DOES LOSS OF PRH ALTER THE SENSITIVITY OF DASATINIB IN BREAST TUMOUR CELLS

By

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I dedicate this thesis in memory of my beloved mother Manjit Kaur who will always be my shining light in times of need.

ABSTRACT

Breast cancer is the most common type of cancer to cause death in the UK (Cancer Research UK, 2014). Current therapeutic treatment has achieved significant success in reducing mortality rates but there are still a minority of patients for whom treatment is unsuccessful. A form of targeted therapy for breast cancer is the treatment with the Src/ABL inhibitor, Dasatinib. This project explores whether the transcription factor Proline Rich Homeodomain (PRH) mediates dasatinib sensitivity in breast tumour cells. Here we show that immortalised, non-tumour MCF-10A control cells are more sensitive to dasatinib than MCF-10A PRH knockdown cells Similarly, tumorigenic MCF-7 control cells are more sensitive to dasatinib treatment than MCF-7 PRH knockdown cells suggesting that in both of these cell types reduced viability following dasatinib treatment is mediated in part through PRH. In contrast, no significant difference in dasatinib sensitivity was found between the highly metastatic MDA-MB-231 control cells and MDA-MB-231 PRH knockdown cells. However, MDA-MB-231 control cells are more sensitive to dasatinib treatment than MCF-7 and MCF-10A control cells. We further investigated the molecular mechanism by which dasatinib sensitivity is mediated through PRH in the MCF-7 control and MCF-7 PRH knockdown cells. This thesis therefore demonstrates the potential role of PRH as a biomarker for dasatinib treatment of luminal breast cancer patients.

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Firstly, I would like to thank Dr Sheela Jayaraman for the advice, support and guidance I have received throughout my experimental and theoretical work for this project. I would also like to take this opportunity to express my gratitude towards my co-supervisor, Dr Rachael Kershaw whose fantastic experimental expertise and knowledge has taught me what I know today in the laboratory. I would also like to point out that without this remarkable supervision partnership, this project would never have been possible and am sincerely grateful for their input.

Secondly, I would like to thank my family who have always taught me that there are no shortcuts to any place worth going, by instilling in me a very strong work ethic. They will always be my greatest support network and without their countless best wishes, prayers and blessings this work would never have been possible.

Lastly, I would like to thank my friends for their encouragement and for always being the provider of fun times.

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INTRODUCTION

1.1   Breast Cancer

Breast cancer is the most common cancer to cause death amongst western women. In 2011 49, 936 women were diagnosed in the UK with invasive breast cancer (Cancer Research UK, 2014). The breasts primarily function as apocrine milk-producing glands. Figure 1.1 illustrates the structure of the breast which consists of alveoli in which lactocytes can be found. Lactocytes function to secrete milk. The clustering of the lactocytes form a lobule and the ducts act as transporters of milk that secrete milk through the nipple (Help Fight Cancer, 2011). Cancer within the lobules is known as lobular carcinoma and can become invasive when the wall of the lobule has been penetrated, indicating that the surrounding tissues of the breast have been invaded. Ductal carcinoma in situ (DCIS) occurs when the cancer has developed from the ducts of the breast and spread to surrounding areas of the breast tissue. (Cancer Research UK, 2012).
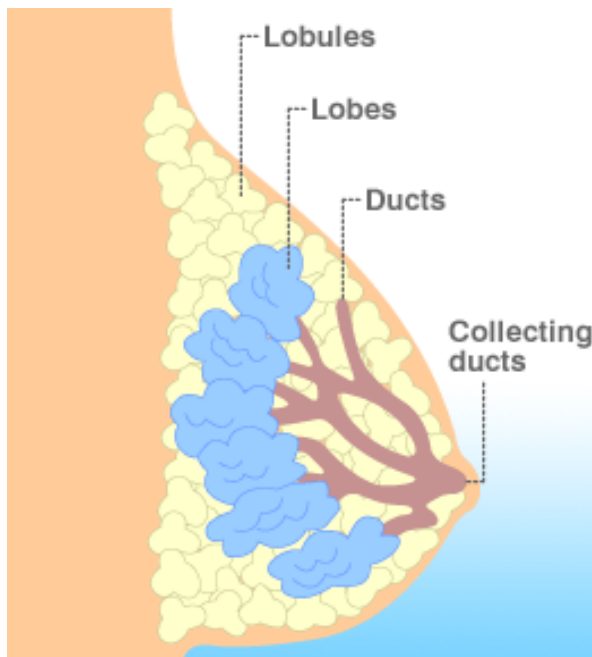
Figure 1.1: Anatomy of the breast. The breast consists of lobules and ducts that predominately function to produce and secrete milk. Taken from (Help Fight Cancer, 2011) (**http://www.helpfightcancer.com/learn/**).

1.2   Subtypes of Breast Cancer

The heterogeneous nature of breast cancer has led to the classification of various subtypes: basal (triple-negative), luminal (hormone-receptor-positive) and the Human Epidermal Receptor (*HER2*) (Schnitt, 2010). Basal tumours are progesterone (PR), oestrogen (ER) and *HER2* negative and have a more aggressive nature compared to other tumour-types; basal tumours also have a poor prognosis (Schnitt, 2010). Luminal tumours are ER/PR positive where approximately 70% are invasive and respond well to endocrine therapy. Finally, *HER2* tumours are ER/PR negative and are generally high grade tumours (Schnitt, 2010). Gene expression profiling and hierarchical clustering has led to the identification of seven additional subtypes: luminal A, luminal B, luminal C, *HER-2-*enriched (*HER-2+*), claudin-low, basal-related and normal breast-related (Kittaneh, *et al*, 2013). Table 1 highlights the main features for each subtype.

Table 1.1: Recently classified breast tumours. Taken from Kittaneh, *et al*, 2013

Breast Cancer Subtype	Features
Luminal A	Over express ER-regulated genes Under express proliferation-related genes Sensitive to endocrine therapy
Luminal B	Higher expression of proliferation-related genes Genetically unstable- harbour *TP53* mutations Higher risk of relapse
Luminal C	Distinguish from luminal A/B by their high expression of a set of genes with unknown function. For example transferrin receptor (CD71)
HER-2+	High expression of *HER2* proliferation genes Low expression of luminal clusters. For example luminal cytokeratins (CK) CK7.
Claudin-Low	Over expression of EMT-related genes. For example vimentin. No expression of luminal differentiation markers Exhibit metaplastic and medullary differentiation
Basal-related	ER^-/PR^-/*HER2* $^-$ High frequency BRCA1 mutations High histological grade Not synonymous with triple negative (TN) sub-group, TN are more heterogeneous

1.3   Breast Cancer Therapy

The identification of such sub-types has led to the advancement in generating therapeutic methods where great clinical success in patient management has emerged. BRCA1 and BRCA2 are proteins involved in DNA double strand repair by homologous recombination (Karami, and Mehdipour, 2013). Sufferers with BRCA1 and BRCA2 mutations have a reduced survival. Risk based screening has aided family members with the BRCA1 and BRCA2 mutations to control their risk in having cancer, for example by preventative mastectomy (Hall and Easton, 2013). Similarly, the administration of the monoclonal antibody Herceptin (*Trastuzumab)* to patients with over expressed *HER2* signalling has significantly increased patient survival (Slamon, 2001). At present, the first line of treatment for metastatic breast cancer sufferers is Herceptin and Tamoxifen for non-metastatic breast tumour sufferers. However, many patients have developed Herceptin resistance, further investigation has revealed great clinical benefit of antibody drug conjugates (ADC), such as *Trastuzumab*-DM1 (Hughes, 2010). This drug contains a special linker that combines the main carrier *Trastuzumab* to the fungal toxin DM1, which is a potent cytotoxic chemotherapeutic drug (Lewis-Phillips, *et al*, 2008). This form of targeted drug delivery has been shown to overcome Herceptin resistance in *HER2* expressing cells via endocytosis (Hughes, 2010).

Targeted therapy has led to the discovery of novel therapeutic targets in breast cancer, for example administration of the cyclin-dependent kinase (CDK) inhibitor, Flavopiridol. Breast tumour cells are sensitised *in vitro* to TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis, by initiating earlier events within the apoptotic pathway (Munagala, *et al*, 2011). Dasatinib (Sprycel) is a dual Src-ABL kinase inhibitor approved for the treatment of imatinib-resistant/ intolerant BCR-ABL+ leukaemia (Kantarjian, *et al*, 2010). Findings from ongoing-phase II clinical trials have shown dasatinib to have little reduction in anti-tumour activity in metastatic breast cancer patients, as a monotherapy (Herold, *et al*, 2011 and Finn, *et al,* 2011). However, administration of dasatinib in conjunction with chemotherapy agents like paclitaxel, leads to increased tumour stability in patients which have already received taxane treatment (Munagala, *et al*, 2011). Paclitaxel is a microtubule-stabilising drug that binds to the β-tubulin subunit causing cell death due to mitotic arrest (Zasadil and Weaver, 2012 and Zasadil, *et al*, 2014). However

it still remains unclear whether there are suitable biomarkers that can identify a cohort of patients that will benefit from dasatinib treatment. With the evolution of diagnostic and preventive techniques in breast cancer management, this project explores the potential therapeutic benefit of dasatinib targeted therapy, directed towards breast tumour cells expressing high levels of the transcription factor PRH.

