THE ROLE OF PIM-1 IN BREAST CANCER METASTASIS

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Abstract

Breast cancer is the most common cancer in women. Despite advances in treatment options, the spread of breast cancer to distant organs (metastasis) remains the major cause of morbidity and mortality in breast cancer patients. This is attributed primarily to the impairment of function in affected organs. Thus, there remains a vital need for better-targeted treatments that more effectively inhibit the development or progression of metastases.

Pim-1 is a serine/threonine survival kinase that has been implicated in the development of metastasis in several haematological and solid cancers. However, little is known about its role in breast cancer. In our laboratory, we previously identified Pim-1 as upregulated in brain metastatic 4T1Br4 syngeneic mouse cells and tumours compared to parental 4T1 cells. This led us to propose that Pim-1 may play a role in mediating breast cancer brain metastasis. Therefore, the overall objective of this project was to examine the expression and functional role of Pim-1 in breast cancer metastasis, with a focus on organ-specific metastasis.

We interrogated public databases to show that Pim-1 expression is low to absent in normal breast tissue and increased in breast tumour tissue. Furthermore we show that the murine (4T1Br4) and human (MDA-MB-231Br) brain metastatic breast cancer cell lines and tumours demonstrate the highest expression of Pim-1 mRNA and protein.

To investigate the function of Pim-1 in breast cancer metastasis we tested the impact of inhibiting Pim-1, either by gene knock down using short hairpin RNAs or the pharmacological inhibitor SGI-1776, on the ability of 4T1Br4 and MDA-MB-231Br cells to migrate and invade in vitro. 4T1Br4 cells displayed increased migration and invasion propensity after Pim-1 knock down and this was coupled with a decrease in β4 integrin expression. Conversely, MDA-MB-231Br cells showed a decreased ability to migrate and invade after Pim-1 KD, as well as decreased cell surface expression of β1 and β3 integrins.
Treatment with SGI-1776 dose-dependently decreased the ability of both 4T1Br4 and MDA-MB-231Br cells to migrate and invade, decreased cell surface expression of β3 integrin in 4T1Br4 cells, and both β1 and β3 integrins in MDA-MB-231Br cells.

To examine the effect of Pim-1 inhibition in vivo, we assessed the metastatic spread of Pim-1 knock down MDA-MB-231Br cells in an experimental metastasis assay. After intracardiac injection of Pim-1 knock down cells, we observed a reduction in the number of circulating tumour cells and decreased bone metastasis, indicating a functional role for Pim-1 in breast cancer metastasis to the bone. Data from brain metastasis in this model were inconclusive.

In summary, results from this project highlight the importance of Pim-1 in breast cancer metastasis and provide evidence that Pim-1 contributes to the migration and invasion of breast cancer cells both in vitro and in vivo, possibly via regulation of integrin expression, and indicate that Pim-1 is a relevant therapeutic target for the treatment of metastatic breast cancer.
Declaration

This is to certify that:

1. This thesis comprises only my original work towards the MPhil

2. Due acknowledgement has been made in text to all other materials used, and

3. This thesis is less than 50 000 words in length, exclusive of tables, figures, bibliographies and appendices.

............................
Lara Jupp
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The undertaking of a research project is undoubtedly a journey, filled with many trial and tribulations, as well as every pitfall and roadblock you could possibly imagine. However, the successes and new discoveries make it all most definitely worthwhile and are all the sweeter for it. Most of all, I could not have completed this thesis without the help and support of some very important people.

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<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>4EBP2</td>
<td>EIF4E-binding protein 2</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-minimal essential medium</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-dependent protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signalling kinase 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BCL2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CTAK1</td>
<td>Cdc25C-associated kinase-1</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold value</td>
</tr>
<tr>
<td>CTCs</td>
<td>Circulating tumour cells</td>
</tr>
<tr>
<td>DAB</td>
<td>Diamnobenzydine</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DDFS</td>
<td>Distance disease free survival</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagles medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotides</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiating factor 4E</td>
</tr>
<tr>
<td>ER(+)</td>
<td>Oestrogen receptor (positive)</td>
</tr>
<tr>
<td>ETOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HER2/ErbB2(+)</td>
<td>Human epidermal growth factor receptor 2 (positive)</td>
</tr>
<tr>
<td>HAc</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>IDC</td>
<td>Infiltrating ductal carcinoma</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>KD</td>
<td>Knock down</td>
</tr>
<tr>
<td>KID-1</td>
<td>Kinase induced in depolarisation</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
</tr>
<tr>
<td>LN+</td>
<td>Lymph node positive</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>Me OH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MMTV</td>
<td>Murine mammary tumour virus</td>
</tr>
<tr>
<td>MMLV</td>
<td>Maloney murine leukaemia virus</td>
</tr>
<tr>
<td>mTORC1</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>-------------</td>
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</tr>
<tr>
<td>NBF</td>
<td>Neutral buffered formalin</td>
</tr>
<tr>
<td>NFATC</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD scid gamma</td>
</tr>
<tr>
<td>P21</td>
<td>Cyclin dependent kinase inhibitor 1</td>
</tr>
<tr>
<td>P27</td>
<td>Cyclin dependent kinase inhibitor 1B</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(adenosine diphosphate–ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein phosphatase 2A</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PyMT</td>
<td>Polyoma virus middle T antigen</td>
</tr>
<tr>
<td>RIPA buffer</td>
<td>Radioimmunoprecipitation assay buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RTA</td>
<td>Relative transcript abundance</td>
</tr>
<tr>
<td>RTB</td>
<td>Relative tumour burden</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real time quantitative PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium docdecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TNBC</td>
<td>Triple negative breast cancer</td>
</tr>
<tr>
<td>TrkB</td>
<td>Tropomyosin receptor kinase B</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WBRT</td>
<td>Whole brain radiotherapy</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-related integration site</td>
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Literature review

1. Breast cancer

1.1 Overview

Breast cancer is the most common cancer for women in the world. In 2011, estimates put breast cancer deaths at over 508 000 worldwide (Global Health Estimates, WHO 2013). Despite the widely held belief that breast cancer is a disease of the first world, 53% of cases and 62% of deaths are found in the developing world (GLOBOCAN, 2012). In fact, incidence is on the rise in developing countries, presumably due to extended lifespan, and adoption of the ‘Western’ lifestyle. In Australia, 13 567 females were diagnosed with invasive breast cancer in 2008, which equates to 37 new cases every day (Breast cancer incidence Australia, 2012). The number of breast cancer cases diagnosed more than doubled between 1982 and 2008 and it is projected that this will increase further to 17,210 new cases in 2020, equating to 47 new cases every day (Cancer incidence projections, 2012). A slight 2.2% decrease in the five-year breast cancer mortality rate has been observed between the mid-1990s and 2007. This has been attributed to the earlier detection of breast cancer due to organised mammographic screening programmes introduced in Australia between 1989 and 1994 (AIHW, 2009; AIHW, 2012). However, this figure does not take into account the not uncommon recurrence of breast cancer after the five-year mark. Thus, despite this small decrease in mortality, there is a vital need to develop novel targeted therapies to combat this pernicious disease, as the thousands of deaths attributed to breast cancer every year create an enormous socioeconomic burden.
1.2 Breast cancer classification

Breast cancer is a heterogeneous disease and as such several classification methods have been developed to aid treatment and diagnosis. Tumour-intrinsic factors, including histological markers, genetic alteration and molecular changes have all been used to define breast cancer subgroups.

1.2.1 Histological classification

Breast cancer is a heterogeneous disease; hence diagnosis of breast cancer will be followed by classification based on histological type, grade and other parameters. Histologically, breast cancer is broadly categorised into in situ (non invasive) carcinoma or invasive carcinoma. In situ carcinomas are divided into either ductal carcinoma in situ (DCIS) or the less common lobular carcinoma in situ (LCIS) (Malhotra, Zhao et al. 2010). However, the incidence of lobular carcinoma is increasing at a faster rate and is more strongly linked to hormone receptor expression than ductal carcinoma (Li, Uribe et al. 2005). Invasive carcinomas are comprised of many subtypes of which, infiltrating ductal carcinoma (IDC) is the most prevalent and accounts for 70-80% of all invasive breast cancers (Malhotra, Zhao et al. 2010).

Both in situ and invasive tumours are graded on three parameters: tubule formation, nuclear polymorphism and mitotic rate, which are added together to give the tumours a differentiation score between 3 and 9 (Fabbri, Carcangiu et al. 2008). A score of 3-5 means the tumour is grade 1 and well differentiated. A score of 6-7 denotes a grade 2 tumour and moderately differentiated. Finally, a score of 8-9 denotes a poorly differentiated grade 3 tumour (Figure 1). Generally, poorly differentiated tumours with a high grade carry the bleakest prognosis (Ben-Porath, Thomson et al. 2008).
Breast cancer is firstly divided into in situ carcinoma and invasive carcinoma. In situ carcinomas are then classified as either ductal or lobular. Invasive carcinomas are further classified as tubular, ductal lobular, invasive lobular, infiltrating ductal, mucinous, medullary and infiltrating ductal. From (Malhotra, Zhao et al. 2010).

1.2.2 Molecular classification

More than most other cancers, breast cancer often shows familial clustering. This clustering has enabled breast cancer to be further classified into six molecular subtypes based on gene array profiling (Fabbri, Carcangiu et al. 2008). These are comprised of five ‘intrinsic’ breast cancer subtypes as well as a normal breast-like subtype. Breast cancers are first divided into those that express the oestrogen receptor (ER) and those that do not. The ER expressing tumours are further clustered into luminal A and luminal B tumours based on the level of oestrogen receptor expression. ER negative tumours are further divided into three groups; HER2/ErbB2 overexpressing (HER2+), basal-like (which includes the triple negative subtype), and the recently characterised claudin-low subtype of tumours (Sørlie, Perou et al. 2001, Malhotra, Zhao et al. 2010, Prat, Parker et al. 2010, Prat and Perou 2011). These subtypes differ in their incidence, survival time and response to conventional therapy. Poorer outcome
is seen among the hormone receptor negative subtypes (triple negative and HER2+) compared to the hormone receptor high luminal groups (Carey, Dees et al. 2007) due to the widespread availability and use of targeted hormonal therapies such as selective ER modulators (i.e. tamoxifen and raloxifene), as well as selective ER downregulators (i.e. fulvestrant) or aromatase inhibitors that block the production of oestrogens, which have all shown great clinical benefit (i.e. Letrozole) (Mouridsen, Gershanovich et al. 2001, Howell, Robertson et al. 2004, Dowsett, Cuzick et al. 2010). Indeed, the prophylactic use of tamoxifen or raloxifene in high risk individuals has been linked to a 47% reduction rate of invasive breast cancer and a 50% reduction rate of non-invasive breast cancer across all age groups (Vogel 2009).

1.2.3 Luminal breast cancers

Both luminal A and luminal B tumours are characterised by expression of the ER and progesterone receptor (PR), with luminal A tumours expressing the highest levels of the ER and other luminal specific genes such as ESR1, GATA3 and FOXOA1 (Sørlie, Perou et al. 2001, Network 2012). Luminal A and B tumours exhibit the lowest number of mutations compared to the other subgroups, and carry a better prognosis as they retain some of the major tumour suppressor genes RB1 and TP53 (Network 2012). Recent studies have indicated these tumours show significantly better relapse free survival as well as overall survival, with luminal A showing the best prognosis overall (Sotiriou, Neo et al. 2003). Since they also retain expression of ER, patients with luminal type tumours often receive adjuvant anti-estrogenic treatment such as tamoxifen. In a large meta-analysis of 194 trials, tamoxifen treatment decreased mortality by 31% irrespective of chemotherapy or age and also decreased both the 5 year and 15 year rates of relapse, which, combined with chemotherapy increases to a 5 year survival increase of 51% (Group 2005).
1.2.4 HER2+ breast cancer

HER2 (also known as ErbB2) is a member of the epidermal growth factor family of receptors that act along with their ligands to mediate cell-cell interactions both during development and adulthood (Yarden and Sliwkowski 2001). Upon ligand binding, the ErbB receptors form homo- or hetero-dimers and signal through tyrosine kinase phosphorylation. Homodimers of ErbB1, ErbB2 or ErbB4 members of this family exhibit only weak signalling and homodimers of ErbB3 are non-signalling. Heterodimers containing HER2 promote greater cell proliferation and survival that those without and since HER2 is the preferential binding partner of all ERbB receptors, amplification of HER2 expression can lead to dysregulated cell growth (Yarden and Sliwkowski 2001).

HER2+ tumours show amplified expression of the HER2 receptor. This can be due to either increased HER2 gene amplification, or to chromosome 17 aneuploidy, where the HER2 locus is located (Nassar, Khoor et al. 2014). This subtype, comprising 20-30% of all breast cancers, carries poor prognosis and decreased relapse free survival (Slamon, Clark et al. 1987, Slamon, Godolphin et al. 1989). In fact HER2 amplification shows stronger prognostic value than other traditional markers such as hormone receptor status and the involvement of lymph nodes (Slamon, Clark et al. 1987) In addition, HER2 has no identified high affinity ligand and may signal through the activation of multiple growth signalling pathways (Moasser 2007). Therefore, blocking HER2 activation may inhibit several discrete oncogenic pathways. Recently, a humanised monoclonal antibody targeting the extracellular domain of HER2 has been developed. This antibody, trastuzumab, was first approved in 1998 as the first-line treatment in combination with paclitaxel for the HER2 positive metastatic breast cancer (Slamon, Leyland-Jones et al. 2001). Further studies have validated the merit of trastuzumab as an adjuvant treatment for HER2-overexpressing breast cancer for decreasing both initial morbidity, and rate of recurrence (Romond, Perez et al. 2005). However due to its large molecular weight trastuzumab has poor blood brain barrier (BBB) permeability, and although trastuzumab shows benefit in treating systemic disease, the brain remains a sanctuary site for cancer cells.
This is of special significance as patients with HER2 positive breast cancer show higher incidence of brain metastases compared to cancers that do not overexpress HER2 (Leyland-Jones 2009). Currently whole brain radiotherapy is used to try and disrupt the BBB, however as discussed below, this is not an ideal treatment and conveys significant side effects (McTyre, Scott et al. 2013). Therefore there is an urgent need to develop BBB-permeable pharmaceutical agents that can completely eradicate these cancer cells from the body.

1.2.5 Triple negative breast cancer

Triple negative breast cancer (TNBC) defines a subgroup of patients with tumours lacking expression of ER, PR and HER2. These tumours comprise the majority of the ‘basal-like’ subgroup of breast tumours (Foulkes, Smith et al. 2010). This group shows comparatively higher gene expression levels of keratin 5/6, metallothionein 1X and fatty acid binding protein 7 (Sotiriou, Neo et al. 2003). TNBC tumours also show enrichment in cell surface markers that define breast cancer stem cells, with high CD44, low CD24 and expression of aldehyde dehydrogenase (Morrison, Schmidt et al. 2008). These TNBC tumours are reported to comprise 18% of all invasive ductal carcinomas (IDCs) and up to 25% of grade 3 tumours (Banerjee, Reis-Filho et al. 2006). While there is an overlap between TN tumours and basal-like tumours, not all TN tumours are basal-like. 17-40% of basal-like tumours do not have a triple negative phenotype, and up to 20% express ER or HER2 to some degree (Hiller and Chu 2011). There remains a controversy as to how best to classify basal-like TNBC tumours. In a recent study that compared three different classification modalities, out of 142 TNBC tumours, 116 were classified as basal-like using one of the three modalities, but only 13 fell under the basal classification in all three methods (Gazinska, Grigoriadis et al. 2013). Therefore, it is clear that triple negative status should not be used as a surrogate marker for basal-like cancers and care should be taken when defining a tumour as one or the other. TNBC tumours are associated with typically more aggressive tumours with increased metastasis to brain and lung (Lin, Claus et al. 2008, Dent, Hanna et al. 2009). As with HER2-
overexpressing cancers this is also thought to be due to a sanctuary effect. Due to this they are associated with shortened survival times and decreased relapse-free survival (independent of lymph node status) compared to the other subtypes (Sørlie, Perou et al. 2001, Van De Rijn, Perou et al. 2002). Patients with TNBC have the highest risk of recurrence within the first 5 years of diagnosis, whereas in other tumour types the risk of recurrence remains constant over time (Dawson, Provenzano et al. 2009). Part of the reason for this is TNBC tumours generally respond poorly to conventional chemotherapy and if they do respond, they quickly develop resistance (Anders and Carey 2009, Dent, Hanna et al. 2009).

Recently, the link between TNBC and Breast cancer 1 (BRCA1) gene mutation has been observed. It has been found that there is an inherited susceptibility gene to breast cancer and this gene, BRCA1, was mapped to chromosome 17q21 (Hall, Lee et al. 1990) and subsequently cloned when it was found that BRCA1 had several residues that were commonly mutated (Miki, Swensen et al. 1994). In normal cells, BRCA1 is involved in the repair of double strand DNA breaks by co-localising with RAD51 and RAD50 after DNA damage and so functions to protect the integrity of the genome by limiting mutagenic non-homologous repair processes (Moynahan, Chiu et al. 1999). Mutation of BRCA1 destroys its function and so cells deficient or mutated in BRCA1 show increased non-homologous recombination and increased propensity for cancer development (Moynahan, Chiu et al. 1999). BRCA1 mutations convey an inherited increased susceptibility to breast (and ovarian) cancer, with a cumulative risk increase of 87% by age 70 for breast cancer (Ford, Easton et al. 1994). The majority of patients with BRCA1-mutated cancers show a triple negative phenotype with high expression of basal markers (Dawson, Provenzano et al. 2009). However, not all patients with TNBC show BRCA1 mutations. This further highlights the urgent need to identify new biomarkers of TNBC metastasis and to develop novel targeted therapies for this aggressive subtype of breast cancer.
1.2.6 Current treatments for TNBC

The current mainstay of treatment for TNBC is conventional chemotherapy, as there is no ER, PR or HER2 receptors to target with the currently available targeted therapies. Since TNBC patients generally show a poor sustained response to conventional chemotherapy (as the rate of relapse is high), the search is on to discover unique biomarkers in order to develop targeted therapies to selectively target these tumours. A large proportion of patients with TNBC show elevated expression of EGFR, suggesting that these patients may respond to the use of EGFR targeting inhibitors, such as erlotinib, with clinical trials currently underway (Siziopikou and Cobleigh 2007, Maurer, Kalinsky et al. 2013). These include a Phase 1 trial in combination with Metformin (NCT01650506) and a Phase II trial investigating the combination of erlotinib with chemotherapy (NCT00491816). Treating BRCA1 mutated TNBC with cisplatin and carboplatin is a strategy currently being explored as it is thought that BRCA1 pathway mutation is associated with a DNA repair defect that is especially sensitive to these agents. Initial neoadjuvant trials with cisplatin have shown high rates of complete pathological response in patients with TNBC (Silver, Richardson et al. 2010).

Recently, the use of vascular endothelial growth factor (VEGF) inhibitors to treat metastatic TNBC has been explored, as there is evidence of increased expression of VEGF in TNBC patients (Linderholm, Klintman et al. 2009). Along with the increased expression of VEGF, the other main rationale behind the use of VEGF inhibitors is that micrometastases are dependent on angiogenesis for growth, and so blocking angiogenesis may offer greater survival for these patients. However, the actual benefit of adjuvant use of VEGF inhibitors such as bevacizumab has been controversial. Bevacizumab was approved for use in 2008 for the treatment of patients with metastatic breast cancer, but that approval was subsequently revoked in 2011 when further studies showed no overall survival benefit (Barton 2014). The recent phase 3 BEATRICE study, which assessed the promise of chemotherapy with or without bevacizumab treatment, found no difference in overall survival between the groups (Cameron, Brown et al. 2013). However, subsequent biomarker analysis indicated that patients with
high levels of plasma VEGF-2 prior to treatment may benefit from bevacizumab (Cameron, Brown et al. 2013). In three previous randomised phase 3 trials, where progression free survival (PFS) was the main objective bevacizumab showed great benefit and increased PFS significantly, with hazard ratios of 0.48 compared to paclitaxel alone (Gray, Bhattacharya et al. 2009), 0.89 compared to docetaxel alone (Miles, Chan et al. 2010), 0.69 compared to capecitabine alone and 0.64 compared to anthracycline treatment alone (Robert, Diéras et al. 2011). Taken together, these studies suggest that the value of bevacizumab as an adjuvant treatment for metastatic TNBC should not be completely dismissed, but rather requires further studies to define the best clinical settings for its use.

Currently, the use of poly(adenosine diphosphate–ribose) polymerase (PARP) inhibitors is showing promise. PARP is an enzyme involved in base excision after DNA damage in both homologous and non-homologous end joining (Hiller and Chu 2011) and so PARP inhibitors should add value as a neoadjuvant therapy. Indeed, one such inhibitor, iniparib, has completed a phase 2 clinical trial for metastatic TNBC (NCT00938652). In this trial, iniparib was added to a regimen of gemcitabine and carboplatin and demonstrated an increase in the rate of tumour regression (48% vs. 16%), median progression free survival (6.9 months vs. 3.3 months) and an overall median survival rate of 12.2 months vs. 7.2 months in TNBC patients (O'Shaughnessy, Osborne et al. 2009).

While the search is on in earnest for targeted therapies for TNBC (particular metastatic disease), finding one treatment for this heterogeneous subset of tumours is challenging and so more research needs to be done to characterise conserved molecular targets and to design strategies to effectively treat these patients with minimal systemic toxicity.

1.3 Breast Cancer Metastasis

Metastasis is the spread of cells from the primary neoplasm to distant organs. It is a multifactorial process that relies on the coordination of multiple changes occurring both in the primary tumour and in the host tissue (Nguyen, Bos et al. 2009). The process begins with dissociation of several tumour cells
from the primary tumour followed by invasion through the host stroma and dissemination into the circulation (blood or lymph). This involves proteolytic degradation of the basement membrane surrounding the mammary gland by proteases, most notably matrix metalloproteinases (Murphy, Reynolds et al. 1989). The cells must then adhere to the sub-endothelial basement membrane and migrate through capillary endothelial cells, a process termed intravasation (Fidler 2003). Once in the circulation, tumour cells need to interact with endothelial cells and the underlying basement membrane in order to extravasate from the circulation, invade the host tissue, survive, and colonise the site (Liotta and Stetler-Stevenson 1991, Stetler-Stevenson, Aznavoorian et al. 1993, Fidler 2003) (Fig 1.2). Each step in the metastatic process can be rate limiting. Thus, tumour cells need to acquire several functional characteristics in order to accomplish each of these steps. Accordingly, only a small percentage of tumour cells that reach the circulation will survive to colonise distant sites. For example, cells with different metastatic abilities have been shown to extravasate the primary site at similar rates, but that does not mean that they all will survive to a secondary site, indicating that it is a step post-extravasation that is rate limiting (Koop, Schmidt et al. 1996). In fact, the ability of solitary cells to survive, evade immune surveillance and to develop from single cells, to micrometastases and then on to macrometastases may be more important. This stepwise progression of metastasis offers multiple opportunities to target and shut down the progression from primary tumour to distant lesion.
Metastasis is not a random process. Certain cancers seem to metastasise preferentially to certain organs, with breast cancer metastasises most commonly involving the regional lymph nodes, bone marrow, lung, liver and subsequently the brain (Müller, Homey et al. 2001). Several hypotheses have been put forward to explain this organ sensitivity. Paget’s “seed and soil” hypothesis postulates that certain tumour cells (the seed) have a particular affinity for the environment of certain organs (the soil) (Paget 1889, Fidler 2003). While the specifics of this theory have been revised over time, the current definition states that a population of cells is heterogeneous, with cell that have different angiogenic, invasive and metastatic properties. Therefore, different cells will survive to reach metastatic sites where, at this site, the outcome of successful growth depends on interaction between the tumour cell and homeostatic mechanisms within the area (Fidler, Yano et al. 2002). Therefore, the tumour cells that possess the characteristics that best fit the metastatic site will thrive and proliferate. The pathologist James Ewing threw this hypothesis into doubt during the 1920s. He
argued that no single organ is more hospitable to tumour cells than any others and instead proposed the vascular flow hypothesis (Ewing 1928). This hypothesis states that it is the route that the cells take in the vasculature that determines the site of distant metastasis. Following this, breast cancer cells that have reached the vasculature flow through the blood first to the heart and then to the lungs. The cells may become lodged in the capillary beds of the lungs or pass into the systemic circulation and spread to distant sites such as bone and eventually the central nervous system (CNS) (Chambers, Groom et al. 2002). However, while logical, this theory does not fully explain the high rate of bone metastases that are found in breast cancer patients and so it is most likely that the true situation is a combination of both theories. The circulation (blood and lymph) will determine which capillary beds the cells become lodged into and it is then the characteristics of the organ that will determine whether the cancer cell will flourish in that site. Therefore, the propensity of a cell to colonise a specific organ may depend on efficient growth once in the site rather than only the ‘homing’ of the cancer cell to a specific site (Koop, Schmidt et al. 1996, Luzz, Macdonald et al. 1998).

A characteristic of breast cancer metastasis is the relatively long period of latency, often up to a decade after remission of the primary tumour, before disseminated cells begin to aggressively colonise a distant site (Karrison, Ferguson et al. 1999, Nguyen, Bos et al. 2009) In particular, HER2+/ER- and basal tumours show the greatest incidence of relapse (Carey, Dees et al. 2007). The pattern of recurrence follows a double peaked pattern, with metastases presenting at 18-24 months and then at 5-6 years even after chemotherapy (Demicheli 2001).

