃O₇·2H₂O)
- Adjust to volume with ddH₂O and pH 6.0; dilute to 1x before use

**Acid Alcohol**
1% HCl
70% ethanol

Ammonium water
250 mL dH₂O
2-3 drops of ammonium hydroxide

**SDS-PAGE and Western Blotting**

**Resolving gel (8% gel)**
- ddH₂O 4.8 mL
- 4x Lower Gel Buffer (LGB)
- 29:1 Acrylamide
- 10% Ammonium persulfate (APS)
- TEMED

**Stacking gel**
- ddH₂O 2.4 mL
- 4x Upper Gel Buffer (UGB) 1.0 mL
- 29:1 Acrylamide 0.6 mL
- 10% Ammonium persulfate (APS) 24 µL
- TEMED 8 µL

**4x Lower Gel Buffer (LGB)**
- 1.5 M Tris
- 0.4% SDS
  Adjust to volume with ddH₂O and pH 8.8

**4x Upper Gel Buffer (UGB)**
- 0.5 M Tris
- 0.4% SDS (sodium dodecyl sulfate)
  Adjust to volume with ddH₂O and pH 6.8

**Acrylamide**
- 29% Acrylamide
- 1% Bis-acrylamide
  Adjust to volume with ddH₂O and pass through 45µm nitrocellulose filter

**SDS-PAGE running buffer (10x)**
- 0.25 M Tris
- 1.92 M Glycine
- 1% SDS
  Adjust to 1L with ddH₂O; dilute to 1x before use

**Semi-dry transfer buffer**
- 50 mM Tris
- 40 mM Glycine
- 0.0375% SDS
- 20% methanol
  Adjust to 1L with ddH₂O and pH between 9.0 and 9.4
TBST buffer
1M Tris
150 mM NaCl
0.1% Tween-20
Adjust to volume with ddH2O and pH 8.0

5.2 Human and mouse cell lines—Sources & Culture Conditions

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Source</th>
<th>Culture conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP1</td>
<td>Dr. Bruce Elliott</td>
<td>DMEM 10% FBS</td>
</tr>
<tr>
<td>T47D</td>
<td>ATCC Manassas, VA</td>
<td>RPMI 10% FBS</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>ATCC Manassas, VA</td>
<td>DMEM 10% FBS</td>
</tr>
<tr>
<td>MCF-7</td>
<td>ATCC Manassas, VA</td>
<td>RPMI 10% FBS</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Dr. Peter Siegel (McGill University, Montreal, Canada)</td>
<td>DMEM 10% FBS</td>
</tr>
<tr>
<td>MDA-MB-231-1475-LM2</td>
<td>Dr. Peter Siegel</td>
<td>DMEM 10% FBS</td>
</tr>
<tr>
<td>MDA-MB-231-1833-BMI</td>
<td>Dr. Peter Siegel</td>
<td>DMEM 10% FBS</td>
</tr>
</tbody>
</table>
Appendix 1. Effect of Y477F ezrin on wound healing in AC2M2 cells

Appendix 1. AC2M2 clones expressing pCB6 alone or with Y477F ezrin were grown to high density in 10% FBS/DMEM. Confluent cells were wounded by scoring and medium was immediately replaced. Spontaneous cell migration was monitored for 20 hours and phase contrast images were captured (Courtesy of Colleen Schick).
Appendix 2. Effect of Y477F ezrin on growth and morphology of AC2M2 cells in 3D Matrigel cultures

Appendix 2. AC2M2 cells expressing pCB6 (empty vector) or Y477F ezrin(clones A43 and C13) were grown in 20% Matrigel cultures with 10% FBS/DMEM medium for 7 days. Aggregate formation was assessed using phase contrast microscopy. pCB6 cells formed predominantly loose aggregates with branching extensions, whereas the majority of Y477F ezrin cells formed cohesive aggregates with few extensions. (Courtesy of Colleen Schick).
Appendix 3. Immunohistochemical staining of \( \gamma \)-tubulin in pCB6 control and Y477F ezrin mutant tumours

**Appendix 3.** Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fatpad of athymic nude mice. Primary tumours were excised after 21 days, sectioned, and stained by immunohistochemistry for \( \gamma \)-tubulin. Photographs were taken at 4x and 10x magnification. Scale bars indicate 500 and 200 \( \mu \)m respectively (Courtesy of Lee Boudreau).

Two clones of AC2M2 cells expressing Y477F ezrin (A43 and C13) or empty pCB6 vector were engrafted into the mammary fatpad of nude mice (see Fig. 25). A red box indicates area photographed at higher magnification. Photographs were taken at 4x, 40x, and 100x magnification. Scale bars indicate 500, 50, 20 µm, respectively. Strong foci of partial membranous staining pan ezrin were visible in the Y477F tumours, whereas cytoplasmic ezrin expression was evident in both groups. Enlargement of cells with membranous staining is shown in insert.
REFERENCES


36. Carragher NO, Walker SM, Scott Carragher LA, Harris F, Sawyer TK, Brunton VG, Ozanne BW, Frame MC. Calpain 2 and Src dependence distinguishes mesenchymal and


52. Gary R, Bretscher A. Ezrin self association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. Mol Biol Cell, 1995; 6:1061-75.


81. Ng T, Parsons M, Hughes WE, Monypenny J, Zicha D, Gautreau A, Arpin M, Gschmeissner S, Verveer PJ, Bastiaens PI, Parker PJ. Ezrin is a downstream effector of
trafficking PKC-integrin complexes involved in the control of cell motility. EMBO J. 2001; 20:2723-41


86. Yang HS, Hinds PW. Increased ezrin expression and activation by CDK5 coincident with acquisition of the senescent phenotype. Mol Cell. 2003; 11:1163-76.


159. Fincham VJ, Frame MC. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. EMBO J. 1998; 17:81-92.