1.4   Dasatinib

The potent, small molecule is a well-tolerated drug that targets members of the Src family kinases (SFK), c-Kit, EphA2 and PDGFR (Nautiyal *et al*, 2009). The inhibitor functions by binding to the ATP binding site and inhibits differentiation of cells in the blast phase of chronic myeloid leukaemia (CML) (Nautiyal *et al*, 2009). Dasatinib has a greater affinity for the ABL kinase domain compared to Imatinib, therefore has greater specificity. However, the inhibiting potential of Dasatinib is greater against Src (IC_{50} 0.5 nmol/L) compared to ABL (1 nmol/L) (Kantarjian, *et al*, 2010. Figure 1.2 shows that dasatinib has a greater inhibiting potential for Src than for EGFR, although both are direct targets of dasatinib. The inhibitory effects lead to a downstream inhibition of kinases such as AKT, MAPK and proteins that transduce signalling such as STAT5.
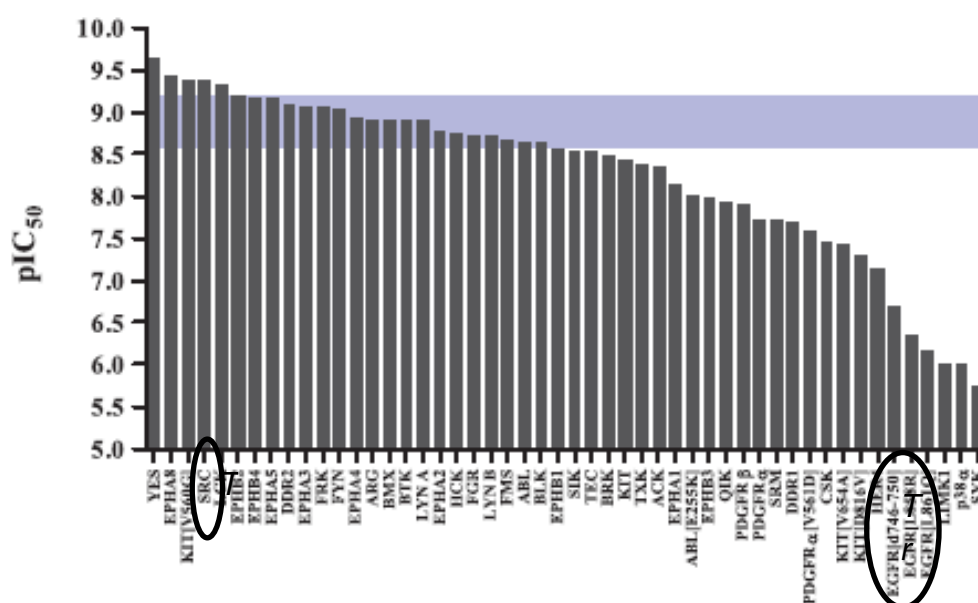
Figure 1.2: Activity-based kinase assay profile of Dasatinib. Top 50 kinases were selected and tyrosine kinase activity was inhibited using dasatinib at an ATP concentration of 1 mM. Taken from Kitagawa, et al, 2012

1.5   Role of Transcription Factors and Chromatin Modifiers in Breast Cancer

There are several transcription factors and chromatin modifiers that have been associated with breast tumorigenesis such as GATA-3, forkhead–box A1 (FOXA1), Special AT-rich Sequence-Binding protein 1 (SATB1), and *TP5*3. Although the transcription PRH is not a well-known tumour suppressor, studies from our laboratory have shown the potential of PRH as a tumour suppressor in breast tumour cells (Kershaw, *et al*, 2013 Oncogene in press and Kershaw, *et al*, submitted).

1.5.1 GATA3

GATA3 is a transcription factor that binds to the consensus motif A/TGATAA/G and functions as a regulator of differentiation during embryonic development (Lowry and Atchley, 2000). Interestingly, GATA3 is a gene regulatory factor which functions to develop immature breast cells into luminal epithelial cells (Ciocca, *et al*, 2009). GATA3 is also associated with regulatory factors such as oestrogen receptor-α (ERα) and FOXA1 (Eeckhoute, *et al*, 2007). Loss of GATA3 expression has also been associated with reduced expression of ER. GATA3 and ER are able to auto-regulate their own expression, leading to increased expression of GATA3 and ER in luminal A breast tumours (Ciocca, *et al*, 2008). In comparison to luminal A tumours, luminal B and basal-like tumours have reduced ER and GATA3 expression (Chou, *et al*, 2013). Although, a large proportion of ER+ breast tumours are known to respond well to hormone therapy, there are a small proportion of tumours that do not. Increased expression of GATA3 has been shown to be a predictor of hormonal response, in conjunction with better prognosis (Chou, *et al*, 2013). GATA3 has also been reported to drive invasive breast cancer cells to undergo reversal epithelial-mesenchymal-transition (EMT), resulting in the suppression of cancer metastasis (Yan, *et al*, 2010). Figure 1.3 highlights the frequency of GATA3 mutations in various breast tumour subtypes.

1.5.2 *TP53*

The tumour suppressor gene p53 (*TP53*) also known as the "guardian of the genome", regulates DNA damage and irregular expression of oncogenes (Horn and Vousden, 2007 and Walerych, et al, 2012). However, altered *TP53* gene expression has been found in approximately 20-40% of breast carcinomas (Dale, 2003). The mis-regulation of *TP53* activity has also been linked with the early development of breast carcinoma and has been reported in DCIS tumours prior to the development of invasive breast carcinoma (Ho, et al, 2000). Cytotoxic stress causes the phosphorylation of *TP53* within the N-terminal domain by many kinases, resulting in the stabilisation of the protein (Tibbetts, *et al*, 1999 and She, *et al*, 2000). Consequently, *TP53* forms a tetramer that binds to DNA and regulates the transcription of a range of *TP53* target genes. These target genes have been shown to be involved in the regulation of the cell cycle, cell proliferation and cell survival (reviewed by McLure and Lee, 1998). Figure 1.3 highlights the frequency of *TP53* mutations in various breast tumour subtypes.
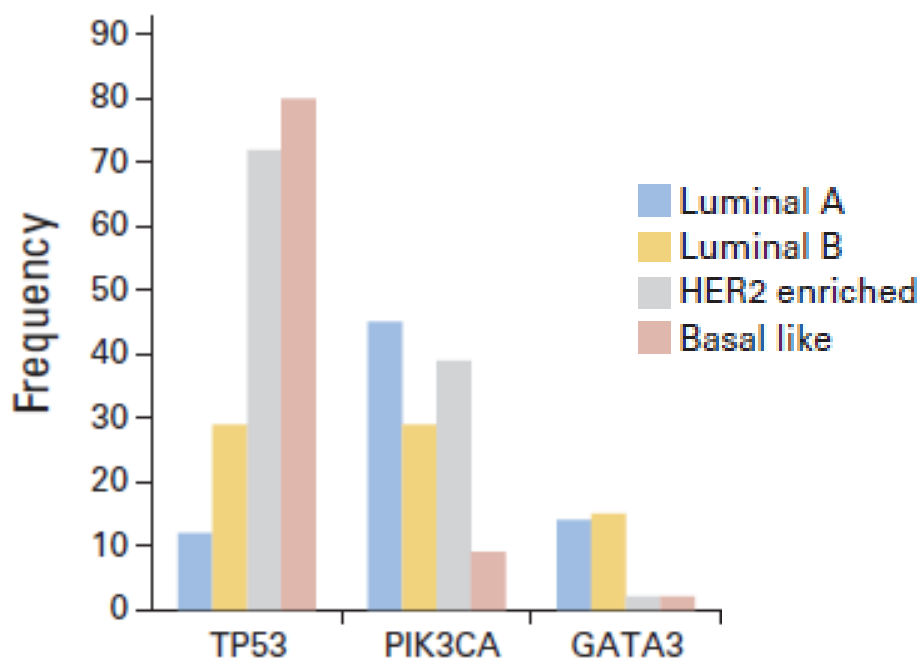
Figure 1.3: Frequency of *TP53* and GATA3 mutations in various subtypes of breast cancer. *TP53* mutations are commonly observed in basal-like tumours but are least likely to be found in luminal A tumours. GATA3 mutations are commonly found luminal B subtypes but are not frequently present in basal-like and *HER2+* tumours. Taken from Ades, *et al*, 2014

1.5.3 FOXA1

FOXA1 is a transcription factor binds to the promoters of genes that are associated with cell signalling and cell cycle regulation (Habashy, *et al*, 2008). FOXA1 possesses growth inhibitory properties and is a marker for various steps in differentiation (Habashy, *et al*, 2008). FOXA1 has also been shown to inhibit metastasis by promoting the expression of E-cadherin (Habashy, *et al*, 2008). FOXA1 binds to its consensus DNA motif which is affected by risk-associated single-nucleotide-polymorphisms (SNPs) that are able to alter the binding affinity of DNA to FOXA1. Upon oestrogen stimulation, ER is recruited to the DNA and its binding is mediated by FOXA1 (Meyer and Carroll, 2012).