**1.3.1 Brain metastasis of Breast Cancer**

CNS metastases are often a late complication and present after metastases in other sites have already appeared. Along with lung cancer and melanoma, breast cancer patients present with brain metastases with a far higher frequency than other cancers with an incidence of 10 to 16% (Weil, Palmieri et al. 2005).
However this figure doesn't take into account the incidence of asymptomatic brain metastases that are discovered only at autopsy which, with a rate of about 15%, brings the incidence of breast cancer brain metastases to a worrying 30% (Miller, Weathers et al. 2003, Cheng and Hung 2007). Brain metastases from breast cancer mainly present in the parenchyma, indicating a vascular method of spread (Tsukada, Fouad et al. 1983), or less commonly, in the leptomeninges, whereby they can arise from multiple pathways including haematogenous spread and infiltration from vertebral metastases (Kesari and Batchelor 2003). The propensity to metastasise to the brain is associated with either triple negative status or HER2 overexpression and correlates with decreased disease free survival and overall survival (Kallioniemi, Holli et al. 1991, Lin, Bellon et al. 2004). Furthermore, HER2 positive patients that are treated with the current best practice agent trastuzumab are at a high risk to progress to brain metastases, due to the inability of trastuzumab to penetrate the blood brain barrier (Bendell, Domchek et al. 2003). Additionally, there are indications that HER2 overexpression can, by an as yet unknown method, increase the incidence of brain metastases (Palmieri, Bronder et al. 2007). Current treatment options for brain metastases are limited and patients are prone to relapse. In one study, patients that received adjuvant treatment had a far higher rate of brain metastases of 50% compared to 26% in patients that did not receive adjuvant therapy (Carey, Ewend et al. 2001). In another study, patients with stage IV breast cancer that were treated with epirubicin and docetaxel showed a 30% incidence in CNS relapse (Crivellari, Pagani et al. 2001).

Corticosteroids are used for symptomatic relief and to reduce oedema but chemotherapy is of limited benefit due to the low permeability of most of these agents through the blood brain barrier and/or the acquisition of drug resistance (Lin, Bellon et al. 2004). Therefore radiation, in particular whole brain radiation therapy (WBRT) has become the predominant therapy.

WBRT is effective in patients with three brain metastases or fewer, especially following surgical resection and can enhance median survival by up to 12 months (Weil, Palmieri et al. 2005, Palmieri, Smith et al. 2007) However the survival rate after one year is 17% and falls to 2% at two years, indicating that further avenues need to be pursued (Mahmoud-Ahmed, Suh et al. 2002).
Furthermore, the side effect profile of WBRT can be severe and include fatigue, cerebral oedema, acute and long-term radiation-induced neurocognitive degeneration (McTyre, Scott et al. 2013). Recently, the concomitant use of stereotactic radiosurgery, that is, the use of single treatment beams of radiation precisely focused at intracranial targets, along with whole brain radiation has been shown to confer similar benefits to surgery and radiation (Andrews, Scott et al. 2004, Linskey, Andrews et al. 2010). Stereotactic radiosurgery alone has shown benefit, with one study reporting a median survival after brain diagnosis of 13.9 months and a median survival after radiosurgery of 7 months (Lederman, Wronski et al. 2001). Another study reported an even more encouraging 13 month median survival after radiosurgery and 18 month mean survival after brain diagnosis (Firlik, Kondziolka et al. 2000). This targeted therapy also limits the development of severe adverse effects as seen in WBRT as a smaller part of the brain is being targeted. However, despite these treatments, the mean 1 year survival from brain metastases is estimated at around 20% (Altundag, Bondy et al. 2007). Therefore, there is an urgent need for novel targeted treatments for brain metastases.

1.3.2 Integrins

Metastatic progression is typically associated with altered tumour cell affinity for the extracellular matrix (ECM), which is in part mediated by changes in number and composition of integrins. Integrins are cell surface transmembrane proteins that are the major receptors for cell adhesion to ECM proteins as well as cell-cell adhesion.

The family of integrin receptors is comprised of 18 α-subunits and 8 β-subunits that assemble to form 25 distinct heterodimeric proteins, each with its own affinity for a specific set of ligands. (van der Flier and Sonnenberg 2001, Hood and Cheresh 2002). The affinity of an integrin for its ligand may depend on the cell type it is expressed on and the specific activating stimuli. Each integrin subunit has multiple domains that change conformation to mediate different allostERIC states in response to ligand binding. The unliganded, low affinity state
is bent or ‘closed’ and changes to a high affinity ‘open’ state where the cytoplasmic domain opens up in a switchblade-like motion (Moschos, Drogowski et al. 2007). Gene knock out studies have revealed the diverse functions of each integrin, which include neonatal development, haemostasis, inflammation and angiogenesis (Hynes 2002). This is due to the ability of integrins to trigger signal transduction pathways, as well as promoting cytoskeletal linkages. Once ligand bound, dimerised and attached to the ECM, integrins cluster together and recruit signalling proteins (mostly talin and focal adhesion kinase) to form structures known as focal adhesions, through which proteins such as kinases and scaffolding molecules are recruited (Desgrosellier and Cheresh 2010). Since integrins lack integral kinase activity, it is through these focal adhesions that integrins mediate signalling events. Downstream signalling cascades include Raf/ERK/MAPK, Src family kinases, PI3K/Akt, NFκB and Jun (Moschos, Drogowski et al. 2007). Some of the signalling pathways activated by integrins are the same as those activated by growth factors such as EGF and are used by normal cells to block apoptosis and stimulate cell cycle progression (Hynes 2002). It is these effects that are exploited by cancer cells. In fact, recently it has been demonstrated that some oncogenes and growth factors require interactions with integrins for tumour progression and initiation (Goodman and Picard 2012).

1.3.2.1 Evidence for integrin signalling and cancer

Increased integrin receptor expression is linked to tumorigenicity and metastasis in several cancers. In solid tumours, ligands expressed by endothelial cells, such as αvβ3, αvβ5, α5β1, α6β4, α4β1, and αvβ6 show increased expression that correlates with disease progression in a variety of solid and liquid tumour types (Desgrosellier and Cheresh 2010). However there are a few integrins that lose expression during malignant transformation, such as α2β1 in breast cancer. Forced re-expression of α2β1 reduced the mobility and migratory ability of Mm5MT cells, indicating that α2β1 integrin could function as a tumour suppressor (Zutter, Santoro et al. 1995).
There is substantial evidence for the roles that integrins play in breast cancer metastasis. MDA-MB-231Br breast cancer cells have been shown to utilise a variety of integrins for metastasis including \( \alpha_v\beta_5 \) for cell proliferation and attachment and \( \alpha_v\beta_3 \) for migration (Sung, Stubbs et al. 1998). These cells also utilise \( \alpha_1\beta_1 \) and \( \alpha_v\beta_3 \) integrins to mediate attachment and invasion into bone (Vloedgraven, Papapoulos et al. 1997). Indeed, \( \alpha_v\beta_3 \) is overexpressed in bone residing metastatic breast cancer cells and also mediates the activation and attachment of osteoclasts to bone matrix, facilitating invasion by breast cancer cells (Liapis, Flath et al. 1996, Takayama, Ishii et al. 2005, Carter, Micocci et al. 2015). Enhanced \( \alpha_v\beta_3 \) expression increases the adhesion and haptotactic migration of 66Cl4 breast cancer cells in vitro (Sloan, Pouliot et al. 2006). There is also a link between \( \alpha_v\beta_3 \) expression and angiogenesis. The expression of \( \alpha_v\beta_3 \) is absent on normal endothelial cells but upregulated during angiogenesis (Brooks, Clark et al. 1994). Increased \( \alpha_v\beta_3 \) expression in 66cl4 breast cancer cells increase metastasis to multiple organs (Sloan, Pouliot et al. 2006, Carter, Micocci et al. 2015).

Levels of \( \alpha_3\beta_1 \) integrin have been shown to be low in normal epithelial cells and primary tumours but increases in metastatic lesions (Natali, Bartolazzi et al. 1993). Studies have also implicated \( \alpha_3\beta_1 \) in promoting adhesion, migration and invasion of metastatic breast cancer cell lines (Tawil, Gowri et al. 1996, Morini, Mottolese et al. 2000). Blockade of the \( \beta_1 \) subunit inhibits the adhesion and migration of T24 human bladder carcinoma cells (Heyder, Gloria-Maercker et al. 2005). Silencing of integrin \( \alpha_3\beta_1 \) decreases adhesion, proliferation and spreading of breast cancer cells, as well as impedes metastasis to lung (Zhou, Gibson-Corley et al. 2014).

Integrin \( \beta_4 \) has been shown to be strongly associated with basal breast cancers and linked to aggressive behaviour (Lu, Simin et al. 2008). Many studies have characterised the ‘switch’ \( \beta_4 \) undergoes during cancer development from an adhesion molecule to a signalling component receptor. During cancer progression, integrin \( \beta_4 \) is mobilised from hemidesmosomes in response to Akt phosphorylation and acts to facilitate migration and invasion (Lipscomb and Mercurio 2005).
Integrin antagonists have entered clinical trials. Cilengitide, an RGD pentapeptide inhibitor of αvβ3 and αvβ5 integrins, has been extensively studied and has entered phase III clinical trials for glioblastoma (NCT00689221), as well as phase II clinical trials for head and neck as well as lung cancer (NCT00842712) (Desgrosellier and Cheresh 2010, Reardon and Cheresh 2011). The anti-αvβ3 antibody Vitaxin, has been tried in late stage disease as an anti-angiogenic factor and in early trials has shown disease stabilization with very limited toxicity (Posey, Khazaeli et al. 2001). Another study showed that using the natural compound curcumin blocks β4 signalling in MDA-MB-231 and MDA-MB-435 breast cancer cell lines and results in decreased cell motility and blocks downstream Akt signalling (Im Kim, Huang et al. 2008). Taken together, these observations indicate that integrins are valid therapeutic targets for multiple cancers, including breast cancer.

1.3.3 Models of breast cancer metastasis

While breast cancer cell lines are useful to test basic parameters and responses to various cancer treatments in vitro, responses that are seen in cell lines are often very different to those that are seen in vivo due to different selective pressures from the tumour micro-environment and immune system, among others. In order to develop and characterise novel cancer treatments, there is a requirement for mouse models that faithfully recapitulate the tumour growth and metastatic progression that is seen clinically.

One such strategy is the use of mouse xenograft models, where human tumour cells from patients are transplanted into immunocompromised mice. The use of this strategy shows merit as it better phenocopies human tumours, tumour stem cell activity and drug responses (DeRose, Wang et al. 2011).

However, although xenograft models are good for assessing events within the tumour, it is becoming increasingly evident that tumour-stroma interactions as well as immune system modulation are just as important. Therefore, a fully clinically relevant mouse model that faithfully recapitulates primary tumour growth and metastasis requires orthologous extracellular matrix (ECM), the species-matched stromal-tumour interactions and a functional immune system.
Syngeneic models can either be transgenic mouse models or transplantable mouse cell lines. There are a variety of transgenic mouse models that rely on transforming factors to drive tumour development. The murine mammary tumour virus (MMTV)-polyoma middle T antigen (PyMT) mice, which carry the oncogenic polyoma middle T virus, develop metastatic mammary adenocarcinoma as soon as 3 months of age (Guy, Cardiff et al. 1992). MMTV-neu mice, which carry an overexpression of the ERBB2 or neu also develop metastatic mammary carcinoma spontaneously, although with a longer latency period of 29-52 weeks (Guy, Webster et al. 1992). Other transgenic mice include those with induced expression of WNT1/p53 or BRCA1/p53, with similar kinetics (Varticovski, Hollingshead et al. 2007). While these transgenic mice develop spontaneous tumours with a full stroma and immune profile, the long latency, variability, incomplete penetrance and reportedly low incidence of metastases, indicates the need for a faster and more reliable mouse model (Varticovski, Hollingshead et al. 2007).

Syngeneic transplanted tumours have the advantage that they can be inoculated into the desired primary site and proceed in a period of weeks rather than months with fully intact stroma and immune systems. One of the best transplantable tumour series in current use is the 4T1 tumour series. Cells from this series were originally isolated from a single autochthonous tumour in a Balb/cfC3H mouse (Dexter, Kowalski et al. 1978). Further cloning isolated subtypes that are non-metastatic (67NR, 168FARN), weakly metastatic to lungs (66Cl4), highly metastatic to lung and liver (4T1) and highly metastatic to bone and lungs (4T1.2) as shown in Figure 1.3 (Aslakson and Miller 1992, Lelekakis, Moseley et al. 1999). As such, the 4T1 series is widely used to study metastatic breast cancer. 4T1 variants have several characteristics that make them ideal models; they are easily transplantable, and metastatic disease develops spontaneously from the primary site in a manner that is similar to human breast cancer (Pulaski and Ostrand-Rosenberg 2001). The primary tumours are also easily resectable to enable comparison to the clinical setting, and they are relatively easy to manipulate in vitro and in vivo. In our laboratory we have inserted the fluorescent markers mCherry and luciferase with relative ease to enable precise quantitation of metastatic burden. More recently in our
laboratory, we have isolated a brain metastatic cell line, denoted 4T1Br4. This cell line was isolated from a rare brain metastasis from a 4T1 tumour in a Balb/c mouse. After serial \textit{in vivo} transplantation, the robust 4T1Br4, brain metastatic cell line was cloned, which forms brain metastases after orthotopic injection in up to 80% of animals.

\textbf{Figure 1.3: Distribution of spontaneous metastases in mice bearing different tumour lines.} Metastasis was measured using RT-qPCR. Average Relative Tumour Burden scores from an experiment using five mice per tumour line. Tumour lines were grouped into three categories based on metastatic burden: non-metastatic (67NR), weakly metastatic (168FARN and 66cl4), and highly metastatic (4T1.2 and 4T1.13). From (Eckhardt, Parker et al. 2005).

1.4 Pim family kinases

1.4.1 Structure and activity of Pim family members

The Pim kinase family is a family of survival kinases that are comprised of Pim-1, Pim-2 and Pim-3. Pim-1 was originally identified as a proviral insertion site of the Moloney Murine Leukaemia Virus in the virus-induced murine T cell lymphomagenesis (Cuypers, Selten et al. 1984). Subsequently the highly homologous human Pim-1 gene was identified (Nagarajan, Louie et al. 1986) and both murine and human Pim-1 genes were found to encode a highly conserved serine/threonine kinase (Padma and Nagarajan 1991); (Hoover, Friedmann et al. 1991). Both mouse and human Pim-1 genes encode two proteins of 33kDa and 44kDa respectively, using an alternative translation initiation site of an upstream
CUG codon (Figure 1.4) (Saris, Domen et al. 1991). The 44kDa isoform possesses an additional proline-rich motif and so may interact with an additional set of proteins, such as the SH3 family of proteins (Linn, Yang et al. 2012). The 33kDa protein is located in the cytosol and nucleus while the 44kDa protein is located in the cytosol and cell membrane (Pircher, Shuqing et al. 2000, Xie, Xu et al. 2005, Linn, Yang et al. 2012). Studies on human recombinant Pim-1 have identified a Pim-1 substrate recognition sequence of (Arg/Lys)3-X-Ser-Thr-X' where X' is neither a basic nor a large hydrophobic residue (Friedmann, Nissen et al. 1992).

Retroviral insertion site cloning revealed Pim-2 activation was a late event in tumourigenesis that can compensate for the loss of Pim-1 in Pim-1 knock out mice (Breuer, Cuypers et al. 1989). Pim-2 is located on chromosome 11, has 51% sequence homology to Pim-1 and through the use of alternate AUG codons has three isoforms with molecular weights of 30 kDa, 38 kDa and 40 kDa (Figure 1.4) (Brault, Gasser et al. 2010). Pim-3 was originally identified as KID-1 (protein Kinase Induced in Depolarisation), and was found to be significantly upregulated in specific areas of the hippocampus and cortex in response to kainic acid and electroconvulsive shock-induced seizures, as well as in PC12 cells (Feldman, Vician et al. 1998). However it was renamed Pim-3 when it was found it showed high sequence homology with the Pim-1 (57.1%) and Pim-2 (44%) (Konietzko, Kauselmann et al. 1999). Pim-3 gene is found on chromosome 22 and in contrast to the other members of the family encodes only a single isoform (Figure 1.4) (Fujii, Nakamoto et al. 2005).

Unlike most serine-threonine kinases, the three Pim kinases are constitutively active once translated, and require no post-translational modifications for activity. This is because they adopt a constitutively active conformation that is stabilised by extensive hydrophobic and hydrogen bonds between the activation and catalytic loops (Qian, Wang et al. 2005). While post-translational modifications are not necessary for activation of Pim kinases, they do contribute to sustaining the absolute level of kinase activity by enhancing the stability and half-life of Pim-1. This means that the activity of Pim kinases depends largely on the absolute levels of Pim protein in the cell. Consequently, their expression is under tight regulation. Pim-1 has a half-life of around 10 minutes in normal tissues, though this can be increased up to 100 minutes in

**Figure 1.4 Pim-1 genes, transcripts and proteins.** Pim genes are located in different locations in the mouse (black numbers) and human (orange boxes). Pim mRNA transcripts are encoded by 6 exons (dark blue boxes). Different protein isoforms are synthesised using alternative translation initiation sites (solid and dashed arrows) and additional codons present at the 5’ end of these mRNAs. Pim protein isoforms have different molecular masses but retain their kinase activity. Pim kinases have no regulatory domain and a white box indicates their highly conserved kinase domain. From (Nawijn, Alendar et al. 2011).

### 1.4.2 Regulation of Pim kinase expression in normal tissues

Pim kinases are growth and survival kinases that are expressed at high levels in developing tissues and decrease in expression in most normal adult tissues. Pim-1 is highly expressed in foetal liver and spleen during rapid development but expression is shut off in the corresponding adult tissues (Amson, Sigaux et al. 1989). Pim-1 is also expressed in normal haematopoietic and lymphoid cells as well as testis and mammary tissue in both mouse and man during embryonic development (Amson, Sigaux et al. 1989, Padma and Nagarajan 1991, Saris, Domen et al. 1991, Wingett, Reeves et al. 1992, Gapter, Magnuson et al. 2006).

However, despite this difference in expression, functional redundancy has been shown in vitro and in vivo as all three share a common phosphorylation consensus sequence (Bullock, Debreczeni et al. 2005). For example, Pim-2 can substitute for Pim-1 in lymphomagenesis (Van der Lugt, Domen et al. 1995). Moreover, mice deficient in one of the family members show no observable phenotype, further indicating functional redundancy (Mikkers, Nawijn et al. 2004). It is not until Pim1-/- Pim2-/- Pim3-/- mice are generated that they begin to show some phenotypic changes. The mice are viable and fertile, however they display reduced size throughout life and reduced hematopoietic cell proliferation in response to growth factors and cytokines (Mikkers, Nawijn et al. 2004). This indicates that inhibition of only one of the Pim kinases may not be sufficient to induce a phenotypic response, and rather, pan-Pim kinase inhibitors may yield the best results.

Indeed, recently the idea of pan-Pim kinase inhibition has gained traction. They have traditionally been difficult to develop due to the fact that Pim-2 has a low affinity for ATP and so requires a very potent inhibitor to block its kinase activity (Garcia, Langowski et al. 2014). However, several pan-inhibitors have recently been developed with promising results. The pan-Pim kinase inhibitor AZD1208 has been investigated and validated in models of acute myeloid leukaemia and Myc driven prostate cancer where it functions as a radiosensitiser (Keeton, McEachern et al. 2014, Kirschner, Wang et al. 2015). AZD1208 entered Phase 1 clinical trials as a treatment for malignant lymphoma (Clinical trials identifier: NCT01588548). LGH447, a compound from Novartis, also demonstrated activity against several models of acute myeloid leukaemia and multiple myeloma (Langowski, Holash et al. 2013), which also culminated in a Phase 1 clinical trial (Clinical trials identifier: NCT01456689) for multiple myeloma where it proved to be well tolerated and exhibited signs of single agent efficacy (Raab, Ocio et al. 2014).

However, although there is merit in the clinical use of pan-Pim inhibitors, there are functions of Pim-1 that are not compensated for by the other isoforms, which are highlighted below. Thus, while the regulation and function of Pim-1, Pim-2 and Pim-3 isoforms in cancer progression and metastasis is discussed in
more detail below, in the interest of teasing apart the specific functions and mechanisms by which Pim-1 is mediating its effects, studies specifically documenting the expression and function of Pim-1 in breast cancer will be highlighted.

1.4.3 Pim kinase induction and functional regulation

The expression of the Pim kinases is for the most part regulated at the transcriptional level. Pim kinase induction is mediated by tyrosine kinase signalling, mostly mediated through the Janus kinase (JAK) receptor (Wernig, Gonneville et al. 2008). The expression of Pim kinases is enhanced by a variety of cytokines, hypoxia, growth factors and other mitogenic signals. Several cytokines including IL2, IL-7, IFNγ and G-CSF bind to JAK, causing activation of two members of the Signal Transducers and Activators of Transcription (STAT) family of factors; STAT3 and STAT5, which both bind to the pim-1/pim-2/pim-3 promoters, leading to an upregulation of Pim kinase transcription (Bachmann and Möröy 2005). Once translated, the degree of each Pim kinase expression is mainly regulated by the stability of the protein, as discussed above. As well as in response to cytokines and growth factors, the mRNA and protein expression of Pim-1 kinase (but not Pim-2 or Pim-3) is also increased under hypoxic conditions. This is due to an inhibition of ubiquitin-mediated proteasome degradation, thus increasing its half-life (Chen, Kobayashi et al. 2009).

The expression of Pim-1 is regulated post-transcriptionally by eIF4E, which enhances the rate of translation of the Pim-1 protein during periods of growth (Hoover, Wingett et al. 1997). Its stability is enhanced through interaction with the heat-shock protein Hsp90 as it protects Pim-1 from ubiquitin-proteasomal degradation, as well as helps to maintain kinase activity (Mizuno, Shirogane et al. 2001, Shay, Wang et al. 2005). Conversely, the serine/threonine phosphatase Protein Phosphatase 2A (PP2A) dephosphorylates Pim-1, which facilitates its proteasomal degradation and decreases the level of Pim-1 protein both in vitro and in vivo (Losman, Chen et al. 2003).
1.4.4 Pim kinases and Cancer

The loss of Pim-1 expression in many adult tissues suggests that inappropriate expression of Pim-1 could contribute to uncontrolled growth and malignant transformation. Indeed, the levels of Pim-1 are markedly increased in many tumours, with highest expression in rapidly growing solid tumours such as the 5637 bladder carcinoma cells compared to slower growing tumours such as HeLa or CaOV (Liang, Hittelman et al. 1996). The restricted expression in normal adult tissues compared to the overexpression seen in cancer cells has led to the suggestion that Pim-1 could be a good specific target for cancer therapy.

Overexpression of Pim-1 in murine lymphoid cells induces a low incidence of lymphomas in a dose dependent fashion, as well as increased susceptibility to chemical and Moloney Murine Leukaemia Virus (MoMuLV)-induced lymphomagenesis (van Lohuizen, Verbeek et al. 1989, Domen, van der Lugt et al. 1993). The long latency and low incidence of lymphoma development in the Pim-1 transgenic mice suggests that Pim-1 alone is insufficient for malignant transformation and so it is classified as a weak oncogene. When Pim-1 transgenic mice were infected with MoMuLV, lymphomas developed much faster, at a latency of 7-8 weeks compared to 3-7 months without viral infection (van Lohuizen, Verbeek et al. 1989). In virus-induced lymphomas, levels of c-Myc were also activated, suggesting that these two oncogenes cooperate to accelerate the development of lymphomas. This has also been demonstrated in prostate cancer, with overexpression of Pim-1 in PC3 prostate cancer cells shown to increase c-Myc levels and vice-versa (Ellwood-Yen, Graeber et al. 2003, Chen, Chan et al. 2005). Pim-1 phosphorylates Histone H3 on Ser10, allowing increased transcription of c-Myc protein, and Pim-1 phosphorylates and stabilises c-Myc on Ser62 thereby resulting in augmented transcriptional activity of c-Myc (Zippo, De Robertis et al. 2007, Zhang, Wang et al. 2008). This indicates a strong relationship between the two oncogenes.

Recently, more studies have focused on the contribution of Pim-2 and Pim-3 kinase to cancer progression. Pim-2 is identified as a pro-viral insertion site, which is associated with the rapid promotion of malignant T cell lymphoma (Breuer, Cuypers et al. 1989) and is arguably the most relevant Pim kinase in the
progression of hepatocellular carcinoma (Weirauch, Kürz et al. 2017). The expression of Pim-3 is markedly enhanced in malignant tumours of the liver, pancreas, colon and stomach compared to the normal organs, with aberrant expression occurring early on in carcinogenesis (Mukaida, Wang et al. 2011).

While the role of Pim kinases has been extensively studied in haematological, prostate and pancreatic cancers, less is known about their role in breast cancer. Recent studies have shown that Pim-2 has an important function in TNBC, promoting tumour progression and metastasis through activation of STAT3 (Uddin, Kim et al. 2015, Cobb, Hunter et al. 2017). Furthermore, there is evidence that Pim-2 can substitute for Pim-1 in the development of breast cancer (Horiuchi, Camarda et al. 2016). The role of Pim-3 in breast cancer is less clear and few studies have documented its expression and specific function. Instead, Pim-3 seems to be implicated in the development of pancreatic cancer, where it promotes cell survival by inhibition of Bad, facilitating cell cycle progression and inducing the expression of c-Myc (Li, Popivanova et al. 2006, Mukaida, Wang et al. 2011).

In contrast, Gapter and colleagues found that Pim-1 was elevated in a range of human breast cancer cell lines, with the most invasive lines showing the greatest expression of Pim-1 (Gapter, Magnuson et al. 2006). *In vitro*, Pim-1 expression is elevated in a large number of TNBC cells and knockdown of Pim-1 (but not Pim-2 or Pim-3) by shRNA decreases cell growth and sensitises these TNBC cells to chemotherapy agents (Brasó-Maristany, Filosto et al. 2016). *In vivo*, the inhibition of Pim-1 by small molecule inhibitors is effective in TNBC xenograft tumours with elevated levels of Pim-1 and Myc (Horiuchi, Camarda et al. 2016). Elevated Pim-1 expression correlates with triple negative status and high clonogenic ability in a cohort of human breast cancer samples and is associated with poor prognosis in TNBC patients (Maristany et al. 2013; Horiuchi, Camarda et al. 2016). Moreover, a recent study indicates that only the long 44kDa Pim-1 isoform appears to be expressed in breast cancer (Tipton, Nyabuto et al. 2016). These studies highlight the need to explore the relationship between Pim-1 and breast cancer progression in more detail, leading to the basic focus of my project.
Figure 1.5: Regulation of Pim-1 and downstream targets. Cytokines or growth factors bind their receptors, and activate Pim-1 transcription through JAK/STAT signalling. Pim-1 is stabilised by Hsp90 and targeted for proteasomal degradation by PP2A. Pim-1 promotes G1/S cell cycle progression by interacting with p21 and CdC25A, and promotes G2/M cell cycle progression by interacting with P27Kip1 and CDC25C. Pim-1 promotes cell survival by inhibiting Bad and FOXO1a/3a and by enhancing the actions of PAP-1 and P100. Pim-1 also contributes to resistance to chemotherapy by modulating ABC transporter activity.
1.5 Downstream targets of Pim-1

1.5.1 Pim-1 and cell cycle progression

Accelerated progression through cell cycle checkpoints enhances the proliferation rate of cancer cells and is often dysregulated by oncogenes, including Pim-1. Pim-1 enhances cell cycle progression at several stages of the cell cycle. In particular, several mediators of the G1 to S cell cycle checkpoint are targets of Pim-1 (As summarised in Figure 1.5). Cdc25A phosphatase is phosphorylated and inactivated by Pim-1, initiating G1 to S progression (Mochizuki, Kitanaka et al. 1999). Pim-1 also phosphorylates and inactivates the cyclin-dependent kinase inhibitor p21, causing it to dissociate from its binding partner PCNA and translocate from the nucleus into the cytosol (Zhang, Wang et al. 2007). Inactivation of p21 removes its inhibitory block on the cell cycle and allows the resumption of the G1 to S transition. Recently, Pim-1 has been shown to both decrease the expression and inactivate the activity of the cyclin-dependent kinase inhibitor p27Kip1 (Morishita, Katayama et al. 2008, Lin, Beharry et al. 2010). As p27Kip1 binds and impedes the activity of cyclin dependent kinase 2, inhibition of p27Kip1 further promotes the G1/S transition. The inverse relationship between Pim-1 and p27Kip1 has been correlated with poor prognosis in leukaemia, as well as prostate and breast cancers (Catzavelos, Bhattacharya et al. 1997, Morishita, Katayama et al. 2008, Lin, Beharry et al. 2010). In addition to regulating the G1/S transition, Pim-1 facilitates the G2 to M transition by phosphorylating both Cdc25C-associated kinase-1 (CTAK1) as well as the Cdc25C phosphatase itself, which allows Cdc25C to positively regulate the cell cycle switch to M phase (Bachmann, Hennemann et al. 2004, Bachmann, Kosan et al. 2006). Pim-1 also phosphorylates the nuclear mitotic apparatus, causing recruitment and formation of mitotic spindles, a process that is vital for the correct completion of mitosis (Bhattacharya, Wang et al. 2002).
1.5.2 Pim-1 as a pro-survival protein

One of the main mechanisms by which Pim-1 promotes survival is by regulating the activity of Bcl2 family proteins. The Bcl2 family of proteins is comprised of three groups. Firstly, the BH3 proteins (BIK, EGL-1, BIM, BMF, NOXA, BID, BAD, BNIP3 and Beclin-1) that sense cellular stress and then activate the second group of pro-apoptotic proteins Bax or Bak which permeabilise the mitochondrial membrane, causing caspase activation (Shamas-Din, Kale et al. 2013). The third group are the anti-apoptotic proteins Bcl2 and BclXL which inhibit both BH3 proteins and Bax/Bak (Shamas-Din, Kale et al. 2013). Pim-1 (and Pim-2) inactivates the pro-apoptotic protein Bad by phosphorylating Ser112, Ser136 and Ser155, causing inhibition of apoptosis (Aho, Sandholm et al. 2004, Macdonald, Campbell et al. 2006).

Other anti-apoptotic actions of Pim-1 have been documented. This includes inhibiting the activities of the pro-apoptotic forkhead transcription factors FOXO1a and FOXO3a by phosphorylation and inactivation (Morishita, Katayama et al. 2008). Pim-1 also contributes to pro-survival signalling by activating c-Myb, p100 and PAP-1. C-Myb is a proto-oncogene which functions as a DNA-binding transcriptional activator and is elevated in several proliferating cell types as well as several types of human tumours (Leverson, Koskinen et al. 1998).

Pim-1 also acts to promote survival under hypoxic conditions. Under these conditions Pim-1 expression is increased and acts to prevent the loss of mitochondrial membrane potential, thereby inhibiting caspase activation and apoptosis (Chen, Kobayashi et al. 2009). During stressful conditions several intracellular mitogen activated protein kinase (MAPK) pathways are activated to facilitate apoptosis. One of the important players in these cascades is apoptosis signalling kinase 1 (ASK1). Once phosphorylated, ASK1 activates MKK4/MKK7 and MKK3/MKK6, which phosphorylate and activate JNK and p38 MAPK respectively, leading to apoptotic signalling (Dhanasekaran and Reddy 2008, Gu, Wang et al. 2009). This occurs either through the upregulation of pro-apoptotic genes (via c-Jun), or by altering the activities of mitochondrial proteins via phosphorylation, leading to caspase-3 activation and apoptosis (Dhanasekaran...
Pim-1 phosphorylates ASK1 on Ser83, which inhibits its kinase activity and so decreases the extent of cell death following a hypoxic challenge (Gu, Wang et al. 2009).

Pim-1 also promotes survival by altering cellular metabolism under stressful conditions. Expression profiling showed that reduction in Pim-1 levels altered the cellular redox state of the cell by reducing certain intermediates in the glycolytic and pentose phosphate pathways, leading to abnormal oxidative phosphorylation in the mitochondria (Song, An et al. 2014). Pim-1 KO mice also show decreased levels of the antioxidants superoxide dismutase, glutathione peroxidase and peroxiredoxin 3, the loss of which lessens the ability of the cell to deal with reactive oxygen species and oxidative stress. In mouse embryonic fibroblasts, Pim kinase knockout or pharmacological inhibition blocks mammalian target of rapamycin (mTORC1) signalling by activating its negative regulator AMP-dependent protein kinase (AMPK) (Beharry, Mahajan et al. 2011). This increased the ration of AMP: ATP and resulted in a reduced rate of protein synthesis and decreased cell proliferation.

1.5.3 Pim-1 promotes migration and invasion of cancer cells

Recently there has been increasing evidence that Pim-1 signalling promotes the migration and invasion of cancer cells, though the precise mechanisms still remain incompletely understood. Pim-1 is a regulator of CXCL12 and CXCR4 – mediated homing and migration. CXCL12 along with its chemokine receptor CXCR4 mediates homing and migration signalling in leukemic stem cells and other cells in the bone marrow niche, a mechanism well exploited in both solid and liquid cancers (Croker and Allan 2008). In particular, CXCR4 expression correlates with metastatic propensity in breast cancer, with highly metastatic MDA-MB-231 cells expressing higher levels compared to non-metastatic MCF7 cells (Dewan, Ahmed et al. 2006). Furthermore, inhibition of CXCR4/CXCL12 signalling in breast cancer decreases the metastatic propensity of the cells (Liang, Wu et al. 2004).

Pim-1 phosphorylates CXCR4 on Ser339, and Pim-1 knock out decreased cell surface expression on CXCR4 as well as impaired the ability of cells to
migrate towards a CXCL12 gradient (Beharry, Mahajan et al. 2011). Mouse embryonic fibroblasts deficient in all three Pim isoforms showed decreased growth in vitro and decreased bone invasion in vivo by up to 70% compared to wild type mice (Narlik-Grassow, Blanco-Aparicio et al. 2012).

Silencing of Pim-1 in prostate or squamous carcinoma cells decreases the rate of migration and invasion (Santio, Vahakoski et al. 2010). This was validated with the use of the inhibitor DHPCC-9 in a dose-dependent fashion. The authors speculated that this effect could be due to NFATc signalling, an established target of Pim-1. However, considerably more work needs to be done to elucidate the precise mechanisms by which Pim-1 promotes migration and invasion both in vitro and in vivo.

1.6 Overall objectives, hypothesis and aims

The overall objectives of this study are to characterise the prognostic value and functional contribution of Pim-1 to breast cancer metastasis. We hypothesise that tumour cell expression of Pim-1 contributes directly to breast cancer metastasis, and that Pim-1 could be a useful prognostic marker.

The specific aims of this thesis are:

1. To investigate the expression of Pim-1 in normal and tumour tissue in order to evaluate the merit of Pim-1 as a prognostic factor.

2. To examine whether Pim-1 regulates proliferation and integrin-dependent adhesion, migration and invasion of breast cancer cells in vitro.

3. To test the effect of inhibiting Pim-1 on breast cancer metastasis in vivo.

The results of our investigation are presented in the following two chapters concluding with a summary of our overall findings and a discussion of the potential implications for the treatment of breast cancer patients and for future research avenues.
2. Materials and Methods

2.1 Materials

2.1.1 General chemicals and reagents

Laboratory reagents used in this thesis were obtained from commercial sources as follows: Acetone, acetic acid (HOAc), methanol (MeOH), absolute ethanol (EtOH), chloroform, and hydrogen peroxide from Merck, USA. Ethylenediaminetetraacetic acid (EDTA), tris and phenol were from Amresco, USA. Kaleidoscope pre-stained protein standards, polyvinylidene fluoride (PVDF) membrane and Tween-20 were from Bio-Rad Laboratories, Richmond, CA, USA. Bovine serum albumin (BSA) from Roche, Basel, Switzerland. Sodium chloride (NaCl) and hydrogen chloride (HCl) from Biochemicals, AUS. Alpha-Modified Minimum Essential Medium (αMEM), Dulbecco’s Modified Eagle Medium (DMEM), foetal bovine serum, 1% penicillin/streptomycin, L-glutamine, 0.5% trypsin/EDTA from Gibco, USA. Insulin, trypsin, red blood cell lysis buffer, trypan blue, isoamyl alcohol, sodium citrate tribasic (citrate buffer), Triton-X 100, Hanks balanced salt solution, HEPES, β-mercaptoethanol from Sigma-Aldrich, USA. Matrigel from BD pharminogen, USA. SDS and proteinase K from Promega, USA. dNTPs from Invitrogen, USA. ABC kit and Vectashield from Vectalabs, USA. Neutral buffered formalin (NBF) and haematoxylin from Australia Biostain, Australia. 3,3’-Diaminobenzidine (DAB) from Dako, USA, Collagenase 1 from Worthington, USA, DNase 1 from Roche, Switzerland, Taqman Fast Universal PCR master mix, Applied Biosystems, USA, Enhanced chemiluminescence (ECL) western blotting, Amersham/GE Healthcare, UK, Super RX Fuji medical X-ray film, Fuji, Japan, Sorenson’s solution Amber Scientific, Australia.
### 2.1.2 Antibodies

Monoclonal antibodies against the following antigens used for flow cytometric, western blot and immunohistochemical analyses were obtained from commercial sources as follows:

**Table 2.1 Antibodies and concentrations used.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalogue number</th>
<th>Supplier</th>
<th>Application</th>
<th>Concentration/dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ab845</td>
<td>Abcam</td>
<td>Western blotting</td>
<td>1/10 000</td>
</tr>
<tr>
<td>Rabbit anti-human Pim-1</td>
<td>ab85898</td>
<td>Abcam</td>
<td>Western blotting, IHC</td>
<td>WB 1/1000</td>
</tr>
<tr>
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<td>MAB1997</td>
<td>Chemicon</td>
<td>Flow cytometry</td>
<td>1/100</td>
</tr>
<tr>
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<td>559073</td>
<td>BD Pharmingen</td>
<td>Flow cytometry</td>
<td>1/100</td>
</tr>
<tr>
<td>Hamster anti-mouse β3 integrin</td>
<td>553347</td>
<td>BD Pharmingen</td>
<td>Flow cytometry</td>
<td>1/50</td>
</tr>
<tr>
<td>Hamster IgG1 isotype</td>
<td>550344</td>
<td>BD Pharmingen</td>
<td>Flow cytometry</td>
<td>1/100</td>
</tr>
<tr>
<td>Rat anti-mouse β4 integrin</td>
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<td>Jackson Immuno</td>
<td>Flow cytometry</td>
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<tr>
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<td>Lifespan biosciences</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Antibody Type</td>
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<td>Supplier</td>
<td>Application</td>
<td>Dilution</td>
</tr>
<tr>
<td>----------------------------</td>
<td>----------------</td>
<td>-----------------------</td>
<td>----------------------</td>
<td>----------</td>
</tr>
<tr>
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<td>Flow cytometry</td>
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<td>Biorad</td>
<td>Western blotting</td>
<td>1/1000</td>
</tr>
</tbody>
</table>

Abcam (Cambridge, UK); Chemicon; BD Pharmingen, (San Diego, CA, USA); Biorad, (Hercules, CA, USA); Jackson Immuno Research (West Grove, PA, USA); Invitrogen (Carlsbad, CA, USA).

### 2.1.3 Plasmids

For gene knock down studies, the mammalian lentiviral vector pLKO.1 TRC_005 cloning vector and pGipz lentiviral vector were used as depicted in Figure 2.1. pLKO.1 TRC_005 was obtained from the Broad Institute (Cambridge, MA, USA) and pGIPZ was obtained from Dharmaco, Inc. (Lafayette, CO, USA).
Figure 2.1 Schematics of the cloning vectors used to knock down Pim-1.
A) The pLKO TRC_005 vector (Broad Institute, Cambridge, MA) was used to knock down Pim-1 in 4T1Br4 cells and stably transfected cells were selected using puromycin (5 μg/ml). B) The pGipz vector (Dharmacon, Lafayette, CO) was used to knock down Pim-1 in MDA-MB-231Br cells, and cells that had stably incorporated the vector were also selected using puromycin (5 μg/ml).
2.1.4 Cell lines

The tumour lines 67NR, 66Cl4 and 4T1 were provided by Dr Fred Miller (Karmanos Cancer Institute, Detroit, US). These lines are clonal populations of cells derived from a spontaneous mammary carcinoma arising in a Balb/cfC3H mouse (Dexter, Kowalski et al. 1978). The 4T1 line was further sub-cloned to isolate the 4T1.2 cell line in our laboratory (Lelekakis, Moseley et al. 1999, Eckhardt, Parker et al. 2005). The 4T1BM2 cell line was isolated from an in vivo serial selection of spontaneous 4T1 bone metastases (Kusuma, Denoyer et al. 2012). The 4T1Br4 cell line was isolated from brain by serial in vivo selection in our laboratory (Dr Normand Pouliot, unpublished).

The 67NR, 66Cl4, 4T1 and 4T1.2 mouse tumour lines have been transfected previously with a plasmid containing the neomycin resistance gene to enable discrimination of tumour cells from endogenous mouse tissue in a quantitative pCR assay (Eckhardt, Parker et al. 2005). The 4T1BM2 and 4T1Br4 cell lines have been transfected previously with a plasmid containing the mCherry gene to enable discrimination of tumour cells (Denoyer, Potdevin et al. 2011). The human MCF7 and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA). The MDA-MB-231Br cell line was provided by Joan Massague (New York, NY).

2.1.5 Tissue sections

Paraffin sections and cryosections of human breast tissues were obtained from the Peter MacCallum Cancer Centre Tissue Bank.
2.2 Methods

2.2.1 Cell culture methodology

2.2.1.1 Culturing mammary epithelial cell lines

Mouse mammary epithelial lines were maintained in α-minimal essential media (α-MEM) supplemented with 5% foetal calf serum (FCS) and 1% penicillin-streptomycin (P/S). Human mammary epithelial tumour cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM), 10% FCS and 1% P/S. Packaging cell lines were cultured in DMEM, 10% FCS and 1% P/S. All cells were incubated at 37°C with 5% CO2. Cells were passaged when sub-confluent and maintained for a maximum of four weeks.

Prior to experimentation, cell viability was assessed by trypan blue exclusion. Murine cells were detached using 0.01% ETDA in phosphate buffered saline (PBS) to dislodge adherent cells. For human breast cancer cell lines, cells were detached for sub-culture using 0.05% Trypsin-EDTA in PBS. For long-term storage, cells were kept in liquid nitrogen in a 10% DMSO/90% FCS solution.

2.2.2 Molecular Techniques

2.2.2.1 Isolation of plasmid DNA from bacteria

Plasmid DNA was extracted using the Qiagen-prep kit. For small-scale preparation, bacteria were grown in 10 mL Luria broth (LB; 85mM NaCl, 0.5% yeast extract, 1% casein hydrolysate) supplemented with ampicillin (100 μg/ml). For large-scale preparation, 400 ml of bacterial culture were prepared and grown at 37°C for 18 hours with shaking. The overnight bacterial cultures were then centrifuged at 10 000 x g for 10 minutes and plasmid DNA was extracted from bacterial pellet using Qiagen-mini or maxi prep for small and large culture respectively.
2.2.2.2 Isolation of genomic DNA

Tissues were snap frozen in liquid nitrogen and homogenized using the MO BIO Tissue PowerLyser 24 tissue homogenizer (Qiagen) before being digested overnight at 55°C in 500 μl digestion buffer (50 mM Tris-HCl pH 7.5, 1% SDS, 100 mM EDTA, 100 mM NaCl, 200 μg/ml proteinase K). The next day, 350 μl of saturated NaCl solution was added and the tubes incubated on ice for 30 minutes. The mixture was centrifuged at 10 000 x g for 10 minutes and the supernatant was transferred to a new tube. Genomic DNA was precipitated by the addition of 2 volumes of 100% ethanol and centrifuged at 10 000 x g for 5 minutes. Genomic DNA was washed with 70% ethanol twice, air-dried and the DNA pellet was resuspended in 400 μl sterile water. The genomic DNA was then quantified by NanoDrop technology (Biolab), diluted to 10 ng/μl for assessment of metastatic burden or stored at -20°C for long-term storage.

2.2.2.3 Isolation of total RNA

Cell pellets from confluent 10 cm² dish cultures were homogenized in 1 ml Trizol reagent. Homogenised samples were incubated at room temperature for 5 minutes. Two hundred μl of chloroform was added and the sample vortexed. The samples were then centrifuged at 12 000 x g at 4°C for 15 min. The upper, aqueous phase containing RNA was transferred into a fresh tube and precipitated with 500 μl of isopropanol. The samples were mixed by inversion, incubated at room temperature for 10 min and centrifuged at 10 000 x g for 10 min. RNA pellets were washed with one volume of 70% ethanol, centrifuged at 7 500 x g at 4°C for 5 min, and briefly air dried before resuspending in 40 μl of Diethylpyrocarbonate (DEPC) water. The concentration and purity of RNA were quantitated by NanoDrop technology.
2.2.2.4 Reverse Transcription

Reverse transcription was performed using M-MLV reverse transcriptase, RNase H Minus M-MLV RT (H-) (Promega). The cDNA was transcribed from 2 μg of RNA. Two μl of 5 μM oligo dTVN primers were added to the RNA and DEPC water was added to bring the total reaction volume to 10 μl. The reaction mixture was incubated at 70°C for 5 min and then placed on ice for 5 min. To this, the following was added (final concentration shown) 1x M-MLV RT (H-) buffer, 0.5 mM dNTPs, 200 U M-MLV RT (H-), 0.01 M DTT, 1 U/μl RNAsin inhibitor and DEPC water to bring the total reaction volume to 100 μl. The reaction mixture was kept at room temperature for 10 min and incubated for a further 50 min at 42°C. cDNA was kept at -20°C for long-term storage.

2.2.2.5 Assessment of mRNA expression levels utilizing SYBR Green I technology

A SYBR Green I (Applied Biosystems) assay was used to determine the relative levels of Pim-1, Pim-2 and Pim-3 compared to that of the housekeeping gene rps27a. Primers were designed using Primer Express version 2.0 program (Applied Biosystems). Forty-five μl of pCR reaction consisting of 25 μl of 2x SYBR Green I master mix, 5 μl cDNA and water was prepared. The pCR reaction mixture was then split into four tubes, each containing 9 μl. To these, a mixture of Pim-1, Pim-2, Pim-3 or rps27a forward and reverse primers (0.1 μM) were added. Total reaction volume was 10 μl. Standard cycling procedures were employed (enzyme activation at 55°C for 2 min, 95°C for 1 min and 55°C for 10 sec repeated for 81 cycles). pCR was performed on the StepOne Plos (BioRad). Specific amplicon formation with each primer pair was confirmed by dissociation curve analysis. Gene expression was measured relative to the expression of rps27a using the following formula: Relative transcript abundance (RTA) = 10 000/2^{(CtGENE-Ctrps27a)}. CT being the cycle threshold value.
2.2.2.6 Assessment of metastatic burden using a multiplex Taqman® assay

Real time RT-PCR using Taqman® chemistry (Applied Biosciences) was used to determine the relative metastatic burden in mouse organs after injection of tumour cells into the left cardiac ventricle. Genomic DNA isolated from tissues (section 2.2.2.2) was subjected to multiplexed real time RT-PCR to detect the cycle threshold (Ct) for vimentin gene (total mouse cells and tumour cells) and mCherry (tumour cells only). By comparing the Ct values of vimentin and mCherry (ΔCt), a score for relative tumour burden was calculated using the following formula: Relative Tumour Burden (RTB) = 10 000 x 1/2

CorrΔCt. CorrΔCt is a ΔCt value that includes correction for the differences in mCherry copy number in each tumour line, as determined by multiplexed real time RT-PCR. Multiplex PCR reactions consisted of approximately (final concentrations shown) 20 μg genomic DNA, 1x Taqman universal PCR master mix, 50 nM vimentin forward and reverse primer mixture, 50 nM vimentin probe, 50 nM mCherry probe and 50 nM mCherry forward and reverse primers mixture. Total PCR reaction was 10 μl. Cycling was performed according to the manufacturers instructions (enzyme activation at 50°C for 2 min, 95°C for 15 min, 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 min, followed by generation of melting curve, 95°C for 1 min, 55°C for 1 min, and 55°C for 10 sec repeated for 81 cycles). All real time RT-PCR experiments were performed on a 7900HT Fast Real-Time PCR system (Applied Biosciences).

2.2.2.7 Pim-1 knock down by shRNA

To characterise the functional effects of Pim-1 in metastatic breast cancer cells we stably silenced Pim-1 expression using short hairpin RNAs (shRNA). The hairpins were cloned into a plasmid expression vector (pLKO TRC_005 for murine cells and pGIPz for human cells) as shown in Table 2.1 and introduced into the cells by lentiviral infection. Cells with the stably integrated hairpins were selected in the presence of puromycin and analysed for Pim-1 mRNA and
protein expression along with cells with a non-silencing control hairpin. MDA-
MB-231Br and 4T1Br4 cells were assessed, with one control KD clone and two
Pim-1 KD clones (Pim-1 shRNA 1 and Pim-1 shRNA 2) for each cell line (Table
2.2). Once the cells were infected with the hairpins, single cell cloning was
carried out and the clones with the greatest Pim-1 knock down from each
construct were isolated for further use. Both Pim-1 mRNA and protein levels
were ascertained to demonstrate the level of knock down after lentiviral
infection.

To ensure that the shRNA constructs were specific to Pim-1 and that they
did not target Pim-2 or Pim-3, we used the BLAT sequence alignment tool (Kent
2002) to map where the shRNA targeted the genome (Fig 2.2)

Table 2.2: Pim-1 knock down by shRNA. Forward and reverse sequences of
shRNA constructs.

<table>
<thead>
<tr>
<th>Forward Oligo Sequence</th>
<th>Reverse Oligo Sequence</th>
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<tr>
<td>Mouse Pim-1 shRNA 1</td>
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<tr>
<td>Mouse Pim-1 shRNA 2</td>
<td>CCGGGCGGAGAAATCTGGAACCTTTACTCGAGTAAGTTC</td>
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Figure 2.2: BLAT sequence alignment
The BLAT sequence alignment tool was used to identify the region of the Pim-1
gene targeted by the shRNA. A) Murine Pim-1 shRNA 1 and shRNA 2 sequences
(black squares) mapped to the mouse genome with the long 44kDa Pim-1
isoform in dark blue and the short 33kDa Pim-1 isoform in light blue.
2.2.3 Protein techniques

2.2.3.1 Detection of Pim-1 protein by western blotting.

Cells (2x10^6) were seeded in 10 cm² dishes in α-MEM. After 24 hours, cells were washed twice with ice cold PBS and then lysed in Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS). Lysates were clarified by centrifugation (10 000 x g, 15 min at 4°C). Protein concentration was determined using the Pierce BCA protein assay kit (Biorad). Forty micrograms of each cell lysate were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane.

Western blots were performed using standard methods (Burnette 1981). Briefly, proteins were transferred from the SDS-PAGE gel to PVDF membranes in protein transfer buffer (0.19M glycine, 0.25M Tris base, 20% methanol) at 100 volt for 1 hour. The membrane was blocked with 5% (w/v) non fat dried milk in PBS, 0.05% Tween 20 for 1 hour and incubated with rabbit polyclonal anti-Pim-1 (1:1000) antibody overnight at 4°C. The membrane was washed 3 times with wash buffer (0.1% Tween 20 in PBS) for 5 minutes and incubated for 1 hour with an appropriate HRP-conjugated secondary antibody in PBS containing 3% BSA and 0.05% Tween 20. The membrane was washed three times as above followed by protein detection using enhanced chemiluminescence (ECL) reagents (Amersham) and Super RX film (Fujifilm) according to the manufacturers instructions.