1.5.4 SATB1

SATB1 is a protein which binds to the AT-rich regions of the DNA sequences (Hanker, *et al*, 2010). SATB1 is a transcriptional activator that is able to regulate cellular lineage and gene expression. The protein has also been associated with genes such as c-myc, where proliferation mechanisms are directly regulated by SATB1 (Hanker, *et al*, 2010). Silencing SATB1 gene expression in MDA-MB-231 breast tumour cells, decreases cellular polarity and reduces the invasive properties of MDA-MB-231 cells (Hanker, *et al*, 2010).

Expression of SATB1 has been linked with poor prognosis amongst breast tumour patients (Hanker, *et al*, 2010). Recent experimental data has shown SATB1 to be involved in the re-programing of chromatin modelling and transcriptional activity to induce growth of phenotypically aggressive breast tumours (Han, *et al*, 2008). This investigation was able to discover a new model for gene regulation where expression of SATB1 modifies the gene expression profile of the tumour, proving to serve as a potential therapeutic target (Iorns, *et al*, 2008). SATB1 functions by the tethering of hundreds of gene loci onto its regulatory network, where chromatin modifying and transcription factors are assembled (Iorns, *et al*, 2008). The presence of SATB1 in the nuclei of MDA-MB-231 cells is of great prognostic value in patients, independent of their lymph node status (Iorns, *et al*, 2008).

1.6   PRH

Proline-Rich-Homeodomain (PRH) is also known as the haematopoietically expressed homeodomain (HHex) and PRH is crucial in the haematopoietic system (Soufi, *et al*, 2008). PRH is a transcription factor that has a length of 270 amino acids and molecular weight of 30 kDa and PRH regulates cell differentiation, proliferation and migration (reviewed by Soufi, *et al*, 2008, Noy, *et al*, 2010 and Kershaw, *et al* 2013). The protein has three domains: N-terminal domain, homeodomain and the C-terminal domain as illustrated in figure 1.4. PRH regulates cell proliferation via protein-protein interactions that influence the cell cycle (Soufi, *et al*, 2008).It is able to regulate cellular development by modulating gene expression through transcriptional and post-transcriptional mechanisms (Soufi, *et al*, 2008). PRH has also been found to be expressed in organs of adult cells such as the thyroid (D'Elia, et al, 2002) and liver (Denson, *et al*, 2000), where it is vital in regulating the cellular proliferation and differentiation of these cells (Soufi, *et al*, 2008). The mis-localisation and altered subcellular distribution of PRH has been associated with malignancies such as leukaemia (George, *et al* 2003), breast (Puppin, *et al*, 2006) and thyroid cancer (D'Elia, *et al,* 2002).
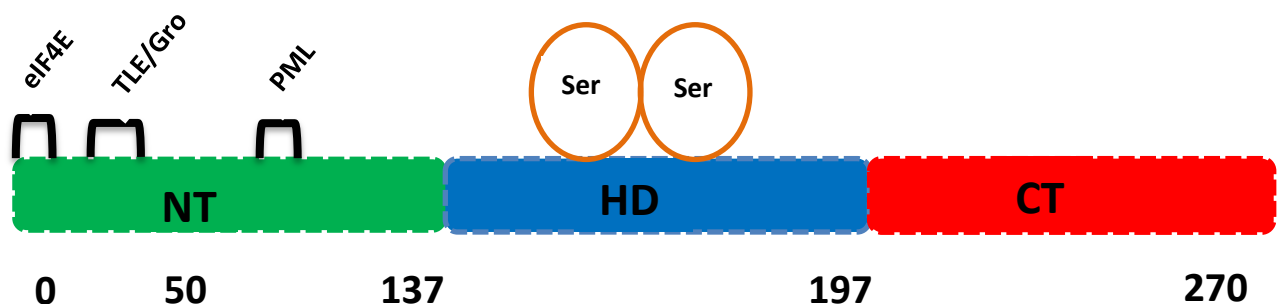
Figure 1.4: Structure of PRH. PRH consists of three major domains, the proline-rich N-terminal domain (green), the DNA binding site, homeodomain (blue) and the acidic C-terminal domain (red). The different domains also contain various sites where PRH is able to interact with DNA and proteins such as TLE. The homeodomain is the site of PRH phosphorylation where the two serine residues are phosphorylated by CK2. Adapted from Soufi, *et al*, 2008.

1.6.1 N-Terminal Domain

The N-terminus of the protein (AA 1-137) is proline-rich and fundamentally important in the oligomerisation of PRH (Soufi et al., 2008). This region also contributes to transcriptional repression through the binding of PRH, to the co-repressor Groucho/TLE (transducing-like enhancer) co-repressor family. The N-terminal domain is also involved in interactions with protein kinase CK2β subunit, promyelocytic leukaemic (PML) protein and the translation factor eIF-4E (Soufi, et al, 2008). PML is a co-repressor for transcription factors like the tumour suppressor protein Mad and SATB1 (Soufi, *et al*, 2008).

1.6.2 Homeodomain

The PRH homeodomain is 60 amino acids in length and is able to bind DNA at the consensus binding site 5'-C/TA/TATTAAA/G-3'. Generally homeodomains are present in proteins that form monomers, hetero-dimers and hetero-trimers on the binding of DNA. However, PRH is a very unique protein as it is able to form homo-oligomeric octamers and hexadecamers *in vitro* and in cells (Soufi, *et al*, 2008). This has led to the observation that PRH binds to genes via multiple tandem DNA PRH binding sites through a mechanism that does not involve a well-defined recognition site (reviewed by Kershaw, *et al*, 2012). The binding of DNA does occur through the recognition helix of the homeodomain, positioned in the major groove. The arm of the N-terminus of the homeodomain aids DNA binding by the DNA, by targeting specific base pairs in the adjacent minor groove. The resultant loop interacts with the phosphate backbone of DNA (Soufi, *et al*, 2008). The homeodomain contains two serine residues, amino acids 163 and 177 in human PRH. Phosphorylation of the serine residues by the protein kinase CK2 inactivates PRH, in that it abolishes DNA bindings and decreases nuclear retention (Soufi, *et al*, 2008).

1.6.2 C-Terminal Domain

This domain largely involves transcriptional activation and has been shown to be linked to the activation of the bile transporter gene NTCP (sodium- dependent bile acid co-transporter) (Sato, *et al*, 2004). Through the aid of the homeodomain PRH is able to bind to this promoter and activate transcription. The activation region involves the carboxyl-terminal area, with amino acids 197 to 271 human residues of PRH (Sato, *et al*, 2004 and Denson, *et al*, 2000, reviewed by Soufi, *et al*, 2008). On deletion of the amino acid residue 197 to 216 in PRH, results in a huge decline in promoter activity by approximately 50%. This strongly indicates that the homeo domain is imperative for maximal transcriptional activation (Soufi, *et al*, 2008).

1.6.3 Oligomerisation

Cross-linking studies have revealed PRH forms oligomers within cells (Soufi, *et al*, 2006, Swingler, *et al*, 2004 and Topcu, *et al*, 1999). Pull-down assays have identified two regions within the N-terminus which are involved with self-association; additionally the homeodomain is also involved in self-association (Soufi, *et al*, 2006, reviewed by Soufi, et al, 2008). Analytical ultra-centrifugation (AUC) sedimentation velocity investigations have found that the oligomeric form of PRH binds to DNA (Soufi, *et al*, 2006). The oligomeric form of PRH binds with a relatively high affinity to DNA binding sites (Bess, *et al*, 2003 and reviewed by Soufi, *et al*, 2008). PRH can also cause transcriptional repression whereby it interacts with the Groucho/TLE co-repressor and recruits TLE proteins to a subgroup of promoter targets (Brickman, *et al*, 2000, Zamparini, *et al*, 2006 and Cong, *et al*, 2006). This is depicted in figure 1.5. Oligomeric TLE/proteins can recruit histone deacetylases leading to the binding of TLE proteins to hypo-acetylated chromatin, resulting in a repressive chromatin domain (reviewed by Soufi, et al, 2008).
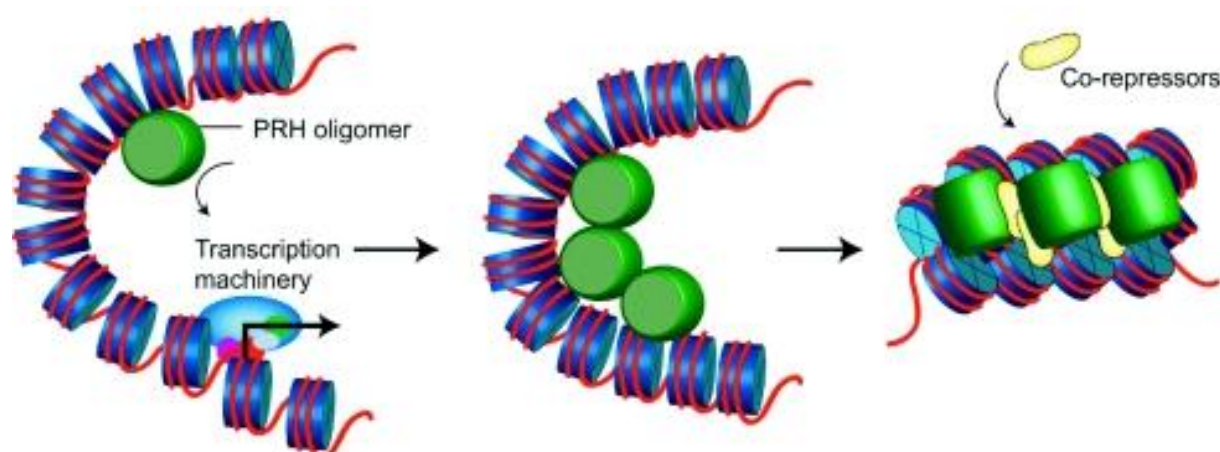
Figure 1.5: Model depicting the oligomerisation states of the PRH protein. The open state of chromatin is able to bind to PRH octamers (green) to various sites within the target promoter. The self-association of PRH along the chromatin eliminates the transcription machinery, resulting in transcriptional repression-repressed state. The condensed state of the chromatin leads to the recruitment of co-repressors such as Groucho/TLE. Taken from Soufi and Jayaraman 2008.