2.2.4 In vitro cell-based functional assays

2.2.4.1 Adhesion assay

Adhesion assays were performed in serum-free medium (α-MEM supplemented with 0.05% BSA, 1 mM pyruvate, 2 mM L-glutamine and 1% P/S) using a calcein labelling method (Chia, Kusuma et al. 2007). Briefly, triplicate
wells of a 96 well plate were coated overnight at 4°C with 100 μl of extracellular matrix (ECM) protein. LM-511, type IV collagen and vitronectin were used at 5 μg/ml, 20 μg/ml and 10 μg/ml respectively. The wells were blocked with PBS, 1% BSA for 1 hour at 37°C and washed twice with PBS before addition of cells. Calcein-labelled tumour cells were resuspended in serum-free medium and added to the wells at a density of 4x10⁴/100 μl/well. The plates were centrifuged at 400 x g for 1 min and incubated for 30 min at 37°C. Non-adherent cells were removed by gentle washing three times with PBS. Adherent cells were lysed with 1% sodium dodecyl sulphate (SDS) and cell adhesion was determined by measuring specific fluorescence in a Biorad Molecular imager FX reader. Specific adhesion was expressed as the percentage of total cell input and calculated from a standard curve made up of 0, 12.5, 25, 50, 75 and 100 μl of calcein-labelled cell lysate derived from the initial cell suspension. Each experiment was repeated three times and the results shown represent the mean % of total cell input ± standard error of the mean (SEM) of triplicate wells. The statistical differences between treatments were analysed using a one-way ANOVA; p<0.05 was considered significant.

2.2.4.2 Invasion assay

Tumour cell invasion towards serum, LM-511 (5 μg/ml), type IV collagen (20 μg/ml) or vitronectin (10 μg/ml) was measured in triplicate Transwell migration chambers (8 μm pore size) (Corning Inc., Life Sciences, Acton, MA). Tumour cells (2x10⁵) were embedded in 100 μl of a 1:1 mixture of serum free α-MEM medium and Matrigel and placed in the upper well of migration chambers. Set gels were topped up with 100 μl of serum free α-MEM to make a total of 200 μl in the upper well. The cells were allowed to invade and migrate towards 10% serum-containing medium (600μl) or ECM protein coated on the underside of the porous membrane in the absence of serum in the media. The number of cells on the underside of the porous membrane was scored after 24 hours (human cell lines) or 18 hour (mouse cell lines) of incubation at 37°C in 5% CO₂. Following incubation, the inserts were washed in PBS and the apical surface of the membrane was wiped with a cotton swab to remove non-invaded cells. Invaded
cells on the underside were fixed in 10% buffered formalin overnight at 4°C and washed in PBS as above. Invaded cells on the underside were permeabilised in 0.1% Triton X-100 for 5 min at room temp and stained with DAPI (0.5μg/ml in Milli-Q water) for 30 min. The inserts were then washed in PBS and the membranes were cut out and mounted onto a glass slide. Three random fields per membrane were photographed on an Olympus BX-61 fluorescence microscope at 40× magnification and the number of invaded cells was counted using Image J version 1.5 software. Invasion assays were repeated three times and the results shown represent the mean number of invaded cells per field ± SEM of nine fields of view/condition. The statistical differences between assay conditions were analysed using a one-way ANOVA; p<0.05 was considered significant.

For invasion inhibition assays, the cells were pre-treated for 30 minutes on ice with the Pim-1 kinase inhibitor at a range of 0.03-1 μM before adding to the upper wells of the Transwells.

2.2.4.3 Migration Assay

Haptotactic migration of tumour cells towards LM-511 (5 μg/ml), type IV collagen (20 μg/ml) and vitronectin (10 μg/ml), and chemotactic migration towards serum was measured as described for the invasion assays section 2.2.4.2, with the exception that tumour cells (2×10^5/100 μl/well) were placed directly into the upper wells of migration chambers. Tumour cell migration towards the ECM proteins was measured after 3 hours or for migration towards serum, 4 hours of incubation at 37°C in 5% CO₂.

For migration inhibition assays, the cells were pretreated for 30 min on ice with SGI-1775 at concentrations of 0.03-1 μM, before adding to the upper wells of the Transwells.
2.2.4.4 Proliferation Assay

The proliferation of human and mouse cell lines were measured over 5 days in complete α-MEM 5% FCS (mouse cell lines) or DMEM 10% FCS (human cell lines). Cells were seeded at a density of 100/well in six replicate wells of a 96 well plate per time point. At the end of each time point, tumour cells were fixed in 50 μl of 50% trichloroacetic acid. Tumour cells were then washed in water and stained with 100 μl of SRB dye (0.4% sulfrhodamine B in 1% acetic acid) for 30 minutes at room temperature. Unbound dye was washed with 1% acetic acid and specific cell attachment was analysed by adding 100 μl of 10 mM unbuffered Tris (hydroxymethyl) methylamine to solubilize the bound dye. Cell proliferation was quantitated by absorbance at 550 nm on VersaMax plate reader.

To measure the effect of SGI-1776 on tumour cell proliferation, cells were seeded in 6 replicate wells per time point and incubated for 4 hours at 37°C. The medium was removed and fresh medium containing a final concentration of 1 μM SGI-1776 was added. Proliferation was measured over 5 days and quantified by SRB staining as described above.

2.2.5 Animal husbandry and in vivo assays

2.2.5.1 Mouse maintenance

Mice were maintained in a specific pathogen –free environment with food and water freely available. Female 6-8 week old Balb/c mice were obtained from the Walter and Eliza Hall Institute, Melbourne. NOD scid gamma (NSG) mice were bred in house. Mice were monitored every second day for signs of distress or ill health. All procedures involving mice accorded with the National Health and Medical Research Council animal ethics guidelines and were approved by the Peter Mac animal ethics and experimentation committee (AE EC #509).
2.2.5.2 Experimental metastasis assay

NSG mice were anesthetised by isoflurane inhalation and the tumour cells (2×10^5 cells in 0.1 ml saline) were inoculated into the left ventricle of the heart. Mice were monitored every second day for signs of distress and sacrificed on day 21 by anaesthetic overdose. Lungs, brains and both femurs, liver and spine were excised, trimmed of excess connective tissues, snap frozen in liquid nitrogen and processed for quantitation of metastatic burden by TaqMan® assay (see section 2.2.2.7). Prior to sacrifice, approximately 1 ml of blood was collected by cardiac puncture for estimation of viable circulating tumour cells (CTCs) in colony formation assays. Briefly, plasma was removed by centrifugation at 400 × g for 5 minutes followed by red blood cell lysis by hypotonic shock (0.82% NH4Cl, 0.1% KHCO3, 0.038% EDTA). Remaining cells were resuspended in 1 ml of complete medium and plated at densities of 500 μl, 100 μl or 50 μl in 10cm² dishes and allowed to form colonies at 37°C for 10 days. CTC colonies (>50 cells) were fixed and stained with a solution of crystal violet, counted and measured. The statistical differences between groups was analysed using a one-way ANOVA; p<0.05 was considered significant.

2.2.6 Histology and immunostaining methods

2.2.6.1 Tissue fixation methods

Tissues were fixed in 10% buffered formalin at 4°C for 24 hours and processed for paraffin embedding.

2.2.6.2 Haematoxylin and eosin staining

Tissue sections of 4 μm thickness on poly-L-lysine coated slides were dewaxed in histolene for 10 min followed by re-hydration in an ethanol series (100%, 90% and 70% ethanol) and then into deionised water. Sections were stained with Mayers haematoxylin, washed in deionised water and in blue Scott’s
tap water. Tissues were counterstained in eosin, dehydrated in ethanol followed by histolene, mounted in DPX and cover slipped. Basic staining and sectioning of tissue specimens was performed by the Microscopy and Imaging Core Facility (Peter MacCallum Cancer Centre, Melbourne).

2.2.6.3 Pim-1 immunohistochemistry (IHC)

Tissue sections were dewaxed and re-hydrated as above, equilibrated in antigen retrieval buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) and heated by pressure cooker (125°C for 3 minutes, 90°C for 10 minutes). Sections were rinsed extensively in TBS and blocked for 30 min at room temperature in blocking buffer (TBS, 3% BSA, 0.05% Tween 20). Primary antibody against Pim-1 was added and incubated at 4°C overnight under humidified atmosphere. The sections were washed 3 times with wash buffer (TBS, 0.05% Tween 20) and reacted with a biotin-conjugated anti-rabbit secondary antibody (Vector) for 1 hour at room temperature. Unbound antibodies were washed as above and tissues endogenous peroxidases were inactivated in methanol with 0.3% hydrogen peroxide for 30 minutes at room temperature. Specific primary-secondary antibody complexes were detected using the ABC reagent (Vector) and developed using a diaminobenzidine peroxidase (DAB) substrate kit (Vector). All slides were developed in parallel and the reaction stopped before the detection of non-specific staining in control pre-immune serum treated sections. Sections were counterstained with haematoxylin and mounted in DPX neutral mounting medium. Photographs were taken on a BX-60 upright compound microscope (Olympus) at 20x magnification.

2.2.6.4 Flow cytometry

To detect cell surface integrin expression, $5 \times 10^5$ cells were resuspended in blocking medium (DMEM, 2% heat denatured BSA and 2% FCS) and kept on ice for 30 minutes. Blocking medium was replaced with primary antibodies against integrin receptors in labelling buffer (DMEM, 2% FCS) (for antibody concentrations see 2.1.2). As a control replicate, cells were incubated with the
appropriate pre-immune sera or purified isotype-matched antibodies. All cells were treated for 1 hour on ice and washed twice with wash buffer (PBS, 2% FCS) to remove unbound antibodies. The cells were then reacted with the appropriate fluorescein isothiocyanate or allophycocyanin-conjugated secondary antibodies for 45 minutes on ice. Cells were washed twice with wash buffer and resuspended in 500 μl of wash buffer. Viability marker Fluoro Gold was added and cell surface labelling was detected on an LSR flow cytometer (Becton Dickinson, San Jose, CA).

To detect changes in cell surface expression of integrins in response to Pim-1 inhibition, adherent cells were pretreated with 0.03 μM SGI-1776 in complete medium for 4 hours at 37°C before being processed for flow cytometry as above.

2.2.7 Statistical methods

All statistical tests were performed using GraphPad Prism 5.04 (GraphPad software, San Diego, CA). For analysis of repeated measurements a two way ANOVA followed by a Bonferroni Post-Hoc test was performed. Differences between two groups was evaluated using un-paired t-test and a p-value <0.05 was considered to be significant. To compare more than two groups one-way ANOVA followed by a Bonferroni post-hoc test was done. For tissue microarray (TMA) immunohistochemical staining, statistical differences between H-scores were evaluated using Mann-Whitney and Kruskal-Wallis tests. Data are represented as mean ± SEM or triplicates unless otherwise stated in the figure legends.
Chapter 3: The expression of Pim-1 in normal and tumour tissue

3.1 Introduction

Despite the plethora of research into the causes and treatment of metastasis, dissemination of breast cancer to distant organs remains a problem, especially when it colonises the brain. Brain metastasis occurs at a frighteningly high incidence in patients with HER2+ cancer and TNBC (Lin, Bellon et al. 2004, Nam, Kim et al. 2008) and it is still a difficult clinical problem to predict exactly which patients will present with metastasis and where the lesions will present. Therefore, there is an urgent need for the discovery of novel, specific prognostic markers so patients that are at risk of distant relapse can be identified early and monitored before the metastases develop.

In our laboratory, we have developed several clinically relevant syngeneic mouse models of breast cancer metastasis derived from the well-characterised 4T1 mammary tumour line that originated from a single spontaneous tumour in a Balb/cfC3H mouse (Dexter, Kowalski et al. 1978, Aslakson and Miller 1992). When injected into the mammary gland of a mouse, 4T1 cells develop a primary tumour that spontaneously metastasises to the lungs, lymph nodes as well as bone but rarely to brain, and can be used as a clinically relevant model of metastatic disease (Lelekakis, Moseley et al. 1999, Eckhardt, Parker et al. 2005). More recently, we have developed a brain metastatic variant of 4T1, called 4T1Br4. Rare 4T1 cells that had spontaneously metastasised to the brain underwent serial in vivo transplantation to select for cells that metastasise to the brain with high frequency. After four rounds of selection, the 4T1Br4 cell line was isolated. 4T1Br4 tumours give rise to spontaneous metastases in the brain with a metastasis frequency of up to 80%.

Affymetrix gene array profiling was carried out to identify differentially expressed genes in 4T1Br4 versus parental 4T1 tumours. One such gene that showed increased expression in the 4T1Br4 model was Pim-1. Pim-1 is a serine
threonine kinase that has been implicated in cancer. Indeed, the level of Pim-1 markedly increases in many tumours, with highest expression in rapidly growing solid tumours such as 5637 bladder carcinoma compared to slower growing tumours such as HeLa or CaOV (Liang, Hittelman et al. 1996). Pim-1 levels are high during embryonic development in mouse mammary tissues and declines after birth (Gapter, Magnuson et al. 2006). However, unlike most other tissues, the level of Pim-1 expression in the mammary gland epithelia rises again during puberty, pregnancy and lactation, before declining during parturition. This phenomenon is attributed to growth signals from progesterone; the levels of which also rise and fall in the same manner. Immunohistochemical analysis revealed that Pim-1 expression was elevated in both the cytoplasm and nucleus during these stages (Gapter, Magnuson et al. 2006).

Pim-1 has been implicated as a potential prognostic factor for a range of human cancers including prostate cancer, lymphoma and pancreatic cancer (Dhanasekaran, Barrette et al. 2001, Hoefnagel, Dijkman et al. 2005, Reiser-Erkan, Erkan et al. 2008). There have also been some indications that Pim-1 may have prognostic significance in breast cancer. This is based on Pim-1 expression data in a range of human breast cancer cell lines showing that the most invasive lines express the highest level of Pim-1 (Gapter, Magnuson et al. 2006). Pim-1 was also found to correlate with triple negative status and high clonogenic ability in a cohort of human breast cancer samples (Maristany, Grigoriadis et al. 2013). While promising, the significance of these preliminary data needs further investigation in order to ascertain if Pim-1 expression has prognostic significance in regard to metastatic propensity, specific sites of recurrence and/or clinical outcome in breast cancer patients.

The overall objective of this chapter was to document the expression of Pim-1 in normal and cancerous breast tissues as well as to evaluate the potential of Pim-1 as a prognostic indicator in breast cancer patients. We hypothesise that Pim-1 expression correlates with metastatic propensity of mammary carcinoma cell lines and tumours, and that more aggressive and higher-grade tumours have enhanced expression of Pim-1.
Here we surveyed currently available data on the expression of Pim-1 mRNA and protein in normal and tumour tissues using the Protein Atlas database (Uhlén, Björling et al. 2005). The association with Pim-1 with clinical outcome was investigated using the BreastMark prognostic analysis tool, the Kaplan Meier survival analysis tool and the Oncomine database (Rhodes, Yu et al. 2004, Györffy, Lanczky et al. 2010, Madden, Clarke et al. 2013). These databases provided information on mRNA and protein expression of Pim-1 in a range of human tissues and cell lines. However, while useful, the available data was limited with respect to mRNA and protein expression in specific molecular subtypes of breast cancer. To extend current knowledge, we present in this chapter novel data on Pim-1 mRNA and protein expression levels in mouse and human breast tumour cell lines, in mouse and human xenograft breast tumour sections and in a tissue microarray (TMA) of patient-derived primary breast tumour samples in order to fully evaluate the potential use of Pim-1 as a prognostic marker for breast cancer metastasis.

The results presented indicate that Pim-1 expression increases in breast cancer tissues compared to normal breast tissues, albeit at varying levels. Data from these analyses suggest that high Pim-1 expression may correlate with the propensity of breast cancer cells to metastasise to the brain.
3.2 Results

3.2.1 Protein Atlas database analysis

To begin, it was important to assess the data currently available on the expression levels of Pim-1 in normal and cancerous tissues.

Analysis of the Protein Atlas database revealed that the expression of Pim-1 at both mRNA and protein level (using the antibodies HPA003941 (Sigma Aldrich, USA) and CAB017040 (SDIX, USA)) is relatively low in most adult tissues (Table 3.1). However there are notable exceptions to this, with cytoplasmic expression of Pim-1 shown in the liver (Fig 3.1A), male epididymis (Fig 3.1B) and seminal vesicles (Fig 3.1C).

There were only limited data of Pim-1 expression in breast cancer cell lines in the Protein Atlas database (Fig 3.1D). Luminal MCF7 cells express low Pim-1 mRNA (Fig 3.1D) and protein (Fig 3.1D, E) whereas the HER2+ SkBr3 cell line shows no detectable mRNA transcript (Fig 3.1D) and only low level of protein by immunohistochemistry (Fig 3.1D, F). No expression data in TNBC lines was available in the Protein Atlas database.

Immunohistochemical (IHC) staining of normal breast tissues and breast tumours indicate that Pim-1 expression in the normal breast is low and confined to epithelial cells of the mammary gland (Fig 3.1G). Surprisingly, Pim-1 appears low in lobular carcinoma in situ (Fig 3.1H) and in ductal carcinomas (Fig 3.1I, J)

In summary, Pim-1 expression is low in most normal tissues, with some increased expression in tissues with high cell turnover, and negligible in normal breast tissue. In breast carcinoma cell lines, Pim-1 shows moderate cytoplasmic expression and in the human tumours analysed, the ductal carcinoma sections showed a greater proportion of Pim-1 staining than the lobular carcinoma section.
Table 3.1: mRNA and protein expression of Pim-1 in normal organs. Modified from Protein Atlas database.

![Image](http://www.proteinatlas.org/ENSG00000137193-PIM1/tissue)

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<thead>
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<th>RNA Expression (FPKM)</th>
<th>Organ system</th>
<th>Protein Localization (score)</th>
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<tr>
<td></td>
<td>Thyroid gland</td>
<td>h</td>
</tr>
<tr>
<td>N/A</td>
<td>Parathyroid gland</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>Adrenal gland</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>Respiratory system (Lung)</td>
<td>m</td>
</tr>
<tr>
<td>N/A</td>
<td>Nasopharynx</td>
<td>m</td>
</tr>
<tr>
<td>N/A</td>
<td>Bronchus</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>Cardiovascular system</td>
<td>h</td>
</tr>
<tr>
<td></td>
<td>Heart muscle</td>
<td>h</td>
</tr>
</tbody>
</table>
Figure 3.1: Pim-1 expression in normal and breast carcinoma tissue
Using the Protein Atlas tool, the expression of Pim-1 in normal A) liver, B) epididymis, C) seminal vesicle. D) Protein atlas analysis of mRNA and protein Pim-1 expression in breast and female reproductive cell lines, with representative images of E) MCF7 and F) SkBr3 cell line staining. Several normal and cancerous breast images were available for analysis. G) Normal breast tissue from a 52 year old female, H) lobular carcinoma from a 41 year old female, I) ductal carcinoma from an 87 year old female and J) ductal carcinoma from a 50 year old female.

3.2.2 Breastmark, Oncomine and Kaplan Meier database analysis of prognostic significance

The Breastmark prognostic analysis tool (Madden, Clarke et al. 2013) was utilised to investigate the potential associations between Pim-1 mRNA levels and clinical outcome in the datasets available within this tool. We analysed the effect of high Pim-1 expression on distance disease free survival (DDFS) and combined survival (a combination of disease free survival, distance disease free survival and overall survival). We found no statistically significant associations between Pim-1 and clinical outcome, although there were some trends observed. There was a trend towards high Pim-1 expression and decreased combined survival in TNBC compared to ER+ and HER2+ tumours (p = 0.55794) (Fig 3.2A). There was also a trend towards decreased combined survival in lymph node positive (LN+) TNBC cancers and high Pim-1 expression (p = 0.77258) (Fig 3.2B). What is interesting about these two findings is that there is the complete opposite trend with ER+ and HER2+ cancers to the TNBC. The strongest (however non-significant, p = 0.068115) trend was towards high Pim-1 expression and decreased distance disease free survival (DDFS), i.e. distant metastasis free survival, in TNBC (Fig 3.2C).

To further investigate the relationship between high pim-1 expression and TNBC, we utilised the Kaplan Meier survival analysis tool (Győrfy, Lanczky et al. 2010) to assess the prognostic value of Pim-1 in the overall survival of TNBC patients in the microarray datasets available in this tool. We hoped that the increased number of datasets available in this tool would enable us to gain statistical significance. Indeed, we found that TNBC patients with high Pim-1
expression had a significantly worse probability of survival (p<0.0009) compared to TNBC patients with low Pim-1 expression (Fig 3.2D).

Finally, we probed the Oncomine microarray database to assess the available data therein on Pim-1 mRNA levels and tumour characteristics. We interrogated the available public datasets to identify studies that had a significant association with Pim-1 in breast cancer with a threshold of p<0.05. The search identified that there were far more studies that identified Pim-1 as a poor prognostic factor for clinical outcome (21 studies) than a good prognostic factor (5 studies) (Fig 3.3A). We also found 20 studies that all showed Pim-1 as a negative prognostic factor for breast cancer, which included all the studies in 3.3A, minus one DNA dataset that we discarded (Fig 3.3B). Furthermore there was a highly significant correlation between Pim-1 mRNA expression and tumour grade (Fig 3.3C).
Figure 3.2: Prognostic analysis of Pim-1 mRNA expression in breast cancer subtypes
Using the BreastMark mRNA analysis tool: A) high Pim-1 is associated with decreased combined survival in TNBC compared to ER+ and HER2+ tumours. B) High Pim-1 is associated with decreased combined survival in LN+ TNBC compared to ER+ and HER2+ tumours. C) High Pim-1 expression is associated
with decreased DDFS in TNBC compared to ER+ and HER2+ tumours. DDFS: distance disease free survival. D) Using the Kaplan Meier plotter, high Pim-1 is associated with decreased probability of survival in TNBC patients (n=255). Combined: a combination of disease free survival, DDFS and overall survival. LN+: lymph node positive cancer.

Figure 3.3: Oncomine analysis of Pim-1 as a prognostic factor
A) The disease summary for Pim-1 showing the number of breast cancer datasets for each analysis type where Pim-1 levels are significantly correlated (either positively or negatively) with each parameter. Red indicates overexpression/higher DNA copy number and blue indicates underexpression/lower DNA copy number. B) In probing the Oncomine database, we found 20 breast cancer mRNA datasets that associated high Pim-1 with a negative prognostic outcome in breast cancer. C) Using the large Oncomine repository, we found that there is a highly significant association with Pim-1 expression and tumour grade (p=1.15x10^-26).
3.2.3 Expression of Pim-1 mRNA in metastatic breast cancer cell lines

The mRNA levels of Pim-1 was analysed in a range of mouse and human breast cancer cell lines with varying histological and molecular subtypes as well as varying metastatic ability. Since Pim-1 is a survival kinase, we expected that Pim-1 mRNA expression would correlate with metastatic ability as many studies have documented that the ability of the cancer cells to survive both in the circulation and in the secondary site is a rate limiting characteristic in promoting metastasis (Chambers, Groom et al. 2002, Gupta and Massagué 2006, Kang and Pantel 2013, Seguin, Desgroisellier et al. 2015). Protection from anoikis due to anchorage detachment relies on the induction of pro survival proteins and signalling pathways of which TrkB, Wnt2 and Src proteins are well documented (Kang and Pantel 2013). We proposed that Pim-1 could also be implicated in the survival of metastatic breast cancer cells which would be reflected by increased expression in metastatic lines compared to non-metastatic cell lines. This hypothesis was further supported up by the trend towards Pim-1 mRNA expression and decreased DDFS in TNBC (Fig 3.2C).

For both series of mouse and human breast cancer cell lines, the lines with the highest levels of Pim-1 were the brain metastatic triple negative cell lines, 4T1Br4 for mouse (Fig 3.4A) and MDA-MB-231Br for human (Fig 3.4B). Unexpectedly, the non-metastatic 67NR and the weakly metastatic 66Cl4 cell lines had far higher Pim-1 mRNA expression than the highly metastatic 4T1.13 and 4T1.2 cell lines indicating that there is no positive correlation between Pim-1 mRNA expression and metastatic ability (Table 3.2, Fig 3.4A). The poorly metastatic human Hs578T cell line also unexpectedly showed higher Pim-1 expression than either parental MDA-MB-231 or the highly metastatic MDA-MB-231HM cell lines (Table 3.2, Fig 3.4B).
Table 3.2: Molecular subtype and relative metastatic ability of mouse and human breast cancer cells used.
NM: non-metastatic

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Cell line</th>
<th>Molecular subtype</th>
<th>Metastatic ability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>67NR</td>
<td>TNBC</td>
<td>NM</td>
<td></td>
<td>(Aslakson and Miller 1992)</td>
</tr>
<tr>
<td>66Cl4</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td>(Aslakson and Miller 1992)</td>
</tr>
<tr>
<td>4T1Ch5</td>
<td>TNBC</td>
<td>+</td>
<td>(Kusuma, Denoyer et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>4T1Bm2</td>
<td>TNBC</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4T1Ch9</td>
<td>TNBC</td>
<td>++</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td>4T1.13</td>
<td>TNBC</td>
<td>+++</td>
<td></td>
<td>(Lelekakis, Moseley et al. 1999)</td>
</tr>
<tr>
<td>4T1.2</td>
<td>TNBC</td>
<td>+++</td>
<td></td>
<td>(Lelekakis, Moseley et al. 1999)</td>
</tr>
<tr>
<td>4T1Br4</td>
<td>TNBC</td>
<td>++</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td>EO771</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td>(Johnstone, Smith et al. 2015)</td>
</tr>
<tr>
<td>EMT6.5</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td>(Rockwell, Kallman et al. 1972)</td>
</tr>
<tr>
<td>RCSI HER2</td>
<td>HER2+</td>
<td>++</td>
<td></td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Human</th>
<th>Cell line</th>
<th>Molecular subtype</th>
<th>Metastatic ability</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7</td>
<td>ER+ PR+</td>
<td>NM</td>
<td></td>
<td>(Neve, Chin et al. 2006)</td>
</tr>
<tr>
<td>BT549</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td>(Neve, Chin et al. 2006)</td>
</tr>
<tr>
<td>T47D</td>
<td>ER+ PR+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hs578T</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SkBr3</td>
<td>HER2+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231 NI</td>
<td>TNBC</td>
<td>+</td>
<td>Unpublished</td>
<td></td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>TNBC</td>
<td>+</td>
<td></td>
<td>(Cailleau, Olivé et al. 1978)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>TNBC</td>
<td>++</td>
<td></td>
<td>(Cailleau, Olivé et al. 1978)</td>
</tr>
<tr>
<td>MDA-MB-231 Br</td>
<td>TNBC</td>
<td>++</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-MB-231 HM</td>
<td>TNBC</td>
<td>+++</td>
<td></td>
<td>(Chang, Li et al. 2008)</td>
</tr>
</tbody>
</table>
Figure 3.4: Expression of Pim kinase mRNA in breast cancer cell lines

We quantified Pim-1 mRNA expression by qRT-PCR in A) mouse and B) human breast cancer cell lines of varying molecular subtypes and metastatic ability. Expression is relative to rps27 (human cell lines) or rpl5 (mouse cell lines) and is normalised to the levels of 4T1Br4 cells. Comparison of Pim-1, 2 and 3 mRNA expression by qRT-PCR in the above C) mouse and D) human cell lines. Expression is relative to rps27 (human cell lines) or rpl5 (mouse cell lines). Data represents mean ± SEM of three replicates. * denotes p<0.05 compared to all other cell lines as determined by one-way ANOVA with Dunnetts post-hoc test.