1.6.5 PRH Phospho-protein

Phosphorylation of PRH reduces its DNA binding ability and blocks transcriptional repression by PRH, at many PRH-dependent target genes (Soufi, *et al*, 2009). PRH binds specifically to the β subunit of CK2 leading to the phosphorylation of PRH by CK2 subunits at serine residues 163 and 177 (Soufi, *et al,* 2009). A mutant PRH protein containing glutamic acid residues instead of serine residues at the phosphorylation sites within the PRH homeodomain, has a reduced ability to bind tightly within the nucleus (Noy, *et al,* 2012). This signifies that a decrease in nuclear retention of PRH occurs upon phosphorylation. The phosphorylation of PRH leads to the formation of its truncated product, PRHΔC, by the proteasome. The PRHΔC protein functions as a trans-dominant negative protein relative to endogenous PRH by sequestering the PRH co-repressor protein (TLE) (Noy, *et al,* 2012). Thus, PRH phosphorylation is a potent mechanism of inactivation of PRH and a potential mechanism for the regulation of cell proliferation.

1.7 Role of PRH in Haematopoiesis

PRH is present in species such as *Xenopus* and zebra fish (Ho, *et al*, 1999 and Jones, *et al*, 1999). PRH mRNA was identified in extra-embryonic tissues that are involved in the anterior-posterior axis formation (Thomas, *et al*, 1998 and reviewed by Soufi, *et al*, 2008). The protein has been found within embryonic cells and mesodermal tissues that give rise to haematopoietic and vascular progenitors (Keng *et al*, 1998 and Bogue *et al*, 2000). Loss of *Prh* within the haemangioblast causes a decrease in the differentiation of haematopoietic cells (Guo, *et al*, 2003). Moreover, haematopoietic stem cells exhibit high levels of PRH but PRH expression is reduced during differentiation (Manfioletti, *et al*, 1995).

1.8 PRH and Cancer

Aberrant subcellular localisation of the PRH protein has been associated with cancers such as leukaemia (Topisirovic, et al, 2003) breast (Puppin, *et al*, 2006) and thyroid (D'Elia, et al, 2002). The expression of the fusion protein PRH-NUP98 has been shown to induce acute myeloid leukaemia (AML) and is thought that PRH-NUP98 functions as a trans-dominant negative protein in wild-type PRH and activates genes that PRH normally represses. (Jankovic, *et al*, 2008). Nuclear localisation of PRH has also been shown to be reduced in breast and thyroid cancers, as PRH expression has been found in nuclear and cytoplasmic normal breast thyroid carcinomas (Puppin, *et al*, 2006 and D'Elia, *et al*, 2002). Furthermore PRH expression is decreased in less differentiated hepatocarcinomas that are known to be more aggressive (Su, *et al*, 2012).

1.8.1 PRH and Leukaemia

Past research has revealed that within CML, eIF4E is involved in the modulation of cellular growth (Topisirovic, *et al*, 2003). eIF4E associates with PML and transports nucleo-cytoplasmic specific transcripts which contribute to the transformation of cells in CML (Topisirovic, *et al*, 2003). PRH is a tissue-specific inhibitor of eIF4E dependent cyclin- D1 mRNA transport and growth of PML, by causing cell cycle arrest and reducing cell proliferation (Topisirovic, *et al*, 2003). Interestingly, eIF4E binds to PRH but the functional significance of this inactivation is not yet known

(Bordin, *et al*, 1999). PRH has been observed to have a punctuate pattern within the nucleus and cytoplasm of the leukemic K562 cell line, where PRH and PML both possess an inhibitory growth control function (Bordin, *et al*, 1999).

1.8.2 PRH and Breast Cancer

The mis-localisation and alteration in the sub-cellular distribution of PRH is reported to be associated with the development of breast tumorigencity (Puppin, et al, 2006). PRH is reported to be predominantly cytoplasmic in breast tumour cells and is also sequestered in the nucleolus of MCF-7 cells (Puppin, *et al*, 2006). However, generation of an MCF-7 PRH knockdown cell line has shown that cells have increased proliferation and survival. This has led to the conclusion that some endogenous PRH remains active within the nucleoplasm of MCF-7 cells (Noy *et al.*, 2010), despite the nucleolar localisation (Puppin, *et al,* 2006*)*. Overexpression of PRH inhibits cell migration and invasion of breast tumour cells (Kershaw *et al.,* 2013). Moreover, PRH regulates the transcription of the genes involved in the Vascular Endothelial Growth Factor (VEGF) pathway, within the MCF-7 cell line (Noy, *et al*, 2010).

1.9   PRH and Growth Control in K562 cells

Dasatinib does not directly bind to CK2 but dasatinib treatment blocks the formation of the phosphorylated and inactive form of PRH. This inhibition of CK2 is attributed to the decreased Src signalling as CK2 and Src protein interact directly (Noy, *et al*, 2012). Knockdown of PRH in K562 cells show increased VEGF signalling as PRH represses the transcription of multiple genes that encode the proteins of the VEGF signalling pathway (Noy, *et al*, 2012). Dasatinib treated K562 cells have decreased levels of phosphorylated PRH (pPRH). By inference it is thought that there is more active PRH in cells to re-establish the repression of genes encoding the VEGF signalling pathway, resulting in decreased leukemic cell survival (Noy, *et al*, 2012).

1.10   Tyrosine Kinase- Src

Src belongs to a major family of non-receptor protein tyrosine kinases: Lyn, Fyn, Yes, Lck, Fgr, Hck, Blk and Yrk. Figure 1.6 shows the structure of Src. The 60-kDa tyrosine kinase can be activated by the focal adhesion kinase (FAK) or the Crk-associated substrate (CAS), involved in integrin signalling (Roskski, 2004). Activation can also occur via cell surface receptors such as the epidermal growth factor (EGFR). Such molecular interactions cause disruptions in the intramolecular interactions within Src; forming an open conformation of Src (Ishizawar and Parsons, 2004). This facilitates the interaction with various substrates such as downstream signalling molecules. Full activation of Src occurs through the auto-phosphorylation of the tyrosine residue Y419 which is preserved in the catalytic domain.
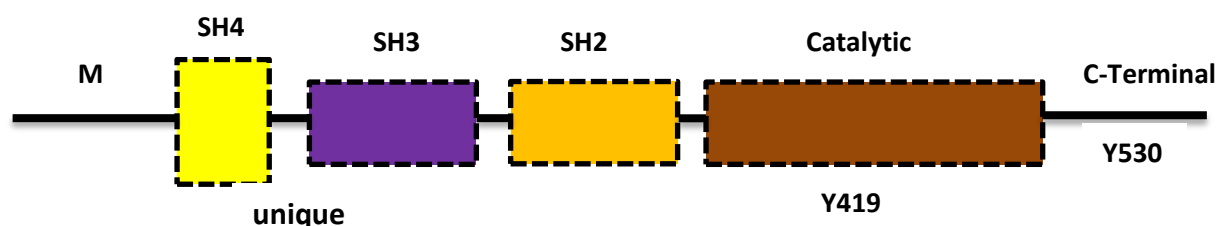
Figure 1.6: Structure of Src. Src consists of the myristylation site (M), Src homology (SH) domains, catalytic domain, unique domain and the c-terminal negative regulatory tyrosine residue. The unique domain is known to vary the most between the Src family kinases. Taken from Finn, *et al,* 2008 and Sicheri and Kuriyan, 1997.

1.10.1 Role of Src in Breast Tumour Cells

Elevated activity of Src has been observed in breast cancer where it plays a critical role in the crosstalk between growth-promoting pathways, like the EGFR pathway (Ishizawar and Parsons, 2004). Src mediates mitogenic effects of oestrogen directly and indirectly, this is important for evaluating the benefits of steroid hormone receptors. This is strongly supported by experimental evidence where Src- deficient cell lines do not activate the mitogen-activated protein kinase by oestrogen. Src is also a critical intermediate by promoting downstream effects of receptor tyrosine kinase activity (RTKs), such as EGFR and is commonly related to the therapeutic target *HER2* (Ishizawar and Parsons, 2004).

Phosphorylated tyrosine residues are a result of auto- transphosphorylation of the activated tyrosine kinase domain, resulting in the activation of several cell-signalling pathways (Ishizawar and Parsons, 2004). Figure 1.6 illustrates the complexity of the various pathways that Src plays a key role as a regulator. Breast cancer cell lines with increased expression of *HER2* and Src have high levels of phosphorylated Src in conjunction with activation of the mitogen-activated protein kinase (MAPK). This leads to increased levels of tumorigencity compared to cell lines that are EGFR and Src deficient (Ishizawar and Parsons, 2004).

Src functions by mediating the activation of the signal transducer and activator of transcription (STAT) RTKs (Okutani, *et al*, 2001). Inhibition of the Src pathway leads to decreased levels of STAT3 within breast tumour cell lines that express increased levels of EGFR (Ishizawar and Parsons, 2004). Src is also involved in the downstream activation of *HER2* and the potential role of *HER2-HER3* interactions (Belsches-Jablonski, *et al*, 2001). Over-expression of Src enhances the dimerisation between *HER2-HER3.* Requirement of Src for tumour cell motility and anchorage independent growth is promoted by the dimerised complex HER2-HER3 (Belsches-Jablonski, *et al*, 2001). Increased expression of Src has also been observed in invasive breast tumour cell lines compared to non-invasive, where treatment with a SFK inhibitor decreases in breast tumour invasion and motility (Belsches, *et al*, 2001).
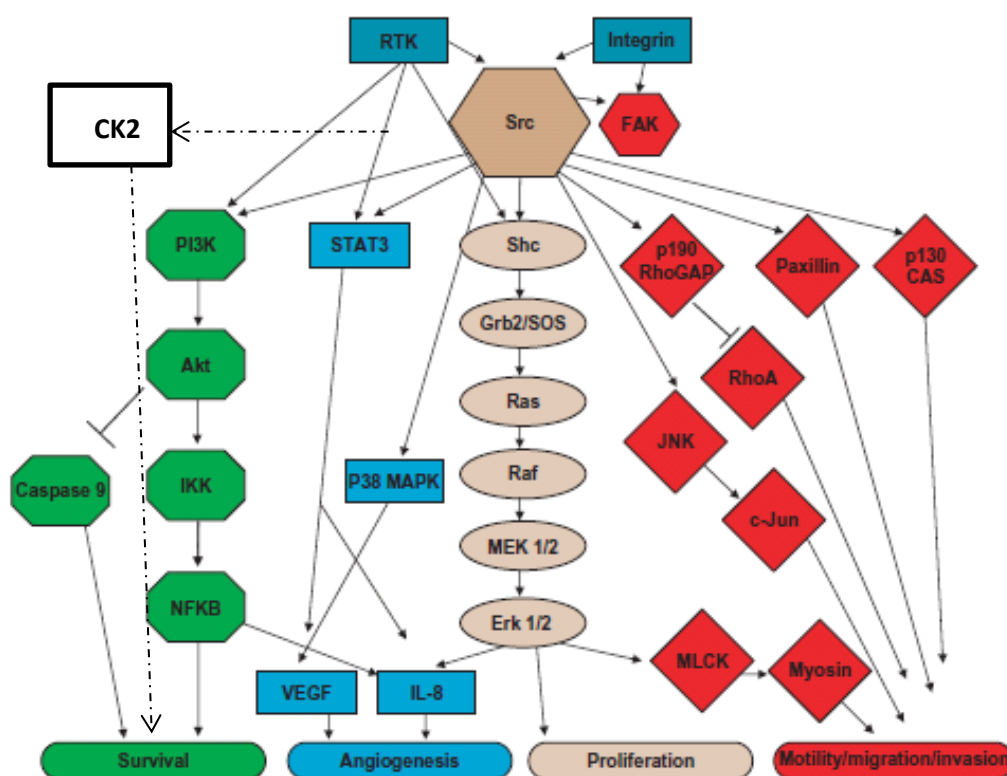
Figure 1.7: Src mediated signalling pathways that contribute to the progression of tumorigenesis. Members of the SFK are intermediates in many different signalling pathways**.** The association of Src with the over-expression of mutated tyrosine kinase receptors, results in constitutively active Src to activate pro-survival pathways (green) and the angiogenic pathways (blue). Increased proliferation occurs when Src is activated by both receptor tyrosine kinases and focal adhesion kinase (FAK) (brown). Crosstalk between the various pathways also occurs with many of the intermediates between these pathways involved in the activation of Src. Adapted from Summy and Gallick, 2006.

1.11   Cell lines

The Jayaraman laboratory has shown that overexpression of PRH in MCF-7, MCF-10A and MDA-MB-231 cells inhibit cell proliferation and cell migration. Thus, PRH is a potential regulator of breast tumorigenicity. MCF-7, MCF-10A and MDA-MB-231 cell lines were used for all experimental work in this project.

MCF-7 cells are derived from a pleural effusion from a 69 year old metastatic breast cancer patient (Levenson, *et al,* 1997). The cells are negative for *HER2* amplification but express wildtype p53 (Subik, *et al*, 2010). MCF-7 cells are of luminal origin, specifically categorised as luminal A and form tightly cohesive structures (Holliday *et al* 2011). MCF-7 cells are characterised with an "epithelial-like" phenotype due to high levels of E-cadherin expression and low levels of Vimentin expression. Matrigel experiments have shown MCF-7 cells to be weakly invasive and to form spherical colonies (Lacroix and Leclercq, 2004).

MDA-MB-231 cells are derived from pleural effusion from a 51 year old female patient and grow as a monolayer. MDA-MB-231 cells are tumorigenic in nude mice and grow to form a poorly differentiated adenocarcinoma. MDA-MB-231 cells also lack oestrogen receptors and exhibit oestrogen-independent growth, giving this cell line a more aggressive phenotype than MCF-7 (Thompson, *et al*, 1992). They are known as triple negative as they lack ER, PR and *HER2* expression but possess a basal-like phenotype (Lacroix and Leclercq, 2004). The cells also express a mutated form of p53 (R280K) enhancing tumour survival (Hui, *et al*, 2006). Due to the MDA-MB-231 cells being highly invasive they form stellate colonies in Matrigel and are characterised as being "mesenchymal-like" (Lacroix and Leclercq, 2004).

MCF-10A cells are non-tumorigenic and do not form tumours in nude mice but are unable to undergo anchorage-independent growth in Matrigel (Soule, *et al*, 1990). They are an immortalised cell line derived from normal diploid mammary epithelium (Soule, *et al*, 1990). MCF-10A cells lack SV40 T antigen and *HER2* amplification, they are positive for wild-type p53 expression (Soule, *et al*, 1990). The MCF-10A cell line is a good model for studying normal untransformed breast cells, when grown in 3D culture they form acinar structures that are phenotypically similar to mammary glands (Debnath, *et al*, 2003)