3.2.4 Expression of Pim isoforms in metastatic breast cancer cell lines

The data above indicate that Pim-1 transcript levels were elevated in the 4T1Br4 and MDA-MB-231Br cell lines compared to other metastatic and non-metastatic cell lines. Although the main focus of this study is on Pim-1, we thought it was important to evaluate the comparative levels of all three Pim isoforms. There is high sequence homology (between 44% and 57%) and functional redundancy between all three isoforms (Konietzko, Kauselmann et al. 1999, Brault, Gasser et al. 2010). So it was important to determine the relative
expression levels of each isoform as to determine their possible association with the metastatic potential of each cell line.

In the array of mouse cell lines analysed, Pim-2 expression was far lower than Pim-1 and Pim-3, indeed for the majority of cell lines it was undetectable (Fig 3.4C). Pim-3 on the other hand showed high expression levels in 4T1Br4, similar to Pim-1, and in 4T1Ch5, EMT6.5 and RCSI Her2, Pim-3 levels were higher than Pim-1 (Fig 3.4C). Interestingly, in the panel of human cell lines, the levels of all three isoforms were relatively even, with the exceptions of MDA-MB-231Br, in which the expression of Pim-1 was far higher than the other isoforms, and MDA-MB-231HM, in which Pim-2 showed slightly higher expression than the other isoforms (Fig 3.4D).

To determine if Pim-1 mRNA expression was correlated with protein expression in these cell lines, the expression of Pim-1 protein was analysed by western blotting. In accordance with what was found by Tipton et al, only the long 44kDa isoform was observed in these breast cancer cells (Tipton, Nyabuto et al. 2016). While not a perfect correlation, the trend seems to hold strong at the protein level, with both 4T1Br4 and MDA-MB-231Br cell lines showing elevated protein expression compared to the other lines (Fig 3.5A, 3.5B). Taken together, the expression of Pim-1 in metastatic breast cancer seem to be similar at both mRNA and protein levels and again confirms the trend that Pim-1 levels are highest in brain metastatic triple negative cell lines. No clear correlation was observed between Pim-2 or Pim-3 expression and brain metastatic potential in mouse and human lines.
3.2.5 IHC detection of Pim-1 in tumours derived from mouse and human mammary carcinoma cell lines

The results from the RT-PCR and western blotting indicated that Pim-1 expression levels correlate with brain metastatic ability. Here we went on to examine the protein expression of Pim-1 at the tumour level. So that we were able to analyse the prognostic significance of Pim-1 at the tumour level. So that we were able to develop an IHC protocol compatible with detection of Pim-1 in formalin-fixed paraffin-embedded (FFPE) tissues. To do this we developed an IHC protocol using 10% buffered formalin fixation and a citrate buffer pH 6.0/pressure cooker antigen retrieval method that gave good specific detection of Pim-1 in FFPE tissues.

As expected, the Pim-1 expression varied amongst all tissues irrespective of metastatic ability and molecular subtype. The mouse cell lines all showed some degree of cytoplasmic staining of varying intensity, and the brain
metastatic 4T1Br4 tumour section showed by far the greatest amount of cytoplasmic staining, with high nuclear staining additionally (Fig 3.6A). The 4T1Bm2 tumours also expressed some nuclear Pim-1, but not to the levels seen in 4T1Br4 tumours (Fig 3.6A). This suggests that Pim-1 may be working through a mechanism that requires nuclear translocation in this cell line.

Staining of Pim-1 was also mainly cytoplasmic in the human cell line tumours, with luminal MCF7 and HER2 positive SkBr3 showing weaker staining than the other cell lines (Fig 3.6B). The MDA-MB-231Br tumour sections show the strongest cytoplasmic staining, with some nuclear staining as well, although this isn’t as strong as the 4T1Br4 sections. However, it is interesting to note that both brain metastatic tumours showed high nuclear Pim-1 expression compared to all other lines.
Figure 3.6: IHC detection of Pim-1 protein in primary tumours from mouse and human xenografts.
Pim-1 protein expression was examined by standard immunohistochemistry in A) mouse and B) human xenograft tumours of varying histological subtypes as indicated. Arrows indicate nuclear localisation. Brown staining: Pim-1; blue staining: haematoxylin counterstain.
3.2.6 Analysis of a Tissue Microarray (TMA)

A tissue microarray was obtained from the Peter MacCallum Cancer Centre Tissue Bank. It contained 57 samples, of which 48 were breast tumours, with 33 matched normal tissues and 5 population normal controls. Patient characteristics are summarised in Table 3.3. The TMA was carefully stained with the optimised IHC protocol, then photographed and analysed by a medical pathologist. Sections were assigned an H-score (Histological Score) (Goulding, Pinder et al. 1995) and statistical differences between H-scores were evaluated using Mann-Whitney and Kruskal-Wallis tests as appropriate. His findings, summarised in Table 3.4, were unexpected. The analysis showed that low Pim-1 lesions are more associated with high-grade tumours (p= 0.035) and that Pim-1 high cancers are more often HER2+ (p=0.0072). Both these observations are statistically significant. Another, interesting, however non-significant trend (p = 0.057) the pathologist found was that Pim-1 high cancers are less likely to be ER positive. The representative images shown in Fig 3.7 show IHC detection of population normal controls, TNBC tumours, ER+ and PR+ tumours and HER2+ tumours.

Table 3.3: Characteristics of patient samples in TMA cohort

<table>
<thead>
<tr>
<th>Patient characteristics (n=48)</th>
<th>No. of patients</th>
</tr>
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<tbody>
<tr>
<td>ER+ PR+ (n=25)</td>
<td>Stage</td>
</tr>
<tr>
<td>I</td>
<td>4 (16%)</td>
</tr>
<tr>
<td>II</td>
<td>16 (64%)</td>
</tr>
<tr>
<td>III</td>
<td>5 (20%)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Her 2+ (n=11)</td>
<td>Stage</td>
</tr>
<tr>
<td>I</td>
<td>3 (27%)</td>
</tr>
<tr>
<td>II</td>
<td>7 (63%)</td>
</tr>
<tr>
<td>III</td>
<td>1 (9%)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
<tr>
<td>TNBC (n=12)</td>
<td>Stage</td>
</tr>
<tr>
<td>I</td>
<td>1 (8%)</td>
</tr>
<tr>
<td>II</td>
<td>8 (66.7%)</td>
</tr>
<tr>
<td>III</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>
Table 3.4: Pathologist analysis of a breast cancer TMA after IHC detection of Pim-1.

<table>
<thead>
<tr>
<th>Correlations</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIM1 Low lesions are associated with more Grade 3 cancers (83% vs 44%)</td>
<td>p=0.035</td>
</tr>
<tr>
<td>PIM1 High cancers are more likely HER2 positive (47% vs 5%)</td>
<td>p=0.0072</td>
</tr>
</tbody>
</table>

Figure 3.7: Representative images of sections from a tissue microarray (TMA) with IHC detection for Pim-1.

Pim-1 protein was assessed by standard immunohistochemistry in a TMA of human breast cancer tissues with different molecular and histological subtypes.
3.3 Discussion

The aim of this chapter was to evaluate the available data on Pim-1 expression, and prognosis in breast cancer in silico, and to extend and contextualise these findings by investigating the expression of Pim-1 in breast cancer cell lines and tissues. We hypothesised that Pim-1 expression would be low in most adult tissues and that the expression of Pim-1 would increase in cancerous tissue and that the level of expression of Pim-1 would correlate with the metastatic propensity of the cell lines analysed.

From our in silico analysis, we found that Pim-1 expression is low to undetectable in normal cells of the body with the notable exception of organs with high growth rates and that Pim-1 is associated with increased tumour grade and decreased distance free survival. Importantly, we demonstrated for the first time that Pim-1 expression is highest in brain metastatic breast cancer cell lines and tumours.

3.3.1 In silico database analysis

Data from Protein Atlas, showed very low mRNA and protein expression levels of Pim-1 in almost all adult tissues (including the breast), apart from a few tissues such as the nasopharynx, cerebral cortex, liver, male seminal vesicles, and epididymis (Fig 3.1A). Pim-1 is a growth and survival kinase and so is expressed at high levels in many organs during foetal development. Pim-1 is highly expressed in foetal liver and spleen but expression is shut off in the corresponding adult tissues (Amson, Sigaux et al. 1989). Pim-1 is also expressed in normal mature haematopoietic and lymphoid cells as well as testis in both mouse and man during embryonic development (Amson, Sigaux et al. 1989, Padma and Nagarajan 1991, Saris, Domen et al. 1991, Wingett, Reeves et al. 1992, Gapter, Magnuson et al. 2006). These expression levels again fall once the mature organs are formed, which indicates that these organs do not require Pim-1 for survival at maturity.
Since there are a few tissues that still express Pim-1 in the mature tissue, Pim-1 must still play a vital function within them. These tissues all have a high cell turnover and so require a large amount of growth signalling to carry out their functions. Pim-1 is a well-established differentiation and proliferation regulator, as it can impact on cell growth (through NFATC1, eIF4E, 4EBP2), survival (through activating Myc, ASK1 and BCL2) and cell cycle proliferation (by inhibiting p21, p27, C-TAK1) (Tursynbay, Zhang et al. 2016). Therefore, these tissues with high Pim-1 expression may be utilizing Pim-1 growth signalling in order to carry out the processes required to sustain their high growth rate. There have been reports of expression of Pim-1 in the mature testis of both mouse and man (Eichmann, Yuan et al. 2000), but the reports of Pim-1 expression in the mature liver indicate only sparse staining in the polymorphonuclear cell fraction (Amson, Sigaux et al. 1989). Pim-1 is mostly absent in the brain, with low to no expression in the brainstem, cerebellum, cortex and hippocampus with increased expression in the brain during periods of high activity such as during seizure (Feldman, Vician et al. 1998).

All available literature points to very low levels of Pim-1 in most adult tissues, with the notable exception of the testis. So it was very surprising to see that the Protein Atlas cohort of tissues showed definite expression in the liver, cerebral cortex and nasopharynx at both the mRNA and protein level. Evidently further investigation into the expression patterns of Pim-1 in these tissues is required in order to determine the role of Pim-1 in homeostatic signalling pathways in these tissues.

The Protein Atlas analysis of breast cancer cells and tumours was limited. There were only two breast cancer cell lines analysed, the ER+ luminal MCF7 and the HER2+ SkBr3 (Fig 1G, 1H). Both cell lines showed cytoplasmic staining of Pim-1, which is in accord with the study by Gapter et al, who indicated that all breast cancer cell lines expressed some Pim-1 compared to control breast tissue (Gapter, Magnuson et al. 2006). However, neither of these cell lines is particularly metastatic, and since there was no TNBC cell line, it is difficult to draw any conclusions from this cohort.
Pim-1 has been implicated as a prognostic factor for a range of human cancers. It has been established that Pim-1 is a robust biomarker for prostate cancer, as a large microarray has revealed that Pim-1 expression is significantly correlated with poor outcome (Dhanasekaran, Barrette et al. 2001). Elevated Pim-1 also predicts poor outcome in mantle cell lymphoma, B-cell lymphoma and diffuse B-cell lymphoma (Hsi, Jung et al. 2008, Narlik-Grassow, Blanco-Aparicio et al. 2014), as well as pancreatic malignancies (Reiser-Erkan, Erkan et al. 2008). However, there is only limited evidence that Pim-1 has value as a prognostic marker in breast cancer. In one study, Pim-1 was found to correlate with triple negative status, high clonogenic ability and high migratory ability in a cohort of human breast cancer samples (Maristany, Grigoriadis et al. 2013). Another study proposed Pim-1 as an ER target, and also correlated Pim-1 expression with high tumour grade and increased malignancy (Malinen, Jääskeläinen et al. 2013). Therefore it was important to analyse other larger databases to see if any such correlations could be confirmed.

Analysis of the KM plotter database found a statistically significant correlation between high Pim-1 expression and decreased probability of overall survival in TNBC patients \( p=0.0009, \ n=255 \) (Fig 3.2D). Unfortunately no significant correlations could be found in the BreastMark database. We postulate that once reason for this is that in most of the analyses, the sample size was small, and so although there were some trends, there was unfortunately insufficient power in this dataset to achieve statistical significance.

There were however, some strong trends found in the BreastMark database. High Pim-1 expression was associated with decreased combined survival in TNBC but not HER2+ or ER+ cancers (Fig 3.2A). There was also a trend found between high Pim-1 expression and decreased combined survival in lymph node positive TNBC (Fig 3.2B). By far the strongest trend was that of high Pim-1 and shorter DDFS in TNBC \( p = 0.068115 \), but not in ER+ or HER2+ cancers (Fig 3.2C). These results imply an association with Pim-1 and TNBC, which is in accord with the findings in the study by Maristany et al, which found that Pim-1 expression correlated with triple negative breast cancers (Maristany, Grigoriadis et al. 2013). Both studies linked Pim-1 to increased metastatic ability, showing that high Pim-1 expression promoted migration of cells and was
associated with malignancy. Another interesting observation was that, while Pim-1 high tumours were associated with poor survival in TNBC, it seemed that Pim-1 LOW tumours were trending towards poor survival in ER+ and HER2+ tumours. Pim-1 expression was trending towards increased combined survival, DDFS and DDFS in LN+ tumours for both ER+ and HER2+ tumours (Fig 3.2A, 3.2B, 3.2C), although the trend was more pronounced for HER2+ tumours. Again, due to the low number of samples, none of these trends were statistically significant, but it does allow for the possibility that the prognostic significance of Pim-1 is dependent on the subtype of cancer.

3.3.2 mRNA and protein analysis of Pim-1 expression

Due to the Protein Atlas data that showed expression of Pim-1 in both breast cancer tumour cells analysed, we hypothesised that the mouse and human breast cancer cell lines we analysed would all show some Pim-1 expression at the mRNA and protein level. We also expected that the amount of Pim-1 expression would correlate to TNBC status, to metastatic ability, or both.

The mRNA and protein data from a range of mouse and human breast cancer cell lines indicates that all breast cancer cells contain some Pim-1 expression, albeit at different levels. This finding is consistent with that reported by Gapter and colleagues, who showed that Pim-1 mRNA is present in a range of breast cancer cell lines (Gapter, Magnuson et al. 2006). They concluded that there was no relationship between mRNA and protein levels, which indicates each cell line has a different method of Pim-1 regulation. Our data, while not a perfect correlation, does show a reasonably consistent relationship between Pim-1 mRNA and protein levels, especially with the brain metastatic lines 4T1Br4 and MDA-MB-231Br having higher expression than the rest of the cell lines analysed. The mRNA data obtained by Gapter et al was obtained using an unquantitated northern blot, which is a far less accurate method than the quantitative PCR method employed in this study. Since the majority of Pim-1 regulation is at the transcriptional level and that Pim-1 undergoes very little post-translational modification, it is expected that the mRNA and protein levels would correlate to some degree (Hoover, Wingett et al. 1997).
It was expected that cell lines that were more metastatic would show higher expression of Pim-1. However, this was not observed. In fact, there was no correlation between Pim-1 expression and metastatic ability. Similarly, there was no clear correlation between Pim-1 expression and molecular subtype. In the cohort of human breast cancer cell lines, the Hs578T cell line, which shows limited metastatic ability in vivo, had a far higher Pim-1 expression than the highly metastatic MDA-MB-231 and MDA-MB-231HM lines (Fig 3.3B). In the cohort of mouse breast cancer cell lines, the very weakly metastatic 67NR and 66Cl4 cell lines had higher mRNA expression of Pim-1 than the highly metastatic 4T1.13 line (Fig 3.3A). These three cell lines are all TNBC cell lines, and the fact that they have such variable Pim-1 expression further indicates that there is no correlation between Pim-1 expression and molecular subtype. Indeed, even the literature cannot come to a consensus, with one study linking Pim-1 to TNBC and the other linking Pim-1 to ER+ cancer (Malinen, Jääskeläinen et al. 2013, Maristany, Grigoriadis et al. 2013).

One interesting observation was that in the western blots; only the larger 44kDa band was seen in any of the cell lines (Fig 3.5A, 3.5B). This particular antibody has been validated and shown to recognise both isoforms by western blot indicating that the 44kDa isoform may be more relevant to breast cancer progression. Our data are consistent with a recent study showing that in breast cancer, only the large 44kDa protein is expressed (Tipton, Nyabuto et al. 2016).

There is evidence that the 44kDa protein is located in the cytosol and membrane while the 33kDa protein is located in the cytosol and nucleus (Xie, Xu et al. 2005). The 44kDa protein also encodes and extra proline rich PXXP motif at the end terminus, which enables this larger protein to have more binding interactions, such as with the SH3 domain of Etk, and the ABC transporter pump ABCG2 (Xie, Xu et al. 2005, Xie, Xu et al. 2008). However in breast cancer, since there is only the 44kDa isoform expressed, there is a possibility that this isoform may also undergo nuclear translocation.
3.3.3 TMA Analysis

The TMA analysed was comprised of 56 samples of which 48 were breast tumours. The TMA also included matched control samples and population normal controls. The TMA was comprised of 25 ER+, PR+ samples, 11 HER2+ samples and 12 TNBC samples (summarised in Table 3.3). After IHC detection, the section was given to an expert pathologist to quantitate the intensity and breadth of staining of each sample. The results were unexpected, with the most significant prognostic associations being that Pim-1 low cancers are associated with higher grade (p = 0.035) and high Pim-1 expression with the HER2+ phenotype (p = 0.0072) (Table 3.4). These results were unexpected since all previous results point to an association with Pim-1 and higher tumour grade, and there had been no previous indication that Pim-1 expression is associated with HER2 positivity.

From this analysis, another surprising observation emerged. Pim-1 high cancers were the most likely to be ER+, Stage 1 or 2 and Grade 2 tumours, although this did not reach statistical significance (p = 0.057) (Table 3.4). This contrasts with our in vitro analysis from which we expected Pim-1 high tumours to be TNBC and of a high grade. Furthermore, a recent well powered study published after completion of our experimental work found that Pim-1 is disproportionately increased in primary TNBC and is associated with poor clinical in TNBC (Horiuchi, Zhou et al. 2016), an outcome that is in agreement with our original hypothesis.

One limitation of this TMA is the small number of samples. This means it is difficult to get sufficient power to see a true indication of Pim-1 expression in each group of tumours, as 11 or 12 per group is not really enough samples to be able to see this. A larger investigation with a bigger and more robust cohort of samples is needed to be able to ascertain the true and complete expression pattern of Pim-1 in breast cancer.

In conclusion, we have established that Pim-1 expression correlates with tumour grade and decreased distance free survival in breast cancer. And, for the
first time, we have established that Pim-1 expression correlates with brain metastatic propensity in both mouse and human breast cancer cell lines and tumours at the mRNA and protein level. Further in depth investigation is needed to validate and ascertain the functional role that Pim-1 may be playing in these brain metastatic cell lines.
Chapter 4: Function of Pim-1 in breast cancer metastasis

4.1 Introduction

Pim-1 is a growth and survival kinase that is expressed at high levels in developing tissues but decreases in expression in most normal adult tissues (Amson, Sigaux et al. 1989). This downregulation of Pim-1 expression suggests that inappropriate expression of Pim-1 in adult tissues could contribute to uncontrolled growth and malignant transformation. As described in Chapter 1, previous studies have reported that the level of Pim-1 is markedly increased in many tumour types, including pancreatic and prostate cancers, squamous cell carcinoma, gastric carcinoma, colorectal carcinoma, liver carcinoma, liposarcoma and bladder cancer (Shah, Pang et al. 2008, Guo, Mao et al. 2010, Nga, Swe et al. 2010). Moreover, Pim-1 overexpression appears to be an early event in prostate carcinogenesis (Xu, Zhang et al. 2005, Cibull, Jones et al. 2006) and consequently Pim-1 expression is correlated with poor outcome (Dhanasekaran, Barrette et al. 2001). Data in breast cancer is more limited and the findings from our own investigation described in Chapter 3 are consistent with observations in other tumour types. Specifically, we found that Pim-1 expression is elevated in breast tumour tissue compared to normal breast tissue and that Pim-1 levels are especially elevated in breast cancer cell lines with brain metastatic propensity. This expression data suggests that Pim-1 may play a functional role in breast cancer metastasis, with a particular emphasis on brain metastasis. There is little currently known about the functional contribution of Pim-1 to breast cancer progression and metastasis compared to other tumour types and so further investigation of Pim-1 in this context is required.

There is evidence that Pim-1 contributes to several functional metastatic processes in leukaemia, lymphoma, pancreatic and prostate cancers. Pim-1 promotes the survival and proliferation of acute myeloid leukaemia, pancreatic and prostate cancer cells which is consistent with its association with poor survival prognosis in patients (Chen, Chan et al. 2005, Saurabh, Scherzer et al.
In sarcoma, Pim kinases are required for bone invasion (Narlik-Grassow, Blanco-Aparicio et al. 2012) and inhibition of Pim-1 decreases the migratory and invasive ability of prostate and squamous epithelial carcinoma cells (Santio, Vahakoski et al. 2010, Santio, Eerola et al. 2015). Several mechanisms and signalling pathways have been linked to Pim-1 dependent migration and invasion in various cancer types, however the precise way in which these Pim-1 substrates interact to promote migration and invasion has not been fully elucidated and mapped. Specifically, in contrast to other solid tumour, evidence for the role of Pim-1 in the metastatic progression of breast cancer in vivo is lacking. In vitro, studies have linked Pim-1 expression to clonogenic ability, migratory ability and increased malignancy in response to oestradiol signalling (Malinen, Jääskeläinen et al. 2013). However, the precise mechanism(s) by which Pim-1 signalling may promote proliferation, migration and invasion of breast cancer cells have yet to be fully studied and revealed.

Therefore, the overall objective of this chapter was to characterise the functional contribution of Pim-1 to breast cancer metastasis. Specifically, the functional consequences and phenotypic changes induced by genetic or pharmacological inhibition of Pim-1 using stable shRNA knock downs of Pim-1 or the selective Pim-1 inhibitor, SGI-1776, was investigated in vitro and in vivo.
4.2 Results

4.2.1 Stable knock down of Pim-1 differentially affects the expression of Pim isoforms in mouse and human brain-metastatic breast cancer cells

To investigate the function of Pim-1 in breast cancer metastasis we first generated mouse and human cell lines with stable suppression of Pim-1 with short hairpin RNAs (shRNA). The Pim-1 hairpins were obtained as viral supernatants from Sigma in the pLKO.1 vector, and from the Victorian Centre for Functional Genomics in the pGIPZ vector, for mouse- and human-specific shRNAs, respectively; both vectors harboured a puromycin-resistance gene (Fig 2.1). The murine 4T1Br4 and human MDA-MB-231Br cells were each infected with one of four Pim-1 shRNA hairpins (Table 2.1) and cells that had expressing the vector were selected for with puromycin. We assessed the knock down of the four different hairpins for each cell line. For each cell line we determined which two out of the four hairpins gave the best knock down of Pim-1 and then performed single cell cloning to isolate the clones that demonstrated the greatest Pim-1 suppression. We chose one clone from each of these hairpins to be studied further. This resulted in two clonal Pim-1 shRNA cell lines for 4T1Br4 cells (denoted shRNA1 and shRNA2) and two clonal Pim-1 shRNA cell lines for MDA-MB-231Br (denoted shRNA1 and shRNA2) cells, along with a control cell line that had incorporated a non-silencing hairpin.

To confirm that the shRNA sequences used were specific to Pim-1 and did not target Pim-2, we used the BLAT sequence alignment tool (Kent 2002) to map where the Pim-1 sequence was targeted by the shRNA. When BLAT was used to search for an alignment, Pim-1 was the only gene that returned and there was no alignment with Pim-2 and Pim-3 (Table 2.1, Fig 2.2). Therefore we are confident that both mouse Pim-1 shRNA 1 and Pim-1 shRNA 2 are likely to specifically target and suppress Pim-1 only.

We measured the impact of Pim-1 suppression on various functions associated with metastatic progression in vitro. Several important functional
parameters were assessed including cell proliferation rate, adhesive ability, and the ability to migrate and invade *in vitro*.

In the murine 4T1Br4 cells, the control cell line harbouring a non-targeting hairpin expressed high levels of Pim-1 and Pim-3 isoforms but very low levels of Pim-2 (Fig 4.1A). 4T1Br4 cells stably expressing shRNA 1 or shRNA 2 showed decreased Pim-1 mRNA levels (~97% inhibition) compared to control cells. Surprisingly, although the sequences were specific to Pim-1 (Fig 2.1), both hairpins also decreased Pim-2 and Pim-3 mRNA expression in the 4T1Br4 cells to undetectable levels (Fig 4.1A).