1.12   Generation of MCF-7, MCF-10A and MDA-MB-231 Knockdown Cell Lines

The inducible MCF-7 PRH knockdown cell line and the MDA-MB-231 PRH knockdown cell lines used for all experimental work were generated by Dr Daniel Roberts in the Jayaraman laboratory (Roberts, 2014). PRH expression was knocked down using specific shRNA sequences against the PRH open reading frame. The PRH shRNA sequences are under the control of the U6 promoter, which contain an engineered lac operator site. A lenti-viral vector integrates the DNA encoding shRNA sequences randmly into the genome and the vector also encodes the lac repressor cDNA and a resistance maker gene (puromycin). Upon integration the lac repressor is expressed along with the resistance marker gene. The lac repressor binds the lac operator repressing expression of the U6 promoter. Upon induction with isopropyl-β-D-1-thiogalactopyranoside (IPTG), the allosteric lac repressor alters its conformation to release itself from the lac operator in the U6 promoter, allowing expression of the shRNA. The resulting pre-shRNA is exported from the nucleus by exportin 5. The enzyme dicer processes the product and the resulting processed shRNA are encompassed within the RNA- induced silencing complex (RISC). The sense strand is degraded whilst the anti-sense strand leads the RISC complex to the mRNA, containing a complementary sequence. RISC functions to cleave either the mRNA or repress translation of the mRNA, resulting in the shRNA to silence its target gene, in this case the PRH genetic sequence. Figure 1.8 depicts this genetic process and figure 1.9 confirms that PRH knockdown was successfully achieved in the MCF-7, MCF-10A and MDA-MB-231 cells.
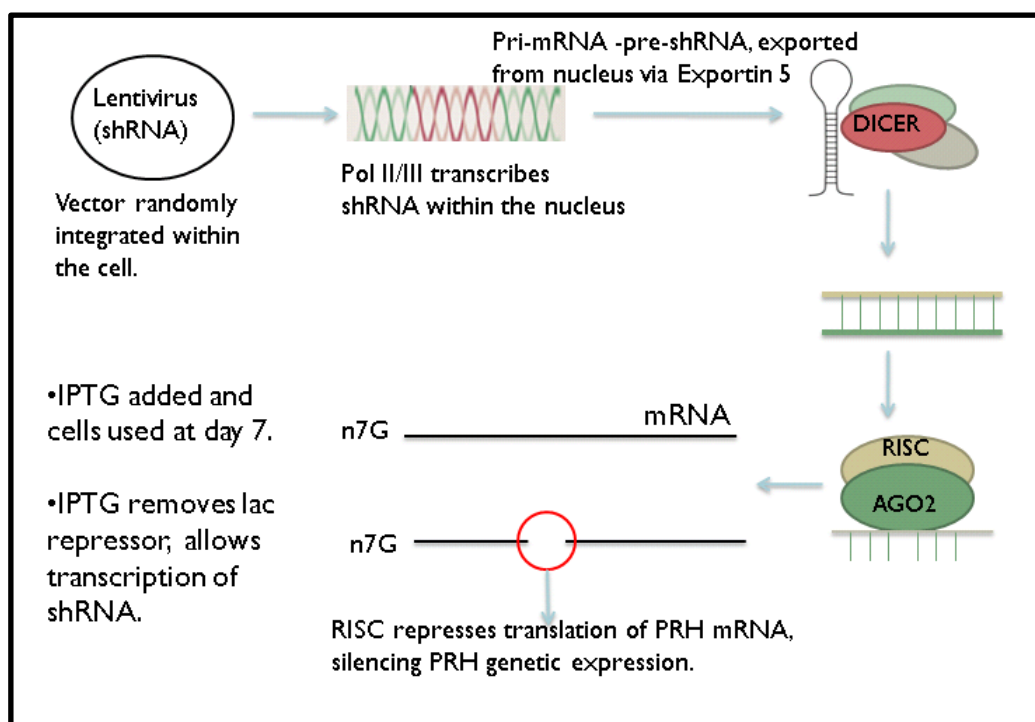
Figure 1.8: Generation of the inducible MCF-7 and MDA-MB-231 PRH knockdown cell line. A lenti-virus containing specific shRNA sequences against the PRH open reading frame, was randomly integrated into the cell. Polymerases II and III transcribed the shRNA. The pre-shRNA was exported via Exportin 5 from the nucleus and processed by DICER. The processed shRNA was encompassed within the RISC complex and shRNA silenced the genetic expression of PRH, resulting in successful PRH knockdown.
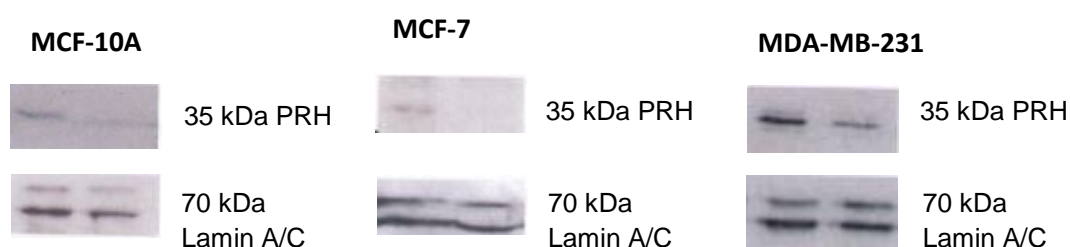
Figure 1.9: Inducible Knockdown of PRH in MCF-10A, MCF-7 and MDA-MB-231 cells. Each cell line was infected with a lenti-virus containing shRNA for either PRH knockdown or against no known mammalian gene, this acted as the control. The infected cells were selected with 0.5 µg/ml puromycin for 7 days; this was followed by induction of shRNA expression using 1mM IPTG. Cells were then pelleted and after 7 days of induction, protein was extracted for separation by SDS-PAGE and probed with the PRH antibody-M6 to confirm successfully knockdown of PRH. Taken from Roberts, 2014.

1.13   Microarray Analysis

A gene expression microarray was carried out by Dr Rachael Kershaw in the Jayaraman laboratory, to compare mRNA expression in the MCF-7 control and PRH knockdown cells (Kershaw, *et al,* submitted). The gene expression profile was assessed by quantitative real-time PCR (qRT-PCR) of genes. Figure 1.10 shows expression levels of cyclin-D2 and Vimentin to validate the microarray.
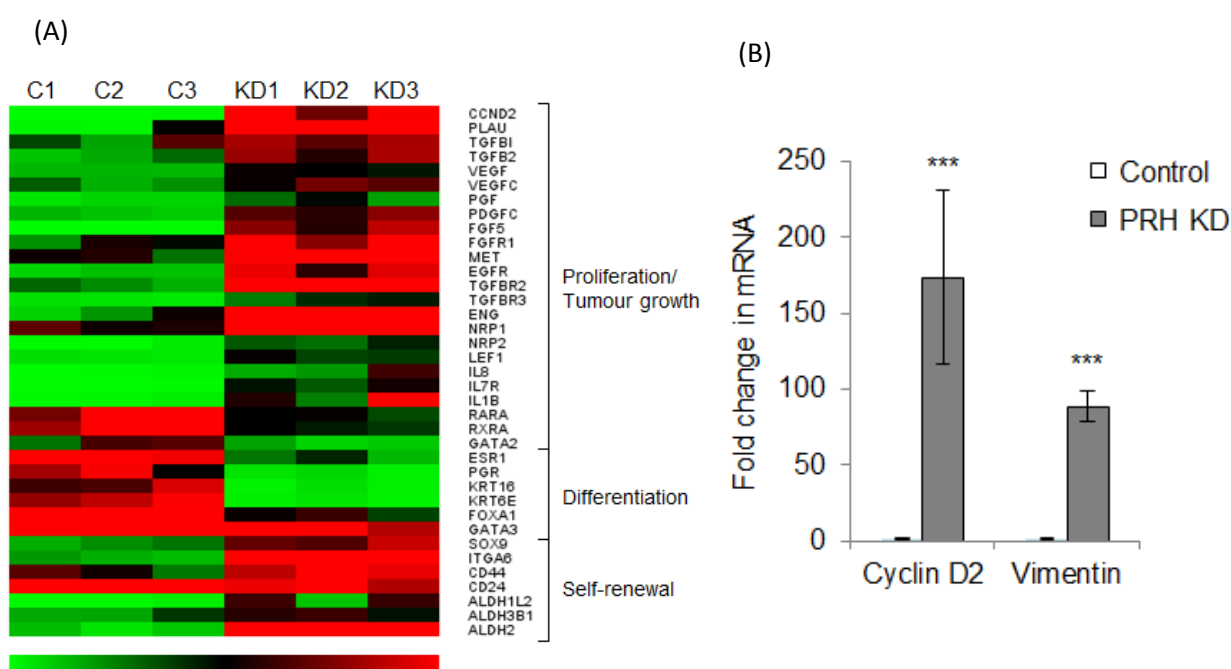
Figure 1.10: (A) Microarray data for the altered gene expression levels detected in the MCF-7 PRH control and knockdown cells. The mRNA expression data for each gene is presented as a matrix where rows represent individual genes and columns represent the individual mRNA samples for three independent control (C1-C3) and PRH knockdown (KD1-KD3) cell lines. The genes selected are genes with known function in cellular proliferation/survival, differentiation or cancer-stem cell renewal. The colour scale at the bottom is the relative level of gene expression. **(B) Relative expression of genes by qRT-PCR: Cyclin D2 and Vimentin.** The expression levels of both genes were normalised to GAPDH mRNA. (Kershaw, *et al*, submitted)

1.13.1   Overview of the MCF-7 PRH Knockdown cell line

The tumorigenic MCF-7 PRH knockdown cell line has increased proliferation, migration and invasion (Noy, *et al*, 2010 and Kershaw, *et al,* 2013). Moreover, unpublished studies in the Jayaraman laboratory have observed the MCF-7 knockdown cells to express epithelial-mesenchymal-transition (EMT) markers such as Vimentin, Slug and Snail. MCF-7 PRH knockdown cells have also been observed to express cancer stem cell markers like CD44+ as well as having the potential to form mammospheres (Roberts, 2014).

1.14 Aims

Since the MCF-7 knockdown cells have cancer stem cell properties it was hypothesised that the PRH knockdown cells may be resistant to conventional chemotherapy treatment and to dasatinib targeted therapy. Therefore the main aim of this project was, to determine whether the highly tumorigenic MCF-7 PRH knockdown cell line is resistant to dasatinib treatment and to further investigate the molecular basis for this resistance. A second aim was to investigate whether the immortalised breast cell line MCF-10A and the highly aggressive MDA-MB-231 cell lines have decreased PRH expression and are resistant to dasatinib treatment.

MATERIALS & METHODS

2.1 Summary of used chemicals and reagents in this project

Table 2.1: Chemicals and reagents

Chemical	Information
Antibodies	Anti-Lamin A/C (H-110) (Santa Cruz sc-20681): Rabbit Polyclonal
	Anti-phospho PRH (YKN5): Rabbit Polyclonal (In house)
	Anti-alpha pan SRC: Rabbit Polyclonal
	Anti-phospho Src (Y416): Rabbit Polyclonal (Cell Signalling Technology #2101S)
	Anti-phospho XRCC1 (S485/T488): Rabbit Polyclonal (Bethyl Laboratories Cat: A300-231A)
	Anti-PRH (M6): Mouse polyclonal
	Anti-mouse IgG HRP conjugated-Goat (Santa Cruz Biotech sc-2005)
	Anti-rabbit IgG HRP conjugated-Goat (Santa Cruz sc-2313)
Bovine Serum Albumin (BSA)	First Link (UK) Ltd. Cat. 40-00-450
Bradford Reagent	Bio-Rad 500-001
Caesin Kinase II Inhibitor VI, TMCB	Calbiochem 218718
Cholera Toxin	Sigma C8052
Complete (protease inhibitor cocktail tablets)	Roche 04 693 124 001
Dasatinib, free base	LC Laboratories D-3307
Dimethyl Sulfoxide (DMSO)	Sigma D8418

DMEM/F12 Ham L-glutamine and 15 mM HEPES	Sigma D8437
Epidermal Growth Factor (EGF)	Peprotech AF-100-15
Ethanol	Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	Sigma E5134
Fetal Bovine Serum (FBS)	Sigma F7524
Glycine	Sigma 241261
Horse serum- heat inactivated	Sigma H1138
Hydrocortisone	Sigma H0888
Insulin Solution Human	Sigma I9278
Isopropyl-β-D-1-thiogalactopyranoside (IPTG)	Bioline 37036
Magnesium Chloride	Sigma M8266
Methanol	Fisher Scientific
Pageruler Plus Protein Ladder	Fermentas- PageRulerTM Plus Prestained Protein Ladder (SM1811)
PBS Tablets	Sigma P4417
Penicillin/ Streptomycin	Sigma P4333
Polyoxyethylene Sorbitan Monolaurate (Tween 20)	Sigma P1379
Ponceau S Solution	Sigma P7170
Potassium Chloride	Sigma P9541
Potassium Phosphate (KH_2PO_4)	Sigma P9791
Puromycin	Gibco by Life Technologies (A1138-03)
RPMI 1640 medium	Sigma R8758
Sodium Azide	Sigma S8032
Sodium Chloride	Sigma S9888
Sodium deoxycholate	Sigma D6750
Sodium dodecyl sulfate (SDS)	Sigma L4509
N,N,N,N-Tetramethylethylenediamine (TEMED)	Sigma T9281
Thiazoly blue formazan [1-4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] (MTT)	Sigma M2003
Trizma Base	Sigma T4661
Trypsin-EDTA (10X)	PAA L11-003
B-mercaptoethanol	Sigma M6250

Table 2.2: Summary of used solutions

Solution	Information
1X PBS	137 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4 Adjusted to pH 7.4

Table 2.3: Summary of solutions for whole cell protein extraction

Solution	Information
RIPA Buffer	50 mM Tris-Cl (pH7.5) 150 mM NaCl 1% v/v Np-40 0.5% v/v Sodium Deoxycholate 0.1% v/v SDS Protease inhibitor tablet
2X Laemmli Buffer	62.5 mM Tris- HCL ph 6.8 25 % glycerol 2% SDS 0.01% Bromophenol Blue

Table 2.4: Summary of SDS polyacrylamide gel solutions

Solution	Information
1X Resolving Buffer	375 mM Tris-Cl (final pH 8.8) 0.1% w/v SDS
1X Stacking Buffer	125 mM Tris-Cl (final pH 6.8) 0.1% w/v SDS
SDS polyacrylamide resolving gel	1X resolving buffer (see above) Acrylamide 0.1% Ammonium Persulfate TEMED
SDS polyacrylamide stacking gel	1X stacking buffer (see above) 4.5% acrylamide 0.1% Ammonium Persulfate TEMED

Table 2.5: Summary of solutions for SDS-Polyacrylamide Page Gel Electrophoresis (PAGE)

Solution	Information
1X Running buffer	25 mM Tris-Cl 192 mM Glycine 0.1% w/v SDS

Table 2.6: Summary of solutions used for the transfer of proteins from SDS-PAGE to Immobilon-P Membrane

Solution	Information
1X Transfer buffer	10 mM Tris-Cl 100 mM Glycine 0.05% w/v SDS 20% v/v Methanol

Table 2.7: Summary of solutions used for antibody detection

Solution	Information
PBS-Tween	1X PBS 0.05% Tween 20
Primary Blocking Solution	3% w/v Bovine Serum Albumin (BSA) 3 mM Sodium Azide in PBS-tween

2.2 Cell Culture Protocols

2.2.1 Adherent Cell Culture

MCF-7 and MDA-MB-231 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium. The medium was supplemented with 10% foetal bovine serum (FBS)-heat inactivated at 56°C for 30 minutes, 100 units/ ml penicillin, 100 µg/ml streptomycin. Induction medium (RPMI+I) was prepared following the same protocol, with the addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and 0.5 µg/ml puromycin at final concentration. Cells were maintained in a humidified incubator at 37°C, within an atmosphere of 20% oxygen, 5% carbon dioxide and 75% nitrogen. For PRH knockdown experiments cells were used only when induced with IPTG for at least 7 days. Cells were checked regularly using the microscope (Leica DMIL/ 090-131.001) for confluence and absence of contaminants. Cells were split when confluent, approximately every two days. The cell monolayer was washed with 1X PBS, followed by trypsinisation with 1X trypsin-EDTA for cell detachment at 37°C for 3 minutes. An equal volume of RPMI was added to the cells and centrifuged at 1000 rpm for 3 minutes (Harrier 18/80-A050169). Cells were re-suspended in RPMI and transferred to a new flask. Both cell lines were grown in Starstedt tissue culture flasks. MCF-10A cells were maintained in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), supplemented with 5 % horse serum (heat inactivated), 20 ng/ml epidermal growth factor (EGF), 0.5 µg/ml hydrocortisone, 100 ng/µl cholera toxin, 10 µg/ml insulin and 100 units/ ml penicillin, 100 µg/ml streptomycin (Sigma P4333). The cells were maintained and split as described above and grown in Falcon tissue culture flasks.

2.2.2 Dasatinib

0.5 g of dasatinib was dissolved in 20 ml DMSO to make a stock concentration 50 mM, by filter sterilisation. 1 ml aliquots were stored at -20°C. Final concentrations of 0.05 µm, 0.1 µm, 0.5 µm, 1 µm and 5 µm were made in the induction medium from the 50 mM stock.

2.2.3 Cell Viability Assays

Cells were counted by trypan blue exclusion, 10 µl trypan blue was mixed with 10 µl cells in medium. The number of viable cells was calculated using the haemocytometer and the formula:

Number of cells/ml = Average count per square x dilution factor x 10^4

Induced cells were seeded at ~5 x 10^4 cells/well in 200 µl induction medium within a 96-well plate and incubated overnight at 37°C. MCF-7 cells were incubated with 0.05 µm, 0.1 µm, 0.5 µm and 1 µm dasatinib for 72 hours, with equivalent volumes of DMSO. 200 µl MTT (0.5 mg/ml) was added to each well and the plate was incubated for 2 hours at 37°C. 100 µl DMSO was added and the plate was placed on a rocking platform to allow complete solubilisation of the MTT crystals. The resulting formazan product produced a colorimetric change, indicative of the total cellular metabolism. The optical density (OD) was measured at 545 nm using the Emax ELISA plate reader. The results were expressed as a relative % of total cellular activity compared with the corresponding untreated controls (expressed as 100%).

2.3　Western Blot Protocols

2.3.1 Extraction of Whole Cell Protein

Whole cell extracts were created from 1 x10^6 dasatinib treated/ DMSO equivalent MCF-7 cells in 6 well plates. Adherent cells were washed with 1 ml 1X PBS, trypsinised with 500 µl 1X trypsin and re-suspended in 500 µl medium. Contents from each well of the 6 well-plate were transferred into sterile 1.5 ml eppendorf tubes and cells were pelleted by centrifugation for 3 minutes at 3000 rpm (Eppendorf 5810R). The supernatant was discarded and a second wash with 1X PBS was performed, followed by centrifugation. The pellet was re-suspended in 100 µl RIPA buffer and incubated on ice for 15 minutes for cell lysis. Cell debris was removed by centrifugation at 13000 rpm (Thermo Scientific Heraeus Fresco 21) at 4°C for 30 minutes. The supernatant was collected into a sterile eppendorf tube and stored at -80 °C.

2.3.2   Determination of Protein Concentration

Protein concentration was determined using the Bradford method (Bradford.M, 1976), BSA was used as a standard and diluted to 0 µg, 2 µg, 4 µg, 6 µg and 10 µg within Bradford reagent (1:5, Biorad). The samples were read spectrophotometrically after blanking the machine with RIPA buffer. Figure 1 was plotted and the gradient used to determine protein concentration, using the Beer-Lambert law. This consists of using the equation: $A = \varepsilon cl$ where absorbance is proportional to the concentration of the molecule being absorbed (c), length of path-light (l) and the molar extinction coefficient (ε). All concentrations of protein samples for SDS-PAGE were calculated using figure 2.1.

Bradford Assay

y = 0.057x - 0.016
$R^2 = 0.9926$

[BSA]
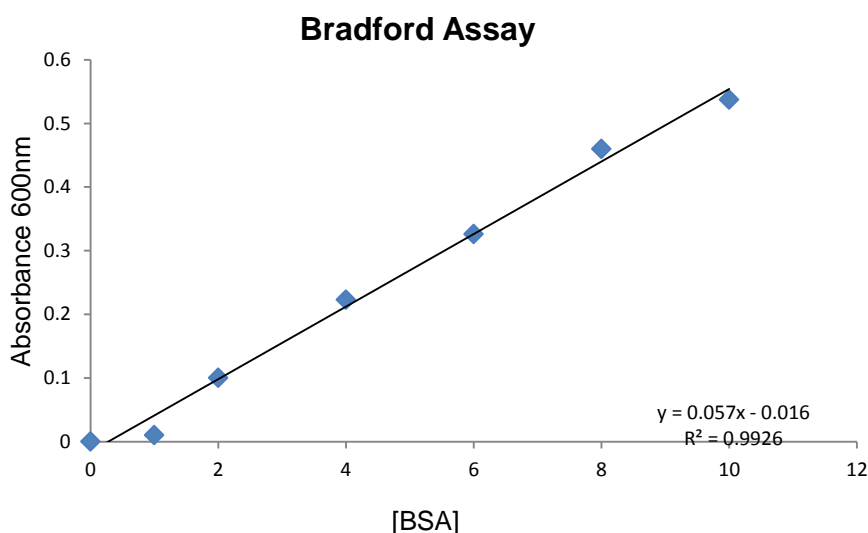
Figure 2.1: Bradford Assay. Used to determine protein concentration by using the Beer-Lambert law $A = \varepsilon cl$.