In human MDA-MB-231Br cells, the control cell line harbouring a non-targeting hairpin expressed high levels of Pim-1, Pim-2 and Pim-3 isoforms (Fig 4.1B). The cells stably expressing shRNA 1 demonstrated almost complete loss of Pim-1 and Pim-2 mRNA and approximately 60% decrease in Pim-3 mRNA expression (Fig 4.1B). MDA-MB-231Br cells harbouring shRNA 2 showed a 6-fold decrease in Pim-1 levels (~ 85% inhibition), whereas the expression of Pim-2 increased approximately 2-fold and Pim-3 decreased to approximately 68% compared to control (Fig 4.1B).

Consistent with Pim-1 mRNA data, western blotting showed a robust knock down in Pim-1 protein levels in both the 4T1Br4 (Fig 4.1C) and MDA-MB-231Br cells (Fig 4.1D).

Taken together, these observations indicate that, while Pim-1 expression was stably decreased after shRNA inhibition, this was accompanied by alterations in expression of the other Pim isoforms.
Figure 4.1: Pim-1 stable suppression differentially alters the expression of Pim isoforms in mouse and human brain-metastatic cell lines

After knock down of Pim-1 by shRNA the mRNA levels of all three Pim isoforms compared to the housekeeping genes rps27a (for 4T1Br4 cells) and rpl37a (for MDA-MB-231Br cells) were measured to give a value of relative transcript abundance (RTA). A) 4T1Br4 cells, B) MDA-MB-231Br cells. The data represents the mean of three separate experiments (n = 3), each performed in triplicates ± SEM. This was correlated with protein expression of Pim-1 by western blotting in C) 4T1Br4 and D) MDA-MB-231Br cells. The lower panels represent quantitation of Pim-1 expression compared to GAPDH by the western blotting using Image J (n=1).
4.2.2 Pim-1 KD has no effect on adhesion and a variable effect on cellular proliferation

Pim-1 is a growth and survival kinase and so impacts on cell proliferation in many tumour types. Sarcomas in Pim-1-deficient mice showed reduced growth rate and absence of proliferation markers, and Pim-1 expressing prostate cancer cells demonstrate enhanced growth and protein synthesis, which decreases upon Pim-1 suppression (Chen, Chan et al. 2005, Hu, Li et al. 2009, Narlik-Grassow, Blanco-Aparicio et al. 2012). The loss of Pim-1 has also been shown to confer a strong adhesive phenotype in endothelial cells (Walpen, Peier et al. 2012). Both proliferation and adhesion are vital processes for the development of breast cancer and subsequent metastasis.

Thus, we assessed if stable suppression of Pim-1 impacted on various cellular responses associated with the metastatic process. We first performed a cell proliferation assay to see if cell proliferation was affected by Pim-1 suppression. There was no difference in cell proliferation rate after Pim-1 KD in mouse 4T1Br4 cells (Fig 4.2A). However in MDA-MB-231Br cells, each Pim-1 hairpin decreased cell proliferation rate, albeit to different degrees (Fig 4.2B).

To test the adhesive abilities of the cells after Pim-1 KD, we tested the ability of the cells to adhere to uncoated (UC) plastic as well as to the extracellular matrix proteins laminin-511, collagen-IV and vitronectin. There was no difference in cell adhesion to any of the substrates tested upon suppression of Pim-1 in either 4T1Br4 or MDA-MB-231Br cell lines (Fig 4.2C, 4.2D).
Figure 4.2: Effect of Pim-1 suppression on the proliferation and adhesion of brain-metastatic cells.

The ability of (A) 4T1Br4 and (B) MDA-MB-231Br cells to proliferate after Pim-1 shRNA was assessed using a 5-day growth assay. Cells were plated at a density of 1000 cells per well and harvested each day for 5 days, stained with SRB and the absorbance was measured at 550nm as described in section 2.2.4.4. The data represents the mean of three separate experiments ± SEM (n=3). ***=p<0.001 as determined by One way ANOVA with Dunnetts multiple comparison test. The ability of (C) 4T1Br4 and (D) MDA-MB-231Br cells to adhere to plastic and extracellular matrix proteins was measured using a calcein-based adhesion assay (Akeson and Woods 1993). Cells were labelled with calcein, allowed to adhere to either uncoated plastic (UC) or an ECM protein (laminin-511, collagen-IV or vitronectin) and the resulting calcein fluorescence was normalised to a standard curve. The data represents the mean of three separate experiments ± SEM (n=3).

4.2.3 Pim-1 KD differentially effects cell migration and invasion in vitro

Migration out of the primary tumour site and invasion into firstly the bloodstream and then to the secondary site are important steps in the metastatic cascade. Therefore we assessed if Pim-1 suppression altered these processes in vitro. For both cell lines we used the Pim-1 shRNA 2 cells. We used the 4T1Br4 Pim-1 shRNA 2 cell line as it had given the most consistent and reproducible data in previous assays. We used the MDA-MB-231Br Pim-1 shRNA 2 cell line as the mRNA levels of Pim-2 and Pim-3 most closely resembled those of the control
cells, so we surmised that was a better cell line to study the specific effects of Pim-1 KD without the confounding influence of differential expression of Pim-2 and Pim-3.

First, we assessed the ability of Pim-1 KD cells to migrate towards different ECM substrates (haptotactic migration). Unexpectedly, the 4T1Br4 Pim-1 KD cells showed increased migration towards all ECM proteins (Fig 4.3A). The opposite effect was seen with the MDA-MB-231Br cells; with the Pim-1 KD cells showing significantly reduced migration towards all ECM proteins (Fig 4.3B).

Similar results were seen with migration towards serum (chemotactic migration). The 4T1Br4 Pim-1 KD cells again showed increased migration towards serum (Fig 4.3C) and the MDA-MB-231Br cells showed reduced migration towards serum (Fig 4.3D). Similarly, when we tested the ability of Pim-1 KD cells to invade through Matrigel towards serum we found the same trend. The 4T1Br4 Pim-1 KD cells showed increased invasion and the MDA-MB-231Br Pim-1 KD cells showed decreased invasion compared to the control cells expressing a non-targeting shRNA (Fig 4.3E, 4.3F).
The ability of Pim-1 KD cells to migrate and invade *in vitro* was assessed using Transwell migration and invasion assays. Cells were seeded in the upper well of Transwell migration chambers and cells that migrated towards the underside of the 8 μM porous membrane that was uncoated (UC) or coated with Collagen-IV, Laminin-511 or Vitronectin were counted after (A) 3 hours for 4T1Br4 cells or (B) 4 hours for MDA-MB-231Br cells. Chemotactic migration towards serum was measured for 4 hours with (C) 4T1Br4 cells or (D) MDA-MB-231Br cells. Invasion through matrigel towards the underside of the Transwell membrane towards serum was measured for either 18 hours for (E) 4T1Br4 cells or 24 hours for (F) MDA-MB-231Br cells. Migration and invasion is expressed as the number of migrated cells/20x field of view and the results show the mean of triplicate wells of three separate experiments ± SEM and images below show representative fields of view images. * = p<0.05, ** = p<0.005, *** = p<0.001 as determined by two tailed t-tests. The knock down hairpin used for these assays was Pim-1 shRNA 2 for 4T1Br4 cells and Pim-1 shRNA 2 for MDA-MB-231Br cells. The data represents the mean of three separate experiments ± SEM (n=3).

### 4.2.4 Pim-1 suppression alters integrin expression

Integrins are cell surface transmembrane proteins that are the major receptors mediating cell attachment to ECM proteins as well as cell-cell adhesion (Hynes 2002). Since integrins are major mediators of adhesion-dependent migration and invasion in cancer cells, and that Pim-1 significantly impacted on these responses (see Fig 4.3), cell surface expression levels of the beta integrins β1, β3 and β4 were analysed by flow cytometry. We decided to analyse these
three integrin subunits as, along with their α-subunit binding partners, they are the receptors for collagen, laminin and vitronectin, which are the same ECM proteins we used in the migration assays (4.3A, 4.3B).

We optimised a flow gating strategy to isolate the cells with β-integrin expression (Fig 4.4). Firstly, cells were gated on morphology to remove debris. Then doublets were excluded so that only the single cells were isolated, followed by the exclusion of dead cells. Finally, the levels of expression of the integrins β1, β3 and β4 integrin were assessed. Isotype antibodies were used as a negative control.

Pim-1 suppression did not drastically change the cell surface expression of β-integrin subunits in 4T1Br4 cells. β1 integrin expression increased slightly from 79.06% in the control cells to 91.29% and 86.75% in Pim-1 shRNA 1 and shRNA 3 respectively (Fig 4.5A). While it is an increase in expression, we do not believe it is significant, as the mean peak fluorescence did not change substantially. Similarly, Pim-1 KD did not impact on the cell surface expression of β3-integrin. Control cells showed 53.29% β3 positive expression compared to 70.24% and 49.52% for the Pim-1 shRNA 1 and shRNA 2 cells respectively (Fig 4.5B). However, in the case of β4 integrin we found a significant decrease in receptor expression in the Pim-1 shRNA 2 cells. The control cells showed 96.11% positive β4 expression, which showed no real change to 99.17% for Pim-1 shRNA 1 and 99.17% positive cells for Pim-1 shRNA 2 (Fig 4.5C). It is worth noting that the KD cells used for the migration and invasion assays were the Pim-1 shRNA 2, as explained above.

Pim-1 KD decreased the cell surface expression of all three β integrin subunits in MDA-MB-231Br cells. The effect of Pim-1 KD was most marked for β1 expression, and reduced β1 mean peak fluorescence by one order of magnitude, from 75.66% β1 high cells in the control subset, which fell to 2.63% and 9.63% for Pim-1 shRNA 1 and shRNA 2 respectively (Fig 4.6A). β3 expression was low in the control cells (9.02% β3 positive cells), and was completely lost upon Pim-1 suppression (0.22% for Pim-1 shRNA1 and 0.31% positive cells for Pim-1 shRNA 2) (Fig 4.6B). Analysis of β4 integrin revealed that ~76% of cells expressed this
receptor and its expression decreased to 42.91% and 48.07% in Pim-1 shRNA 1 and Pim-1 shRNA 2 cells respectively (Fig 4.6C). Western blotting further confirmed that total expression of β1, β3 and β4 integrins were reduced in cells lacking Pim-1 expression (Fig 4.6D).

Figure 4.4: Gating strategy for analysing integrin expression by flow cytometry
Cells were first gated for morphology on side scatter (SSC-A) vs. forward scatter (FSC-A). Single cells were then gated on FSC-H vs. FSC-A. Then viable, integrin positive cells were gated based on exclusion of viability dyes (either 7AAD or Fluorogold) and positive for the specific secondary antibody fluorophore.
Figure 4.5: Integrin expression after Pim-1 KD in 4T1Br4 cells
Cell surface expression of A) β1, (B) β3 and (C) β4 integrins in 4T1Br4 cells were analysed by flow cytometry as described in section 2.2.6.4 after stable suppression of Pim-1 by shRNA (Pim-1 shRNA 1 and Pim-1 shRNA 2) or a non-target control shRNA (control shRNA). Isotype antibodies were used as a negative control.
Figure 4.6: Integrin expression after Pim-1 KD in MDA-MB-231Br cells
Cell surface expression of A) β1, (B) β3 and (C) β4 integrins in MDA-MB-231Br cells were analysed by flow cytometry as described in section 2.2.6.4 after stable suppression of Pim-1 by shRNA (Pim-1 shRNA 1 and Pim-1 shRNA 2) or a non-target control shRNA (control shRNA). Isotype antibodies were used as a negative control. D) Total β1, β3 and β4 integrin protein expression was analysed by western blotting. The data in the bottom panels represents the mean of three separate experiments ± SEM.
4.2.5 Pharmacological inhibition of Pim-1 decreases cell migration and invasion

The results above indicate that genetic suppression of Pim-1 impacts on the ability of 4T1Br4 and MDA-MB-231Br cells to migrate and invade. However, the genetic knock down of Pim-1 either promoted (4T1Br4) or inhibited (MDA-MB-231Br) cell migration and invasion. Furthermore, Pim-1 hairpins differentially altered the expression of other Pim isoforms in mouse and human cell lines suggesting that changes in the expression of other isoforms may also contribute to the functional responses observed (Fig 4.1). It was therefore important to validate the results from gene knock down experiments could be replicated using a pharmacological inhibitor of Pim-1, SGI-1776.

SGI-1776 is an imidazo[1,2-b]pyridazine compound with selectivity for Pim-1 over Pim-2 and Pim-3, with kinase (not cell based) IC\textsubscript{50} values of 7 ± 1.8 nmol/L for Pim-1, 363 ± 27.6 nmol/L for Pim-2, and 69 ± 9.2 nmol/L for Pim-3 (Mumenthaler, Ng et al. 2009). Apart from Pim kinases, SGI-1776 only demonstrates inhibitory action against two other kinases; Flt-3 (kinase IC\textsubscript{50}, 44 nmol/L) and Haspin (kinase IC\textsubscript{50}, 34 nmol/L).

We had previously ascertained the concentration of SGI-1776 necessary to inhibit the proliferation of 50% of the cells (IC\textsubscript{50}) after 24 hours was 4 μM for both 4T1Br4 and MDA-MB-231Br cell lines. Therefore in the next series of experiments, SGI-1776 was used at concentrations ranging from 0.015 μM to 1 μM to make sure that the effects seen by the use of the inhibitor was not due to cytotoxicity. At these concentrations we see little or no effect on cell proliferation and viability.

SGI-1776 significantly and consistently inhibited the chemotactic migration of 4T1Br4 and MDA-MB-231Br cells in a dose dependent fashion. Inhibition of migration was demonstrated at all concentrations tested, from 1 μM down to 0.015 μM (Fig 4.7A, 4.7B). At the lowest concentration tested (0.015 μM), migration of 4T1Br4 was inhibited by 60% compared to control cells (Fig 4.7A). This anti-migratory effect was even greater in MDA-MB-231Br cells as at
0.015 μM of SGI-1776, the lowest concentration used, the migration of these cells was still inhibited by 80% compared to the control cells (Fig 4.7B).

SGI-1776 also demonstrated a dose-dependent decrease in cell invasion at concentrations between 0.125 and 0.015 μM. Both 4T1Br4 and MDA-MB-231Br cells showed comparable sensitivity to SGI-1776, as at the lowest concentration of 0.015 μM, SGI-1776 inhibited the invasion of both cell lines by 30% compared to the control cells (Fig 4.7C, 4.7D).

![Figure 4.7: Inhibition of migration and invasion by treatment with SGI-1776](image)

A) 4T1Br4 and B) MDA-MB-231Br cells were treated with SGI-1776 at the concentrations indicated and allowed to migrate towards serum for 4 hours. Cells were treated with varying concentrations of SGI-1776 and allowed to invade through Matrigel towards a serum gradient for C) 18 hours for 4T1Br4 cells or D) 24 hours for MDA-MB-231Br cells. The data represents the mean of three separate experiments ± SEM. **=p<0.005 compared to control, ***=p<0.001 compared to control as determined by one-way ANOVA with Dunnetts multiple comparison test.
4.2.6 SGI-1776 treatment decreases cell surface integrin expression.

To test whether inhibition of cell migration and invasion in response to SGI-1776 treatment was also associated with changes in integrin receptor expression, cell surface expression of β1, β3 and β4 integrins was examined by flow cytometry. We opted to use 0.03 μM of SGI-1776 and to pre-treat the cells for 4 hours. These conditions were chosen because 0.03 μM was one of the lowest concentrations used in the migration and invasion assays that mediated a robust inhibitory effect, and 4 hours, as this was the duration that the cells were exposed to SGI-1776 in the migration assays.

The expression of β1 integrin in 4T1Br4 decreased very slightly from 99.38% to 91.95% of gated cells after SGI-1776 treatment (Fig 4.8A). Expression of β3 integrin decreased more dramatically from 51.75% to 29.26% after SGI-1776 treatment (Fig 4.8B). However, SGI-1776 treatment had no significant effect on β4 expression (95.47% in control cells versus 96.91% in SGI-1776-treated cells) (Fig 4.8C). Evidently the SGI-1776 treatment is having a greater effect on β3 integrin expression of 4T1Br4 cells than Pim-1 knock down alone.

Treatment with 0.03 μM SGI-1776 for 4 hours decreased β-integrin expression in MDA-MB-231Br cells in a manner similar to Pim-1 KD. Untreated cells expressed 83.43% β1 high expression, which decreased to 65.91% post SGI-1776 treatment (Fig 4.9A). In a similar manner to 4T1Br4 cells, the expression of β3 integrin decreased from 10.07% in the control cells to 4.91% after SGI-1776 treatment (Fig 4.9B). Similarly, β4 integrin expression levels also decreased after SGI-1776 treatment, from 70.51% to 7.93% (Fig 4.9C). SGI-1776 decreased β3 expression in a similar manner in both 4T1Br4 and MDA-MB-231Br cells.
Figure 4.8: The effect of the Pim-1 inhibitor SGI-1776 on β-integrin expression of 4T1Br4 cells.
4T1Br4 cells were treated for 4 hours with 0.03 nM of SGI-1776 or vehicle (control) in α-MEM before being prepared for flow cytometry. Flow cytometry analysis gave quantitation of membrane expression of A) β1 integrin, B) β3 integrin and C) β4 integrin.

Figure 4.9: The effect of the Pim-1 inhibitor SGI-1776 on β-integrin expression of MDA-MB-231Br cells.
MDA-MB-231Br cells were treated for 4 hours with 0.03 nM of SGI-1776 or vehicle (control) in DMEM before being prepared for flow cytometry. Flow cytometry analysis gave quantitation of membrane expression of A) β1 integrin, B) β3 integrin and C) β4 integrin.
4.2.7 Pim-1 KD decreases bone metastasis and CTCs *in vivo*

Pim-1 knock down induced inconsistent proliferative, migratory and invasive responses in the murine 4T1Br4 and human MDA-MB-231Br cells. However, Pim-1 suppression reduced the migration and invasion of MDA-MB-231Br cells (Fig 4.3B, D) and these responses were similarly observed following treatment of the cells with the use of a Pim-1 specific inhibitor in both mouse and human brain metastatic breast tumour cells (Fig 4.7). These observations are consistent with a functional role for Pim-1 in breast cancer metastasis *in vivo*.

To investigate this further, we used an experimental metastasis assay in which MDA-MB-231Br cells expressing a non-targeting control shRNA or Pim-1-targeting shRNA were injected into the left ventricle of the heart of a NOD scid gamma (NSG) mouse. 5 × 10⁴ cells were injected into each mouse and this approach is known to give rise to metastasis in bone, brain and other organs (Eckhardt, Francis et al. 2012). The mice were monitored for signs of metastatic disease (lethargy, ruffled fur, loss of weight, rapid breathing) and sacrificed as a group after 4 weeks when symptoms were apparent. Blood was collected by cardiac puncture and the number of viable circulating tumour cells (CTCs) determined using an *in vitro* colony-forming assay. Relative tumour burden in liver, femur and spine was assessed by genomic qPCR quantitation of the GFP marker gene present only in tumour cells and the reference gene, vimentin, present in all cells (Denoyer, Potdevin et al. 2011) (Fig 4.10). Brains were imaged for GFP fluorescence to detect the presence of brain metastases.

While there was a trend towards decreased liver (Fig 4.11A) and femur (Fig 4.11B) relative tumour burden (RTB) in Pim-1 shRNA groups compared to control group, these differences did not reach statistical significance. However, there was a significant RTB reduction in spine (Fig 4.11C) and this response remained significant when spine and femurs were combined as a measure of total RTB in bone (Fig 4.11D). The number of CTC colonies was also significantly decreased in mice inoculated with Pim-1 shRNA cell lines compared to control (Fig 4.11E), indicating that at four weeks post injection, there were fewer tumour cells in circulation in the Pim-1 KD groups compared to control.
Figure 4.10: Design of the \textit{in vivo} experiment to assess the effect of Pim-1 KD on metastasis.

MDA-MB-231Br control or Pim-1 KD (shRNA 1 and shRNA 2) cells (5x10^4 cells per 100 μl saline) were injected into the left ventricle of the heart. Mice were monitored for signs of sickness. After four weeks, mice were harvested and liver, spine and femur were analysed for GFP signal to determine relative tumour burden by qPCR. Brains were imaged on the Maestro imager for GFP signal to detect the presence of brain metastases. 1 ml of blood was collected by cardiac puncture, red blood cells lysed using a hypotonic buffer, and the remaining cells were resuspended in 1 ml of buffer seeded at low density (100 μl/plate) in culture plates and the number of tumour cell colonies (>50 cells) counted as an indirect measure of viable circulating tumour cells (CTCs).
Figure 4.11: qPCR analysis of relative tumour burden (RTB) and colony assays for viable CTCs
Relative tumour burden in A) liver, B) femur, C) spine and D) combined bone was determined by assessing the gDNA levels of GFP by qPCR four weeks after intracardiac tumour cell inoculation with either control shRNA, Pim-1 shRNA 1 or Pim-1 shRNA 2. Transcript levels of GFP were normalised to mouse vimentin.
E) The number of CTC colonies per 100 μl of blood four weeks after intracardiac tumour cell inoculation. Representative images of CTC colony assays from F) control shRNA, G) Pim-1 shRNA 1 and H) Pim-1 shRNA 2. *=p<0.05, **=p<0.005, ***=p<0.001 compared to control as determined by one-way ANOVA with Dunnetts multiple comparisons test.
We noted during harvest that there was an unexpectedly high incidence of mice developing liver metastases with 7/10 mice presenting with liver metastases in the control group, 8/10 for the Pim-1 shRNA 1 group and 5/10 for the Pim-1 shRNA-2 group (Fig 4.12, Fig 4.13E). Interestingly, many of the mice from the Pim-1 shRNA groups, but not from control group, showed accumulation of bile within the liver, most likely reflecting impaired function. This is illustrated in Figure 4.12, with 4.12A showing livers from control mice, 4.12B from Pim-1 shRNA 1 mice and 4.12C from Pim-1 shRNA 2 mice. The green arrows indicate tumour nodules and the red arrows indicate the presence of these bile nodules.

Figure 4.12: Representative images of livers four weeks after intracardiac tumour cell inoculation
Representative images of livers from mice injected with A) MDA-MB-231Br control shRNA cells, B) MDA-MB-231Br Pim-1 shRNA 1 cells and C) MDA-MB-231Br Pim-1 shRNA 2 cells. Green arrows indicate tumour nodules and red arrows indicate nodules of bile.
Maestro fluorescence imaging showed that 3/10 (30%) of mice from the control shRNA group had brain metastases, compared to 1/10 (10%) and 0/10 (0%) in the Pim-1 shRNA 1 and Pim-1 shRNA 2 groups respectively (Fig 4.13A-D). In addition to brain and liver metastases, metastatic nodules were also observed in the adrenal glands, ovaries, kidneys, heart and spleen of control mice and the incidence was reduced in the Pim-1 shRNA 1 and Pim-1 shRNA 2 groups (Fig 4.13E).

Figure 4.13: Brain metastasis incidence after intracardiac injection.
Brain metastases were visualised using a Maestro imager at necropsy, four weeks after intracardiac injection of MDA-MB-231Br cells. Brightfield images are shown in the top panels and fluorescence images in bottom panels. A)-C) from mice injected with control shRNA cells and D) from a mouse injected with Pim-1 shRNA 1 cell line. E) Table showing the incidence of mice with visceral metastases in each group.
4.3 Discussion

Pim-1 is a growth and survival kinase that has been implicated in metastatic processes in leukaemia, lymphoma, pancreatic and prostate cancers. However, the functional contribution of Pim-1 to breast cancer metastasis has not yet been fully characterised. In Chapter 3, we showed that Pim-1 levels are elevated in breast cancer cell lines with brain metastatic capabilities. Therefore, the aim of this chapter was to characterise the functional contribution of Pim-1 to breast cancer metastasis, and subsequently to further examine the potential functional relationship between Pim-1 and brain metastasis in vivo.

In this chapter we have demonstrated that suppression of Pim-1 by shRNA in MDA-MB-231Br cells reduces the ability of the cells to migrate and invade in vitro and that this corresponds to a decrease in the surface expression of the integrin subunits β1 and β3. We have also shown that, paradoxically, suppression of Pim-1 in murine 4T1Br4 cells increases the ability of these cells to migrate and invade, and these cells show a decrease in β4 integrin expression.

However, after treatment with low concentrations of SGI-1776, both MDA-MB-231Br and 4T1Br4 cells showed decreased ability to migrate and invade and that this corresponds to differential expression of β-integrins. Furthermore, we have shown that Pim-1 KD in MDA-MB-231Br cells reduces their ability to form bone metastases and may impair their ability to migrate in vitro as seen by the decreased number of CTCs.

4.3.1 Pim-1 Knock down by shRNA differentially impacts on Pim-2 and Pim-3 expression.

We used a stable KD of Pim-1 by shRNA in the hope that we could discern Pim-1-specific effects and document its role in BC metastasis. We obtained efficient Pim-1 KD in both 4T1Br4 and MDA-MB-231Br cells but could not obtain a specific Pim-1 KD with 2 hairpins in either cell line without affecting the expression of other isoforms Pim-2 and Pim-3. This effect is most marked in the 4T1Br4 cell line, where both hairpins reduced the mRNA expression of not just
Pim-1, but also Pim-2 and Pim-3 to near undetectable levels (Fig 4.1). This finding was unexpected as the shRNA sequences were designed to be highly specific towards Pim-1, and we have validated that both hairpins that were used targeted Pim-1 only and did not display any sequence alignment towards Pim-2 or Pim-3 (Fig 2.2).

One possibility is that due to clonal selection, we have isolated clones that, for one reason or another, do in fact target the other members of the Pim family. However, this is an extremely unlikely phenomenon due to the fact that we used two different clones from two different hairpins, and as shown in Fig 2.2, each hairpin targets a different section of the Pim-1 transcript. Thus, for both clones to display the same off target effect is extremely unlikely.

With certain families of proteins, there is a degree of co-regulation of expression, meaning that if the expression of one member is downregulated, the expression of the other members is also impacted. It is well accepted that there is a degree of co-regulation between the members of the Pim kinase family (Mikkers, Nawijn et al. 2004). However, this usually means that when the function of one member of the Pim family is impeded, then expression and/or function of the other members actually increases, as shown by the fact that only when all three isoforms are knocked out that mice show an observable phenotype (Mikkers, Nawijn et al. 2004). This indicates that Pim kinases exhibit some functional redundancy, so it is unlikely that the decreased of expression of Pim-2 and Pim-3 is in direct response to the knock down of Pim-1. However, the human MDA-MB-231Br cells had naturally higher basal Pim-2 levels compared to the 4T1Br4 cells and further increased in the MDA-MB-231Br shRNA 2 cells (that were used for subsequent functional testing) after Pim-1 KD (Fig 4.1). This could indicate that, for this particular KD cell line, Pim-2 may have increased expression in response to Pim-1 KD. However, the effect of this slight upregulation of Pim-2 is unlikely to be sufficient alone to compensate for the lack of Pim-1, since this cell line showed significant decreases in migration and invasion after Pim-1 KD (Fig 4.3).
It is well established that Pim-1 expression can be regulated by the relative expression of other kinases, for example, Akt. Pim-1 and Akt have overlapping substrate specificity and similar activities as they both regulate apoptosis, cell cycle progression and cellular metabolism (Amaravadi and Thompson 2005, Bullock, Debreczeni et al. 2005). Mice doubly deficient in both Pim-1 and Akt rarely survive development and those that overexpress both Pim-1 and Akt show predisposition to rapid tumour formation (Hammerman, Fox et al. 2005). Combined inhibition of both Pim-1 and Akt synergistically inhibits prostate cancer growth in vivo (Cen, Mahajan et al. 2013). Together, this indicates that these two kinases are required for many overlapping growth and survival processes. As such, the change in levels of expression or activity of one can impact on the expression of the other. For example, pharmacological inhibition of Akt can induce upregulation of Pim-1 (but not Pim-2 or Pim-3) expression and activity in prostate cancer (Cen, Mahajan et al. 2013). Therefore, there is a possibility that another kinase, such as Akt, may be playing a role in the absence of Pim-1, leading to the unexpected reduction in the other Pim kinases.

Above all, we must consider the obvious differences between mouse and human cancer cell lines. While mouse cancer cell lines are excellent tools to model human breast cancer in vitro and in vivo, there are significant differences in metabolism and sensitivity to transformation (see review by (Rangarajan and Weinberg 2003) and so it is not impossible that mouse and human cells may react slightly differently after Pim-1 KD. Indeed, the mouse 4T1Br4 cells had roughly 4-fold higher basal Pim-1 and Pim-3 levels than the human MDA-MB-231Br cells (Fig 4.1). So, we see a greater magnitude of reduction in Pim-1 and Pim-3 levels in the mouse cells than the human cells after Pim-1 KD. This may mean that related pathways are differentially affected between the cell lines, leading to different downstream signalling, and differences in phenotypic effects.
4.3.2 Pim-KD decreases the ability of MDA-MB-231Br cells to migrate and invade

The decrease in migratory and invasive ability of the MDA-MB-231Br cells with suppressed Pim-1 expression in vitro indicates that the loss of Pim-1 may alter several metastatic properties in these cells.

We expected that Pim-1 shRNA would decrease the ability of our breast cancer cells to migrate and invade as there are several studies linking Pim-1 to migration in cancer. Systemic Pim kinase KO reduces sarcoma growth and bone invasion in the 3-methylchloranthrene model of carcinoma (Narlik-Grassow, Blanco-Aparicio et al. 2012). Pim-2 or Pim-3 KO mice alone showed similar decrease in bone invasion to the triple KO (Narlik-Grassow, Blanco-Aparicio et al. 2012). Overexpression of Pim-3 in SKOV3 ovarian cancer cell lines increased migration (wound healing) via MACC1 (metastasis associated in colon cancer) (Zhuang, Zhao et al. 2014). Transient knock down of Pim-1 by siRNA inhibited migration and invasion of prostate PC3 cells and UT-SCC-12A (Santio, Vahakoski et al. 2010). This study also overexpressed Pim-1 and found that the cells migrated a lot faster than the controls. This observation was further corroborated in vivo (Santio, Eerola et al. 2015).

We have proposed that this decrease in migration and invasion is due to a decrease in cell surface integrin expression, most markedly for β1 integrin, and to a lesser extent, β3 integrin (Fig 4.5). We have found no previous indication that Pim-1 directly interacts with any β-integrin and so this observation may represent an entirely novel interaction in the development of breast cancer metastases. Therefore, in an effort to understand this interaction, we have postulated several pathways and signalling intermediates that could be the missing link between Pim-1 signalling and integrins.

Several potential mechanisms have been postulated for the decrease in migration and invasion upon Pim-1 inhibition. One such is a decrease in
CXCR4/CXCL12 chemokine signalling. The chemokine receptor CXCR4, along with its ligand CXCL12 has been implicated in breast cancer metastasis, with the use of stable RNAi in 4T1 breast cancer cells blocking cell growth and metastatic spread (Smith, Luker et al. 2004). In leukaemia, blocking Pim-1 activity downregulated cell surface expression of CXCR4 by direct phosphorylation of serine 339 (Brault, Gasser et al. 2010). Furthermore, a correlation between Pim-1 overexpression and CXCR4 surface expression was found in cells isolated from leukaemia patients, and treatment with a small molecule Pim-1 inhibitor decreased the cell surface CXCR4 expression in the cells of 4 out of 6 patients (Grundler, Brault et al. 2009). However there has been no link between CXCR4/CXCL12 signalling and integrins so it is unlikely Pim-1 is mediating its actions through this axis.

Another, more likely mechanism linking the inhibition of Pim-1 to the downregulation of integrin expression is the regulation of Nuclear Factor of Activated T Cells (NFATC) transcription factors, in particular, NFATC1. NFATC proteins are transcription factors involved in the development and activation of various cell lineages from lymphocytes to differentiation of cardiac muscle cells (Mancini and Toker 2009). Prior to activation, NFATC proteins are found in an inactive conformation in the cytoplasm, heavily phosphorylated on several serine residues (Mancini and Toker 2009). Dephosphorylation of these sites by calcineurin causes activation, nuclear localization and retention where NFATC binds DNA and exerts its effects. The NFATC proteins were first discovered as an inducible nuclear factor bound to the IL-2 promoter during the activation of T-cells (Shaw, Emmel et al. 1988), but further investigation has found NFATC proteins are expressed ubiquitously throughout the body and their activity is linked to tumour progression.

Importantly, there is evidence that NFATC proteins interact both with Pim-1 and integrins, indicating they may be the mediators behind the downregulation of integrin expression that we have observed after inhibition of Pim-1. NFATC protein activity is enhanced by Pim-1, as Pim-1 phosphorylates NFATC1 on a serine residue distinct to those recognised by calcineurin, causing nuclear translocation and enhancement of transcriptional activity (Rainio,
Pharmacological Pim-1 inhibition by the specific Pim-1 inhibitor DHPCC-9 also decreased NFATC dependent migration of PC3 cancer cells (Santio, Vahakoski et al. 2010).

NFATC proteins are implicated in the regulation of integrin expression. For example, there is evidence for the fact that NFATC1 alters β3 integrin expression. The β3 gene has NFAT binding sites, and also some NFAT/AP-1 binding sites and NFATC directly regulates β3 transcription in osteoclast differentiation (Crotti, Flannery et al. 2006). Reduced NFATC activity is also associated with reduced β1 expression and activation in infantile haemangioma (Jinnin, Medici et al. 2008). There also seems to be compensatory feedback regulation between NFATC proteins and integrins as α6β4 integrin enhances NFATC transcriptional activity and promotes breast cancer invasion (Jauliac, Lopez-Rodriguez et al. 2002). There has long been a link between integrins and Ca2+ established. Integrin stimulation can increase Ca2+ which can induce NFATC activation (Jinnin, Medici et al. 2008). However this study stresses that this shows a link but not causation, hence further study is required to properly elucidate the relationship between integrins and NFATC proteins.

In short, this body of evidence is consistent with a functional relationship between Pim-1, NFATC proteins and β-integrin expression, though further research needs to be done to elucidate the precise mechanism and sites of interaction.

4.3.3 Pim-1 KD increases the ability of 4T1Br4 cells to migrate and invade

It was very surprising to see that while MDA-MB-231Br Pim-1 KD cells showed decreased migratory and invasive capability, the 4T1Br4 Pim-1 KD (specifically Pim-1 shRNA 1) cells showed an increase in the ability to migrate and invade. This corresponded to the slight increase in expression for β1 integrin and a significant reduction in β4 expression (shRNA 2) but no change in expression for β3 in these 4T1Br4 cells after Pim-1 shRNA (Fig 4.5).
The relationship between adhesion and migration shows a bell shape curve as illustrated by (Beckerle 2001) (Fig 4.14). This curve illustrates that migration speed is the net result of three forces 1) protrusion of the leading edge of the cell, followed by 2) formation of adhesion/adhesive complex, generating the required traction for cell movement. Then 3) there is a release of adhesion at the rear of the cell which allows forward movement (Huttenlocher, Ginsberg et al. 1996). Therefore, cell migration speed is greatest at intermediate adhesive strength as, when there is not enough adhesion, no traction can be generated, and when the adhesive strength is too high then the cell cannot release from the cytoskeleton.

There is a large amount of evidence for this bell-shaped curve relationship between strength of adhesion and migration. Human smooth muscle cells migrate most efficiently when substrate protein (fibronectin or collagen IV) is at an intermediate concentration (DiMilla, Stone et al. 1993). Furthermore, it was found that locking α2β3 integrin into a high affinity state decreased migration (Huttenlocher, Ginsberg et al. 1996). Increasing cytoskeletal and focal adhesion organization had the same effect. Additionally, antibody activation of α4β1 integrin decreased haptotactic migration of eosinophils (Kuijpers, Mul et al. 1993). More recently, the loss of Pim-1 was found to confer a hyper-adhesive state on mouse endothelial cells, which correlated with reduction in rate of migration and alteration to the expression of several integrin subunits (Walpen, Peier et al. 2012).
Therefore, this could be an explanation as to why in the 4T1Br4 mouse cells, Pim-1 knock down increases migration and invasion. It is possible that at baseline (control shRNA), these cells have high integrin expression promoting high adhesion and with Pim-1 KD, there is a small decrease in integrin expression resulting in a slight release in the adhesive ability of the cells, which could result in an increase in migration rate.

As the 4T1Br4 Pim-1 KD cells showed an increase in migratory and invasive potential, we hypothesised that there would also be an increase in integrin expression upon knock down. However that was not the case as there was only a slight decrease in β4 integrin and no change in the expression of β1 and β3 integrin. Therefore, this increase in migration and invasion observed must be due to another mechanism.

4.3.4 Pharmacological Pim-1 inhibition by SGI-1776 decreases the ability of 4T1Br4 and MDA-MB-231Br cells to migrate and invade

SGI-1776 is a specific Pim-1 inhibitor with a proven *in vitro* cytotoxic efficacy against several cancer lines including prostate cancer cells, pancreatic cancer, renal cell carcinoma and leukaemia (Chen, Kobayashi et al. 2009, Chen,
Redkar et al. 2009, Mumenthaler, Ng et al. 2009, Chang, Kanwar et al. 2010, Mahalingam, Espitia et al. 2011). Due to these promising results, SGI-1776 was evaluated in a Phase 1 clinical trial for patients with castration-resistant prostate cancer or relapsed/refractory non-Hodgkin lymphoma. However the trial was terminated prematurely due to a narrow therapeutic window and the development of cardiac QT prolongation (irregular heartbeat) in high doses (Foulks, Carpenter et al. 2014). Despite the shortcomings of SGI-1776 as a therapeutic agent, it remains a useful tool to study the pharmacological inhibition of Pim-1 in vitro.

The use of SGI-1776 inhibited the ability of both 4T1Br4 and MDA-MB-231Br cell lines to migrate and invade in a dose-dependent fashion. This observation validates the decrease in migration and invasion after Pim-1 KD in MDA-MB-231Br cells. However, it was surprising to see inhibition of migration in the 4T1Br4 cells after SGI-1776 treatment as Pim-1 KD of 4T1Br4 cells increased migration and invasion.

One possible explanation is that both shRNAs used impacted on the expression of not only Pim-1 but also Pim-2 and Pim-3, whereas SGI-1776 should have only impacted on the activity of Pim-1. Therefore, it is not incomprehensible that there would be different effects observed. If one assumes some functional redundancy or overlap among Pim kinases, it would be expected that the triple inhibition of all three Pim isoforms would have decreased the rate of migration and invasion even more than the inhibition of Pim-1 alone. However, this was not the case.

SGI-1776 treatment impacts only on the activation and activity of Pim-1, whereas knock down by shRNA inhibits the expression of the Pim-1 protein. Therefore, it is conceivable that some protein-protein interactions are retained with the use of SGI-1776, and compensatory pathways (such as upregulation of Akt) may not be activated, as the protein is still present. When Santio and colleagues used both Pim-1 KD (by shRNA) and pharmacological inhibition, they found that the pan-Pim kinase inhibitor DHPCC-9 significantly inhibited migration of PC-3 cells (i.e. blocked the activity of pim kinases), but had no effect on the protein levels of all Pim isoforms (i.e. no effect on expression levels), or on the proliferation and viability of the cells (Santio, Vahakoski et al. 2010).
Transient knock down by siRNA of both Pim-1 and Pim-2 also slowed rates of migration and invasion, but the effect was greater with DHPCC-9, as knock down only slowed migration slightly. This supports the argument that Pim-1 KD and inhibition by SGI-1776 have some differing effects due to different modes of action.

We must also consider the fact that SGI-1776 may be targeting other kinases, while Pim-1 KD by shRNA has been shown to be specific to Pim-1. Along, with Pim-1, SGI-1776 also inhibits Flt-3 and Haspin with kinase IC$_{50}$ values of 44 nmol/L and 34 nmol/L respectively (Mumenthaler, Ng et al. 2009). Flt-3 is a tyrosine kinase expressed on the surface of hematopoietic progenitor cells (Small, Levenstein et al. 1994), and its expression is strongly correlated with survival in breast cancer patients (Ein-Dor, Kela et al. 2004). Haspin is a recently discovered kinase that localises to chromosomes and phosphorylates histone H-3, promoting mitosis and progression through the cell cycle, and is a target of several anti-mitotic compounds for a range of cancers (Higgins 2010, Huertas, Soler et al. 2012). So, inhibition of these kinases may also be impacting on the phenotype of the SGI-1776-treated cells.

4.3.5 Pharmacological inhibition of Pim-1 differentially impacts on $\beta$- integrin expression.

Pim-1 suppression by shRNA in resulted in a decrease in $\beta$4 cell surface expression in 4T1Br4 cells (Fig 4.5), with a concomitant increase in cell migration and invasion (Fig 4.3). Pim-1 suppression resulted in a decrease in both $\beta$1 and $\beta$3 cell surface expression in MDA-MB-231Br cells (Fig 4.6) and a decrease in cell migration and invasion (Fig 4.3). However, in contrast, upon SGI-1776 treatment, both 4T1Br4 and MDA-MB-231Br cells demonstrated a decreased ability to migrate and invade and both these cell lines exhibited a decrease in cell surface expression of $\beta$3 integrin, with MDA-MB-231Br cells also showing a decrease in $\beta$4 cell surface expression (4.8, 4.9).
While it is interesting that shRNA and SGI-1776 treatment yielded variable impacts on β-integrin expression, we believe it is noteworthy that whenever Pim-1 inhibition resulted in decreased migration and invasion, there was also a decrease in β3 integrin expression. β3 has two α-integrin binding partners, αII and αv. αIIβ3 is found mainly on human blood monocytes, granulocytes and leukocytes and is involved in platelet aggregation, among other roles (Burns, Cosgrove et al. 1986), and is therefore unlikely to mediate the effects observed in our study. αvβ3 is found on the surface of endothelial cells and smooth muscle cells, as well as monocytes and platelets (Switala-Jelen, Dabrowska et al. 2004). High αvβ3 integrin expression has been documented in several cancer types including melanoma, prostate, colon and breast, and correlates with poor prognosis (Albelda, Mette et al. 1990, Vonlaufen, Wiedle et al. 2001, Cooper, Chay et al. 2002, Carter, Micocci et al. 2015). In vitro studies have shown that αvβ3 mediates breast cancer cell adhesion to vitronectin, and promotes the survival, migration and invasion of these cells (Bartsch, Staren et al. 2003, Rolli, Fransvea et al. 2003). Vitronectin is one of the ECM proteins that we used to test haptotactic migration in Fig 4.3, and is a large component of the serum we used to test chemotactic migration and invasion in Fig 4.3 and 4.7. Therefore, this decrease in migration seen in response to Pim-1 KD in MDA-MB-231Br cells and in response to SGI-1776 treatment in both 4T1Br4 and MDA-MB-231Br cells could be mediated by αvβ3.

This hypothesis would first be validated by determining if expression of αv as well as β3 is impacted by Pim-1 suppression by using antibodies against both αv and β3 to determine the cell surface expression (by FACS) and total expression (by western blotting).

4.3.6 Pim-1 inhibition by shRNA in vivo

We designed this experiment to test if MDA-MB-231Br cells with Pim-1 KD showed decreased metastatic ability in vivo. We expected that since the Pim-1 KD cells showed decreased ability to migrate and invade in vitro, that the cells may be less metastatic in vivo. The number of CTCs in the blood was markedly reduced after Pim-1 KD. The presence of CTCs in the blood is associated with the...
development of metastases, with greater numbers of CTCs associated with increased number of metastatic sites and a poorer prognosis (Giuliano, Giordano et al. 2014). From this we expected that there would be a decrease in metastases in the mice injected with the Pim-1 KD cells. However, the results were mixed. There was a significant decrease in metastatic burden in spine and in total bone in both Pim-1 KD cell lines, but no decrease in lung, femur or liver metastasis between groups.

In the original paper describing the generation of the “brain-seeking” MDA-MB-231Br cell line, the authors proposed that this cell line metastasises exclusively to brain with up to 100% incidence (Yoneda, Williams et al. 2001). Therefore, the low incidence of brain metastases seen in the control mice was surprising, as was the high incidence of liver metastasis. Since the MDA-MB-231Br line is a brain metastasis model we did not expect to see such an extent of visceral metastases. Subsequent studies by different groups found that between 82.5% and 100% of mice injected with MDA-MB-231Br cells developed brain metastases (Kim, Huang et al. 2004, Heyn, Ronald et al. 2006). However both these studies above used nude mice that lack the ability to generate mature T lymphocytes (Wortis 1971). We used the more immunocompromised NOD scid gamma (NSG) mice. NSG mice lack B cells and Natural Killer cells as well as being deficient in mature T lymphocytes (Greiner, Hesselton et al. 1998). We decided to use the NSG over the nude mice, as we wanted to give the cells the most permissive environment for metastasis and proliferation as it has been shown that MDA-MB-231Br cells metastasise well in NSG mice (Iorns, Drews-Elger et al. 2012). We were hoping that by using NSG we would get a large incidence of brain metastases. In actual fact, the opposite occurred. We believe that it is possible that by using these extremely immunocompromised mice, a large number of visceral metastases (particularly to bone and liver) developed quickly, which caused the mice to become sick before the MDA-MB-231Br cells had enough time to spread to and colonise the brain. This is one possible explanation to why we got such a low incidence of brain metastasis. In future, it would be informative to repeat the experiment using nude mice in parallel with NSGs to
see if there is any difference in visceral metastatic spread, and whether we observe a higher rate of brain metastasis in nude mouse recipients.

Taken together, the findings presented in Chapter 4 suggest a role for Pim-1 in the development and progression of breast cancer metastasis. We have presented evidence that inhibition of Pim-1 may decrease the migration and invasion of breast cancer cells by a mechanism that involved the down regulation of beta integrin expression. This decrease in invasive and migratory potential manifests in our in vivo model by a decrease in metastasis to bone and a decrease in the number of CTCs.
Chapter 5: Summary and Future directions

The overall objective of this research project was to document the expression (Chapter 3) and explore the function (Chapter 4) of Pim-1 in metastatic breast cancer. Below, we summarise the results from our investigation and discuss how they complement and integrate with findings reported by others and how they advance the field. Where appropriate, we propose areas of future investigation that could further validate our observations, address questions that arise from this work and advance our understanding of the function of Pim-1 in breast cancer metastasis and/or its association with brain metastasis.

Expression analyses from various online databases implicated Pim-1 in the development of breast cancer. However, the data available was limited and incomplete, so we aimed to perform a more in-depth analysis of the expression of Pim-1 in metastatic breast cancer. The results from our analysis are supportive of a role for Pim-1 in metastatic progression and for its potential as a prognostic factor for brain metastatic breast cancer. This is based on the observation that highest levels of Pim-1 expression are seen in mouse and human brain metastatic cell lines and tumours. Further investigation will be required to validate this correlation in patients with brain metastases and to clarify the precise mechanisms by which it modulates integrin-dependent migration and invasion of metastatic breast cancer cell lines. Should future studies confirm our observations, Pim-1-targeted therapies could be tailored for this group of patients.

Further investigation into the functional role of Pim-1 in breast cancer cell lines revealed that Pim-1 might play a role in mediating migration and invasion in metastatic breast cancer cells. This is an important finding as migration and invasion are two of the steps required for cancer cells to disseminate and metastasise to distant sites. Furthermore, silencing Pim-1 in breast cancer cells led to a decrease in CTCs and bone metastasis in an in vivo
mouse model. Therefore, targeting Pim-1 could be a viable therapy to reduce metastasis in breast cancer. However, as discussed below, additional work will be required to evaluate Pim-1-targeted therapies.

5.1 Prognostic association of Pim-1 with breast cancer metastasis/brain metastasis

5.1.1 Currently available literature

Survey of the available literature in Chapter 1 indicated that in a variety of haematological and solid cancers, high Pim-1 expression is correlated with increased tumour grade and metastatic ability, especially in concert with Myc (Liang, Hittelman et al. 1996, Chen, Chan et al. 2005, Zhang, Wang et al. 2008). However, the data specifically relating to breast cancer metastasis, molecular subtypes and/or site-specific metastasis were limited. There is some indication that Pim-1 expression correlates with the invasive capabilities of breast cancer cells as well as with a triple negative status (Gapter, Magnuson et al. 2006, Maristany, Grigoriadis et al. 2013), but further studies are required to evaluate and confirm these trends. Herein, we extended these observations and carried a more in-depth analysis of several available databases with the aim of establishing a more definitive relationship between Pim-1 and breast cancer metastatic ability and subtype.

We probed the Protein Atlas database to interrogate the expression of Pim-1 in normal and cancerous tissues. We confirmed that Pim-1 expression is low to absent in normal mammary tissue and that the expression of Pim-1 in the tumour tissue samples is increased compared to normal breast tissue (Figure 3.1). This is consistent with the current view that Pim-1 is low in most adult tissues of the body, and that its expression increases during tumour progression (Amson, Sigaux et al. 1989, Blanco-Aparicio and Carnero 2013). Interestingly, the level of expression varied significantly between tumour subtypes, indicating the
possibility that high Pim-1 expression could be associated with a certain subtype of breast cancer.

We used the BreastMark online database to evaluate the relationship between Pim-1 mRNA expression and clinical outcome. Some interesting trends were observed from this database. The most revealing trend was the strong association between high Pim-1 expression and decreased DDFS in TNBC (Fig 3.2C), which infers that high Pim-1 expression is associated with increased metastatic propensity in TNBC tumours. This finding is in agreement with the findings of Gapter and colleagues as well as Maristany and colleagues (Gapter, Magnuson et al. 2006, Maristany, Grigoriadis et al. 2013). While our own analysis identified a “trend” between high Pim-1 expression and poor clinical outcome in TNBC, the trend did not reach statistical significance. This is likely to be due to the relatively small samples sizes of the datasets analysed. Nevertheless, the associations that we found are supportive of our hypothesis and provide additional information that was not previously available from single studies. Importantly, recent work by Horiuchi and colleagues (published during preparation of this thesis), confirmed that Pim-1 expression is markedly increased in clinical TNBC tumours compared to other subtypes, and is associated with poor prognosis (Horiuchi, Zhou et al. 2016). This is a significant correlation that supports our original hypothesis that Pim-1 is associated with TNBC and breast cancer metastasis.

The Oncomine microarray database revealed a stronger association for high Pim-1 expression as a poor prognostic factor for clinical outcome than as a good prognostic factor for clinical outcome in published studies (21 studies vs. 5 studies, respectively). In particular, we identified a statistically significant association between Pim-1 mRNA expression and increased tumour grade (Fig 3.3). This observation further supports our hypothesis that Pim-1 is associated with breast cancer metastasis, as metastasis occurs with higher frequency in high-grade tumours and is associated with poor clinical outcome (Rakha, Reis-Filho et al. 2010).
5.1.2 Pim-1 is highly expressed in brain metastatic breast cancer cell lines

Contrary to our original hypothesis, Pim-1 mRNA and protein expression did not correlate specifically with tumour cell aggressiveness or metastatic ability since some aggressive and highly metastatic lines (i.e. 4T1.2 or 4T1.13 or MDA-MB-231) did not express high levels of Pim-1 mRNA and protein (Fig 3.4, 3.5). Unexpectedly, we found that the cell lines that expressed highest levels of Pim-1 were the brain metastatic cell lines 4T1Br4 and MDA-MB-231Br. This novel finding was first demonstrated at the mRNA level (Fig 3.4) and confirmed at the protein level (Fig 3.5). To the best of our knowledge, no other study has attempted to correlate Pim-1 expression with brain metastasis. Notably, this association was seen for both the murine 4T1Br4 brain metastatic cells and the human MDA-MB-231Br brain metastatic cells, suggesting a conserved role between these species. Together, these observations suggest a particular role for Pim-1 in breast cancer brain metastasis.

These findings in cell lines prompted us to confirm these observations in vivo. As expected, from the Protein Atlas data, there was a range of staining intensities between the different tumours, with the majority of the staining localising in the cytoplasm for both the murine (Fig 3.6A) and human (Fig 3.6B) tumour sections. In accordance with our mRNA and protein findings in cell lines, the 4T1Br4 and MDA-MB-231Br tumour sections showed markedly increased staining compared to the other, non-metastatic tumours. It is also interesting to note that in brain metastatic tumours there was high nuclear as well as cytoplasmic staining. This raises the possibility that nuclear translocation of the 44 kDa Pim-1 isoform may be required to promote breast cancer brain metastasis, a phenomenon so far only reported for the smaller 33 kDa Pim-1 isoform (Saris, Domen et al. 1991). But, as discussed previously, breast cancer cells express only the larger 44kDa isoform (Tipton, Nyabuto et al. 2016), so the possibility that the 44kDa Pim-1 undergoes nuclear translocation in breast cancer cells will require further investigation. It is, however, supported by the fact that several of the known substrates of Pim-1 are found in the nucleus such
as p100, CDC25a, EIF4a and NFATc proteins, indicating that Pim-1 can regulate transcription and other vital cellular functions from the nucleus (Leverson, Koskinen et al. 1998, Rainio, Sandholm et al. 2002, Bachmann, Hennemann et al. 2004).

To further investigate our hypothesis that Pim-1 expression is associated with brain metastatic breast cancer, we stained a small TMA of clinical breast cancer samples. The results from the TMA revealed unexpected findings, as high Pim-1 reactivity was associated with tumours that were HER2 positive and of Grade 3 stage (Table 3.3). There was no significant association between Pim-1 and TNBC tumours found in this small cohort comprising 48 tumour samples, of which 25 were ER+, PR+, 11 were HER2+ and 12 were TNBC. It is possible that the number of samples may be too low to provide an accurate representation of the pattern of Pim-1 expression in metastatic breast cancer. Therefore, to obtain a thorough and complete picture we require a larger cohort samples comprising both primary tumours and matched metastatic sites.

5.1.3 New questions raised

5.1.3.1 Prognostic use of tumour versus stromal Pim-1

It is well accepted that the stromal components surrounding the tumour play an important role in the growth and metastasis of tumours. It has been proposed that while normal stroma can delay or prevent tumourigenesis, abnormal stromal components can promote tumour growth (Bissell and Radisky 2001). So perhaps the discrepancies between studies on the prognostic significance of Pim-1 expression may depend on whether the high expression is found in the stroma or in tumour cells.

The impact of abnormal stroma on cancer initiation has been documented in breast cancer, with studies showing that irradiation or genetic modification of the mammary gland stroma can confer tumourigenic potential to normal, non-tumourigenic epithelial cells (Barcellos-Hoff and Ravani 2000, Kuperwasser,
Chavarria et al. 2004). Therefore, if we were to stain full tumour sections with associated stroma, we could assess if the tumours with high Pim-1 staining in the stroma versus high Pim-1 staining in the tumour showed any difference in clinical outcome. From this data, we would see if Pim-1 expression in the stroma could contribute to breast cancer metastasis and so would have merit as a prognostic factor.

### 5.1.4 Future strategies

#### 5.1.4.1 Confirm the prognostic value of Pim-1 in metastatic breast cancer

In the present study, we have observed a number of interesting trends that implicate Pim-1 as a prognostic factor for metastasis in TNBC. However, while encouraging, none of these trends reached statistical significance. Therefore, in future, it will be important for us to properly corroborate and confirm the trends we have observed in this study.

In order to gain a fuller understanding of the tumour expression of Pim-1, we would undertake a larger and better-powered TMA analysis. Ideally, this next TMA would contain hundreds of breast tumour samples of all histological and molecular subtypes, with matched brain metastases, as well as information about other metastatic sites. This would enable our analysis to be adequately powered to obtain significant associations and to confirm if high Pim-1 in the primary tumour is associated with brain metastases.

Through our collaborator Prof Sunil Lakhani, we have access to an array of matched pairs of primary breast cancers and brain metastases, as well as metastases to other sites (Da Silva, Simpson et al. 2010). This would give us a better idea of the relationship between intensity/location of Pim-1 expression and metastatic spread, and give us a more definitive picture as to the usefulness of Pim-1 as a prognostic factor for breast cancer metastasis.
5.2 Functions and mechanisms of Pim-1 in breast cancer progression

There is much evidence that Pim-1 contributes functionally to a variety of metastatic processes in cancer, including survival, proliferation, migration and invasion into surrounding tissues (Chen, Chan et al. 2005, Santio, Vahakoski et al. 2010, Narlik-Grassow, Blanco-Aparicio et al. 2012, Santio, Salmela et al. 2016). However, the evidence for the role of Pim-1 in breast cancer has yet to be fully elucidated, with very few studies attempting to address this vital question.

Therefore, we aimed to tease apart the functional role of Pim-1 in breast cancer metastasis by employing stable genetic knock down of Pim-1 with shRNA or the specific Pim-1 inhibitor SGI-1776 and to observe the effect of this inhibition of Pim-1 on several pro-metastatic functional properties.

5.2.1 Inhibition of Pim-1 alters the expression of Pim isoforms in mouse and human breast cancer cells and impacts on metastatic processes.

Suppression of Pim-1 by shRNA resulted in a significant and stable decrease in Pim-1 expression in both the 4T1Br4 and MDA-MB-231Br cell lines to almost undetectable levels at both the mRNA and protein levels (Fig 4.1). We are confident that the level of Pim-1 knock down achieved (85-97%) was sufficient to test the effect of stable Pim-1 suppression effectively.

Pim-1 is widely documented as a growth and survival kinase in many cancer types and Pim-1 KO mice demonstrate reduced growth of prostate cancer and sarcoma (Chen, Chan et al. 2005, Hu, Li et al. 2009, Narlik-Grassow, Blanco-Aparicio et al. 2012). Thus, we expected to see a decrease in proliferation upon knock down compared to control. This was not the case in the 4T1Br4 cells as there was no difference in proliferation rate between the control cells and cells expressing either of the two Pim-1 hairpins (Fig 4.3A). In contrast, there was a significant decrease in proliferation between the control cells and the two KD cell lines in MDA-MB-231Br cells (Fig 4.3B), which is consistent with our hypothesis and the available literature.
Pim-1 KD by shRNA had no effect on the ability of either 4T1Br4 or MDA-MB_231Br cell line to adhere to ECM proteins (Fig 4.3C, D). This is somewhat in disaccord with the study by Walpen and colleagues that found the loss of Pim-1 imposes a hyperadhesive phenotype on endothelial cells (Walpen, Peier et al. 2012). However, they measured adhesive ability as the time to detach cells compared the adhesion assay that we utilised. Nevertheless, it seems that for 4T1Br4 and MDA-MB-231Br cells Pim-1 does not play a functional role in mediating adhesion to ECM proteins.

5.2.2 Inhibition of Pim-1 alters the ability of mouse and human breast cancer cells to migrate and invade in vitro and in vivo

Pim-1 inhibition by shRNA demonstrated different effects on the ability of 4T1Br4 and MDA-MB-231Br cell lines to migrate and invade in vitro. Pim-1 KD of 4T1Br4 cells caused increased migration towards both serum and ECM proteins and increased invasion towards serum compared to the control KD cells (Fig 4.4A, C, E). Increase in migration and invasion upon Pim-1 suppression was unexpected, as all current literature indicates that Pim-1 contributes to, rather than inhibits, migration and invasion in cancer cells (Grundler, Brault et al. 2009, Santio, Vahakoski et al. 2010, Narlik-Grassow, Blanco-Aparicio et al. 2012). The reason for the discrepancies between these studies and ours is unclear but could be related to the repertoire of integrins expressed in difference cell lines and to changes in integrin expression upon Pim-1 knockdown. We found that Pim-1 KD in 4T1Br4 cells also resulted in a slight decrease in β4 integrin expression (Fig 4.6). Since β4 integrin is usually involved in mediating stable adhesion, it is expected that a decrease in β4 integrin would correspond to increased migration as the adhesive forces are lessened (Borradori and Sonnenberg 1996).

Pim-1 KD in MDA-MB-231Br cells induced changes more consistent with findings reported in other tumour lines and demonstrated decreased migration towards both serum and ECM proteins as well as decreased invasion towards serum compared to the control cells (Fig 4.4B, D, F). Decreased migration and
invasion upon Pim-1 suppression was accompanied by a decrease in the cell surface expression of integrins β1, β3, and β4 (Fig 4.7). To the best of our knowledge, change in integrin expression upon Pim-1 KD has not been reported previously. These novel observations indicate that Pim-1 may modulate migratory and invasive responses in part by altering cell surface β-integrin expression in TNBC.

Similarly, treatment with the specific Pim-1 inhibitor SGI-1776 reduced the ability of both 4T1Br4 and MDA-MB-231Br cells to migrate and invade (Fig 4.8) and decreased β3 integrin cell surface expression in 4T1Br4 and MDA-MB231Br cell lines as well as β1 and β4 expression in MDA-MB-231Br cells (Fig 4.9, 4.10). As discussed in section 4.3.3, differential effects of integrin genetic knockdown and pharmacological inhibition have been reported previously (Santio, Vahakoski et al. 2010). Thus, it is conceivable that SGI-1776 treatment, which impacts on the activity of Pim-1, and Pim-1 knockdown, which impacts the protein expression of Pim-1, may induce different responses. For example, blocking Pim-1 activity with SGI-1776 may still permit Pim-1 to act as a protein scaffold for mediating another biological function. Conversely, knock down by shRNA inhibits the expression of the Pim-1 protein and therefore would also prevent other kinase-independent biological functions.

To translate our findings in vivo, we employed an experimental metastasis assay that involves the injection of Pim-1 KD MDA-MB-231Br cells into the left ventricle of the heart and measured the metastatic spread of these cells after Pim-1 suppression. Pim-1 KD decreased the level of metastasis to bone and the number of CTCs (Fig 4.12). Surprisingly, Pim-1 KD had no significant effect on the incidence of brain metastasis (Fig 4.14). However, the number of brain metastases was very low in the control group as well, with only 3/10 mice exhibiting detectable brain metastases. So, even though there was a decreased incidence of brain metastases in the Pim-1 KD groups (1/10 and 0/10 incidence respectively), the difference was too low to reach significance. This low incidence of brain metastases was unexpected as the group who originally described the MDA-MB-231Br cell line reported almost 100% incidence of brain metastases, and no extracranial metastases (Yoneda, Williams et al. 2001). We propose that
the discrepancies between their study and ours may be due to the use of severely
immune-compromised NSG mice that we used compared to the nude mice used
in the other studies. We suggest that the used of these highly immune-
compromised mice allowed rapid development of extracranial metastases prior
to the development of brain lesions. Thus, the mice succumbed to these
extracranial metastases and were sacrificed before brain lesions were
sufficiently developed.

5.2.3 Unanswered questions

5.2.3.1 The contribution of Pim-2 and Pim-3 to breast cancer metastasis

In this thesis we have proposed one mechanism by which Pim-1 may
contribute to breast cancer metastasis, that is, by increasing the rates of
migration and invasion of breast cancer cells. By using a gene knock down
approach our data is consistent with this mechanism in the MDA-MB-231Br cells,
but the results obtained in mouse and human cell lines were confounded by
changes in Pim-2 and Pim-3 expression (in spite of the specificity of the hairpins
used to silence Pim-1).

Given the functional redundancy between the Pim isoforms reported by
others (Fox, Hammerman et al. 2003, Mikkers, Nawijn et al. 2004, Bullock,
Debreczeni et al. 2005, Zhang, Wang et al. 2009), it is likely that Pim-2 and Pim-3
may also play a role in mediating breast cancer metastasis, and it is possible that
the functional responses observed upon Pim-1 knock down may be attributed in
part to changes in Pim-2 and Pim-3 expression. However, it should be noted that
the more consistent results we have obtained with the specific Pim-1 inhibitor
suggest that the effects we observed are clearly mediated in part, if not mostly,
by Pim-1.

To conclusively elucidate the effects of each isoform on the breast cancer
progression and metastasis the use of specific pharmacological inhibitors of Pim-
2 and Pim-3, as well as the use of isoform-specific shRNAs would be required.
Unfortunately, at this stage, there are no commercially available inhibitors
specific to Pim-2 or Pim-3 as all currently available Pim inhibitors are either pan-inhibitors or selective inhibitors of Pim-1 (Anizon, A Shtil et al. 2010).

A more therapeutic approach would be to inhibit all three isoforms by using a pan-Pim kinase inhibitor to ascertain the effects of triple inhibition. For example, CX-6258, an orally available pan-Pim kinase inhibitor has very low IC<sub>50</sub> values for Pim-1, Pim-2 and Pim-3 of 5 nM, 25 nM and 16 nM respectively, making it an efficacious inhibitor of all three Pim isoforms, with negligible activity on other related kinases such as Flt-3 (Haddach, Michaux et al. 2011). CX-6258 demonstrates robust antiproliferative action against a range of cell lines in vitro as well as in models of prostate cancer in vivo (Rebello, Kusnadi et al. 2016). The comparison of pan-inhibitors to specific Pim-1 inhibitors may lead to some important findings on whether inhibiting Pim-1 alone is a viable therapeutic option for the treatment of triple negative breast cancer.

5.2.3.2 Validation of Pim-1 KD experiments

One way that we can validate the findings of our knock down studies is to perform rescue re-expression experiments after knock down to see if re-expressing Pim-1 restores normal function to the cells. This can be done by combining the expression of Pim-1 shRNA along with rescue cDNA in the same vector (Ma, On et al. 2007). The expression of the rescue cDNA is under the control of an inducible promoter, which enables the effect of the gene knock down by shRNA to be completely characterised before induction of the rescue cDNA. This approach would provide fine control of the expression of Pim-1 and could enable us to examine the effect of Pim-1 KD more accurately at different stages of metastatic progression.

Alternately, to overcome compensatory mechanisms that have most likely occurred with stable KD of Pim-1, we could link the Pim-1 shRNA itself to an inducible promoter. The use of the tightly regulated ecdysone-inducible system, which is non-toxic to cells, shows no expression in the un-induced state, and rapid gene suppression upon induction would suit our needs precisely (No, Yao
et al. 1996, Gupta, Schoer et al. 2004). The use of such a promoter would mean that we could induce Pim-1 suppression when required, but since it isn’t a constant knock down of Pim-1, then the compensatory mechanisms that may be occurring with the stable Pim-1 KD would not transpire. This, again, would help us to tease apart the role(s) of Pim-1 at various stages of metastasis.

Another mode of validation would be to then overexpress Pim-1 in cell lines with naturally low basal levels of Pim-1, such as 4T1Ch5 cells (see Fig 3.4A). If the cells in which Pim-1 is overexpressed begin to show functional changes that mimic those of high Pim-1 expressing cells (such as increased migration and invasion), then that would further validate our observations and conclusions.

Clustered regularly interspaced palindromic repeats (CRISPR)/cas9 is a gene editing system that can completely excise a target gene from a genome. It is comprised of a guide RNA to target the gene of interest (CRISPR) and an endonuclease, cas9 (CRISPR-associated protein 9), which causes double-stranded DNA breaks that are repaired by endogenous cellular repair mechanisms (Gori, Hsu et al. 2015, Redman, King et al. 2016). Unlike using shRNAs that affect downstream protein expression, CRISPR/cas9 creates permanent modifications to be made to the genome of the target cells and completely blocks protein production. Therefore, there is unlikely to be compensatory upregulation of other proteins in response to the suppression of Pim-1 protein expression. It could, however, impact on the gene expression of other Pim isoforms or other growth kinases, such as Akt.

5.2.3.3 Validating the function of Pim-1 in breast cancer cell survival

It is well accepted that Pim-1 plays a role in cancer cell survival through inactivation of pro-apoptotic factors including Bad, FOXO1a/3 and ASK1 (Aho, Sandholm et al. 2004, Morishita, Katayama et al. 2008, Gu, Wang et al. 2009), as well as enhancing the anti-apoptotic actions of Myb, p100 and PAP-1 (Leverson, Koskinen et al. 1998). However, there is limited understanding of the precise role of Pim-1 in breast cancer cell survival. Therefore, to elucidate the mechanism by which Pim-1 promotes breast cancer cell survival we would perform the following experiments. We have noted a reduction in the number of
viable colony-forming CTC from Pim-1 shRNA tumour-bearing mice. Whether Pim-1 facilitates the survival of circulating tumour cells in the absence of substrate could be demonstrated more conclusively using anoikis assays in which survival of control and Pim-1 shRNA transduced cells are compared after seeding onto a non-adhesive polyHEMA (poly (2-hydroxyethyl methacrylate)) coated plates (Ivascu and Kubbies 2006). We would then measure apoptotic markers such as Annexin V and cleavage of caspase-3 and caspase-9. This will give an indication as to the ability of Pim-1 KD cells to withstand anchorage-independent cellular stress compared to parental cells.

5.2.3.4 Assessing the functional contribution of tumour vs. stromal Pim-1

Many studies in the literature have investigated the role of stromatumour interactions in the development and progression of metastasis. Indeed, there are indications that the relationship between cancer cells and the surrounding stroma can determine the phenotype of the tumour as the stromal cells, along with ECM components, provide the conditions required for cancer cell growth and metastasis (Micke 2004). In fact, a high stroma/tumour ratio carries a poor prognosis in triple negative breast cancer (Moorman, Vink et al. 2012), indicating that interaction with the stroma can impact greatly on the growth of cancer. As such, it would be worth investigating the relative contribution of tumour and stromal Pim-1 to tumour growth and metastasis. This could be done by comparing tumour growth and metastatic spread of breast tumours in mice with a Pim-1 gene deletion (to assess the role of stromal Pim-1) with tumour cells with Pim-1 KD (to assess the role of tumour Pim-1) or both. If for example, the mice with the stromal Pim-1 KO displayed a far lesser metastatic spread than the tumour cell KD, this would indicate that stromal Pim-1 was more functionally important than tumour Pim-1.
5.2.3.5 Further inhibitor studies

While SGI-1776 is a useful tool to assess the effect of pharmacological inhibition of Pim-1 \textit{in vitro}, its therapeutic use \textit{in vivo} is limited by cardiotoxicity (Foulks, Carpenter et al. 2014). A possible alternative to SGI-1776 is CXR1002, an ammonium salt of perfluorooctanoic acid. With IC\textsubscript{50} values of 40 μM for Pim-1, 170 μM for Pim-2 and 240 μM for Pim-3, it is highly selective for Pim-1 and has shown efficacy against haematological, prostate, lung, and pancreatic tumour models (Barnett, Ding et al. 2010). CXR1002 has also entered Phase 1 clinical trials and does not show the same signs of cardiotoxicity as SGI-1776 (MacPherson, Bissett et al. 2011). Therefore, we propose that this agent would be a useful selective Pim-1 inhibitor to test in brain metastatic breast cancer models such as the 4T1Br4 or MDA-MB-231Br.

While these inhibitors are specific to Pim-1, systemic off target effects are likely to occur if delivered as a free drug. Specific delivery to the tumour site could be achieved through conjugation of the inhibitor to an antibody targeting a specific extracellular receptor. Pim-1 is intracellular so the antibody needs to be targeted against another extracellular receptor, for example, a β-integrin receptor. We have demonstrated that cells that express high levels of Pim-1 also have high levels of expression of certain surface β-integrin subunits and that silencing of Pim-1 decreases the level of these β-integrin receptors. So, by targeting the Pim-1 inhibitor to β-integrin receptors, the inhibitor should selectively target cancer cells with high integrin expression, reducing side effects and systemic toxicity.

5.2.3.6 Validating the mechanism behind the regulation of integrin expression by Pim-1

An important finding from this thesis is that inhibition of Pim-1 decreases the ability of breast cancer cells to migrate and invade and that this inhibition is associated with downregulation of integrin β1, β3 and β4 expression. We believe
that this is the first time a link between Pim-1 and integrin expression has been documented.

In vivo, β-integrins form heterodimers with one α subunit and one β subunit. In this thesis, we have only probed the expression of the β subunits involved. To fully describe the integrins that mediate the changes in migration and invasion in breast cancer cells after Pim-1 KD, we need to confirm the binding partners of β1, β3 and β4. In order to do this, we firstly need to carry out co-immunoprecipitation of the known α-subunits to see if they are associated with the specific β-subunits. As discussed in section 4.3.4, we would start with the αv subunit, as we hypothesise that it is the αvβ3 heterodimer that is largely mediating the metastasis to bone that we observed in vivo.

Furthermore, the precise mechanism in which Pim-1 mediates the downregulation of integrin expression is currently unknown. There are no reports of Pim-1 interacting directly with any integrin subunits. Therefore, to investigate if there is a direct interaction between Pim-1 and integrin subunits we need to confirm if the integrin subunits are directly phosphorylated by Pim-1. Again, we could use co-immunoprecipitation and in vitro kinase assays to directly assess if Pim-1 and each of the β-integrin subunits interact.

Alternatively, Pim-1 could be acting indirectly through modulation of member of the family of transcription factors NFATC as proposed by others (Rainio, Sandholm et al. 2002, Crotti, Flannery et al. 2006, Jinnin, Medici et al. 2008, Santio, Vahakoski et al. 2010) However, this hypothesis needs to be tested more rigorously, as there is currently no known link between Pim-1 and NFATC proteins in metastatic breast cancer. Thus, if we could establish the link, first between Pim-1 and NFATC and then to integrin expression, we may have discovered a novel functional mechanism of Pim-1 in breast cancer metastasis.

We plan to do this by assessing the effect of knocking down and then overexpressing the expression of NFATC proteins to see if there is a corresponding decrease in migration and invasion of the cell lines and a decrease in β-integrin expression. Then we would use the Pim-1 inhibitor SGI-1776 and see if the use of this agent decreases the expression of NFATC proteins. If the expression level of the NFATC proteins were reduced, we would conclude that
the inhibition of Pim-1 has caused this subsequent reduction in NFATC expression. Finally, we would test if an NFATC inhibitor mimics the reduction of integrin expression in the same way as SGI-1776. These data altogether would tell us more conclusively if Pim-1 is signalling through NFATC proteins to cause cell surface downregulation of β-integrins in TNBC.

Whether direct or indirect, modulation of integrin function by Pim-1 could provide novel strategies to inhibit BC metastasis and improve disease outcome in TNBC.

5.2.3.7 Further investigations on metastasis

In this thesis, we have shown that in an experimental model of metastasis, MDA-MB-231Br cells metastasise with high frequency to bone and liver after intracardiac injection. Importantly, Pim-1 knock down reduced the incidence of bone metastasis. This is an extremely interesting finding and one that agrees with our hypothesis that the inhibition of Pim-1 would decrease TNBC metastasis. We propose that Pim-1 regulation of migration and invasion impacts on the ability of tumour cells to escape the primary tumour. Since we only tested the effect of Pim-1 KD in an experimental model, the logical next step would be to test the effect of KD in a spontaneous metastasis assay in which Pim-1 inhibition may have a greater effect on 4T1Br4 metastatic processes. This model involves the orthotopic injection of tumour cells into the mammary fat pad. The tumour is allowed to grow to a certain size, surgically resected, and the resulting spontaneous metastases are allowed to develop until endpoint (Eckhardt, Francis et al. 2012). This model faithfully recapitulates the clinical presentation and treatment of metastases in the patient.

Then, we would assess the effect of another clinically relevant Pim-1 inhibitor on the growth and metastasis of the control MDA-MB-231Br cells, such as CXR1002 as discussed above. In the first instance we would commence treatment when the tumour is palpable, in a neo-adjuvant manner with the aim of reducing tumour growth to a surgically removable size and reduce the chance of metastatic spread. Then, in the next set of experiments we would treat post resection, in an adjuvant approach to kill cells that have already disseminated.
This latter approach mimics the typical clinical scenario and we would observe if CXR1002 inhibits mid to late stage metastasis. This would be especially relevant, as in the current study we found that Pim-1 KD reduced the number of viable CTCs in the blood. This raises the possibility that Pim-1 inhibition is more useful to prevent survival and homing of CTCs to distant organs than to directly inhibit established metastases. By comparing the extent of metastatic burden when treatment is initiated early compared to after tumour resection, we may obtain further insight into the function of Pim-1 in metastasis and the critical steps of the metastatic cascade that is most efficiently targeted by Pim-1 inhibition.

In the current study, the low number of brain macrometastases observed in the control group was unexpected given that these cells have been reported to specifically and efficiently spread to brain (Yoneda, Williams et al. 2001). We relied on the Maestro imager to capture the GFP fluorescence of the MDA-MB-231Br cells and to inform us where metastases occurred. Therefore, we were able to identify large macrometastases easily. This method is not sensitive enough to detect micrometastases and metastases lodged deep within the brain and so future investigation should extend our findings by probing for the presence of these micrometastases to determine if PIM-1 silencing also affects their incidence.

There are two ways in which we would check for potential micrometastases. The first using a sensitive genomic qPCR approach to detect a fluorescent marker gene present only in tumour cells on the whole brains to check the relative tumour burden of the brains (Johnstone, Smith et al. 2015). This would give us a quantitative assessment of total metastatic burden in the brain. However, this approach may be limited by the high cellularity of the brain, which could reduce the sensitivity of detection when the overall burden is low (Dr Normand Pouliot, unpublished observations). Alternatively, whole brains could be sectioned and analysed for the presence of micrometastases by IHC detection of cytokeratin-positive nodules. This is a far more time-consuming method, but a more thorough one and would increase the likelihood of obtaining a more accurate measurement of the number and size of brain micrometastases in each group.
5.3 Conclusion

In this thesis, we have proposed that high Pim-1 expression contributes to the propensity of triple negative breast cancer to spread to brain (and other sites). We propose that Pim-1 contributes to the progression of breast cancer metastasis by facilitating the migration and invasion of the tumour cells into the circulation and that this facilitated by interactions (either direct or indirect) with β-integrins. Furthermore, in our experimental model of metastasis we found that silencing Pim-1 decreased the number of CTCs and bone metastases indicating that Pim-1 may play a role in late stage metastasis.

In summary, although much needs to be done in the future, our study provides further evidence that Pim-1 contributes to breast cancer progression and metastasis.
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