2.3.3 Preparation of SDS-Polyacrylamide Gel

The front and back glass plates (Bio-rad) were clamped into the casting frame which was clamped into the casting stand. The resolving gel was prepared with gentle swirling see table 2.4, and the solution was pipetted between the glass plates. 1 ml isopropanol was added to remove the air-water interface and the gel was left to polymerise for at least 15 minutes. The isopropanol was discarded and the stacking gel was prepared, see table 2.4 This was pipetted on top of the resolving gel to the brim of the plates and a 1 mm comb was inserted into the stacking gel and left to polymerise for at least 15 minutes.

2.3.4 Separation of Proteins by Molecular Weight

All protein samples were heated at 95°C for 5 minutes with 1X Laemmli buffer prior to being loaded onto the gel. The SDS gel was inserted within the separation tank (Bio-Rad Mini-PROTEAN Tetra Electrophoresis System). The protein samples were loaded onto the gel, with PageRuler Plus prestained Protein Ladder (Fermentas SM1811). The gel was electrophoresed at 130V (constant voltage) for 90 minutes.

2.3.5 Transfer of Proteins from SDS-polyacrylamide

Transfer apparatus (Bio-Rad Mini Trans-Blot Cell System) was used where the Immobilon-P membrane (Millipore IPVH00010) was left in methanol for 30 seconds, for hydration and washed in distilled water for 2 minutes. The membrane was equilibrated in 1X transfer buffer for 3 minutes. 3MM Whatman filter paper (Whatman 3030917) was soaked in 1X transfer buffer. Once protein separation had occurred, the gel was removed from between the plates and the stacking gel discarded. The pad and 3MM Whatman filter paper were placed on the cathode side of the transfer cassette, followed by the gel on top, Immobilon-P membrane on top of the gel, 3MM Whatmann filter paper on top and finally the pad on top. The transfer cassette was firmly closed and assembled into the apparatus and the tank was filled 1X transfer buffer. The transfer occurred at 70V (constant voltage) for 1 hour.

2.3.6 Detection using antibodies

The membrane was soaked in methanol for one minute and washed thoroughly in 1X PBS-Tween. The membrane was soaked in Ponceau S solution for five minutes for identification of successful protein transfer and removed with several PBS-Tween washes. The membrane was left overnight in PBS-Tween containing 10% (w/v) milk at 4°C on a rotating platform. The membrane was washed 3 times for 10 minutes in PBS-Tween for removal of milk and then probed for a specific primary antibody, by being incubated in primary blocking solution for one hour. Additional three 10 minute washes in PBS-Tween were performed and a one hour incubation in secondary antibody, diluted in 1X PBS-Tween solution containing 10 % (w/v) milk. Further, three 10 minute washes were carried out. However, when probing for pPRH the pPRH antibody required three 30 minute washes.

The proteins on the membrane were detected using the ECL western blotting detection solutions 1 and 2 (GE Healthcare RPN2106). Both solutions were mixed using a 1:1 ratio and used to soak the membranes, placed on Sarogold wrap for three minutes. Excess ECL reagent was removed and the membrane was wrapped in Sarogold wrap and exposed to ECL hyperfilm (GE Healthcare 28-9068-37) prior to detection using the developer, Xo-graph.

2.3.7 M6 polyclonal antibody

Due to limited availability of antibodies that are able to detect PRH protein expression in breast cells. A novel mouse monoclonal antibody was generated in the Jayaraman laboratory, called M6. The antibody was raised against the human peptide consisting of SPFLQRPLHK amino acids x to x in human PRH sequence. M6 is able to detect the endogenous PRH as a triplet of proteins with a molecular weight of approximately of 37 kD in haematopoietic cells and MCF-7, MCF-10A and MDA-MB-231 breast cells.

2.3.8 Rabbit Polyclonal antibody

In order to detect phosphorylated PRH expression the rabbit polyclonal antibody was used to confirm the phosphorylation of PRH by CK2 (Soufi, *et al*, 2009).

2.4   Puromycin Selection

MCF-10A cells were counted using the method, see 2.2.3 and seeded in a 6 well plate at a density of ~200,000 cells per well and incubated overnight at 37°C. 10 mg/ml puromycin was diluted in DMEM to make final concentrations

0.5 µg/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml. Cells were treated with each concentration until cell death had been observed. Photographs at each concentration were taken, after addition of the puromycin. Cell death was observed between 10 µg/ml and 50 µg/ml so additional 20 µg/ml and 30 µg/ml concentrations were made to treat the cells. Puromycin resistance was observed at 20 µg/ml so cells at this concentration were induced and grown.

2.5   Plasmid

2.5.1 pLKO-puro-IPTG-3xLacO (PRH and control shRNA)

The sequence for PRH shRNA knockdown is CTGTGATCAGAGGCAAGATTT but the control sequence targets no known mammalian gene. WPRE enhances transgene expression and the LacI repressor binds to three LacO sequences that are present in the U6 promoter (Roberts, 2014 and Sigma Aldrich, 2014). This plasmid was used for the generation of inducible MCF-7, MCF-10A and MDA-MB-231 PRH knockdown cell lines see 1.8.

Figure 2.2: Schematic of the pLKO-puro-IPTG-3xLacO Plasmid (Sigma Alrdrich, 2014).

Cppt- Central polypurine tract

hPGK- Human phosphoglycerate kinase eukaryotic promoter

PAC- Puromycin N-acetyl-transferase

WPRE- Woodchuck Hepatitis Post-Transcriptional Regulatory Element

SIN/LTR- 3' self-inactivating long terminal repeat

F1 ori- f1 origin of replication

AMP- Ampicillin resistance gene for resistance bacterial selection

PUC origin- pUC origin of replication

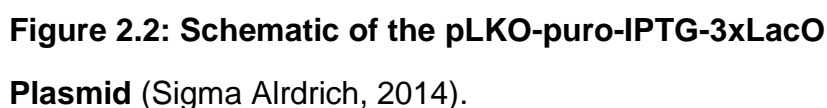**5'LTR**- 5'long terminal repeat

Psi- RNA packaging signal

RRE- Rev response element

LacI- Lac repressor

GSG-F2A Gly-Ser-Gly foot and mouth disease virus IRES 2A

RSV- Respiratory syncytial virus

RESULTS

3.1   Introduction

Previous experimental work from our laboratory has shown that the K562 leukaemic cells treated with dasatinib are more sensitive than K562 PRH knockdown cells (Noy, *et al,* 2012)*.* As outlined in section 1.4, dasatinib inhibits BCR-ABL and Src signalling in K562 cells, resulting in reduced CK2 activity which leads to a reduction in levels of pPRH. A reduction in pPRH protein level results in an increase in the transcriptionally active hypo-phosphorylated PRH protein. This active form of PRH is able to inhibit tumour growth by regulating the proteins involved in the VEGF signalling pathway. Thus, dasatinib reduces cell survival of the K562 leukemic cells which is mediated in part by PRH (Noy, *et al,* 2012). On the basis of these findings we further investigated whether dasatinib sensitivity follows a similar mechanism in reducing breast tumour survival in the MCF-7, MDA-MB-231 and MCF-10A cell lines.

3.2   Cell number is proportional to absorbance, following MTT incubation

To determine whether breast cell viability is decreased with dasatinib treatment, cell viability assays were carried out. A cell viability assay measures mitochondrial activity and cell viability through the assessment of mitochondrial succinate dehydrogenase activity. The enzyme reduces the MTT reagent to formazan (purple), which is then visualised by a colorimetric change and measured spectrophotometrically at 545nm (Gerlier and Thomasset, 1986).

In order to establish that there is a linear relationship between cell number and absorbance at 545 nm, for each cell line. Standard curves were generated for known cell number against absorbance. Initially, MCF-7 cells were seeded in quadruplicate at densities of 1562, 3125, 6250, 12,500, 25000 50,000 and 100,000 cell/well in a 96 well plate. The plate was incubated overnight at 37°C then incubated with the MTT reagent as described in 2.2.3. Figures 3.1 A and 3.1 C show that when cell number was plotted against absorbance, the absorbance was

not proportional to cell number above 50,000 cells for MCF-7 control and knockdown cells. Therefore, standard curves were re-plotted encompassing only the linear range where cell number was proportional to absorbance, shown in figure 3.1 B and 3.1 D. Cell densities for the cell viability assays were selected so that subsequent to the 72 hour dasatinib treatment, cell number was within the linear range for the MCF-7 control and MCF-7 PRH knockdown cells.
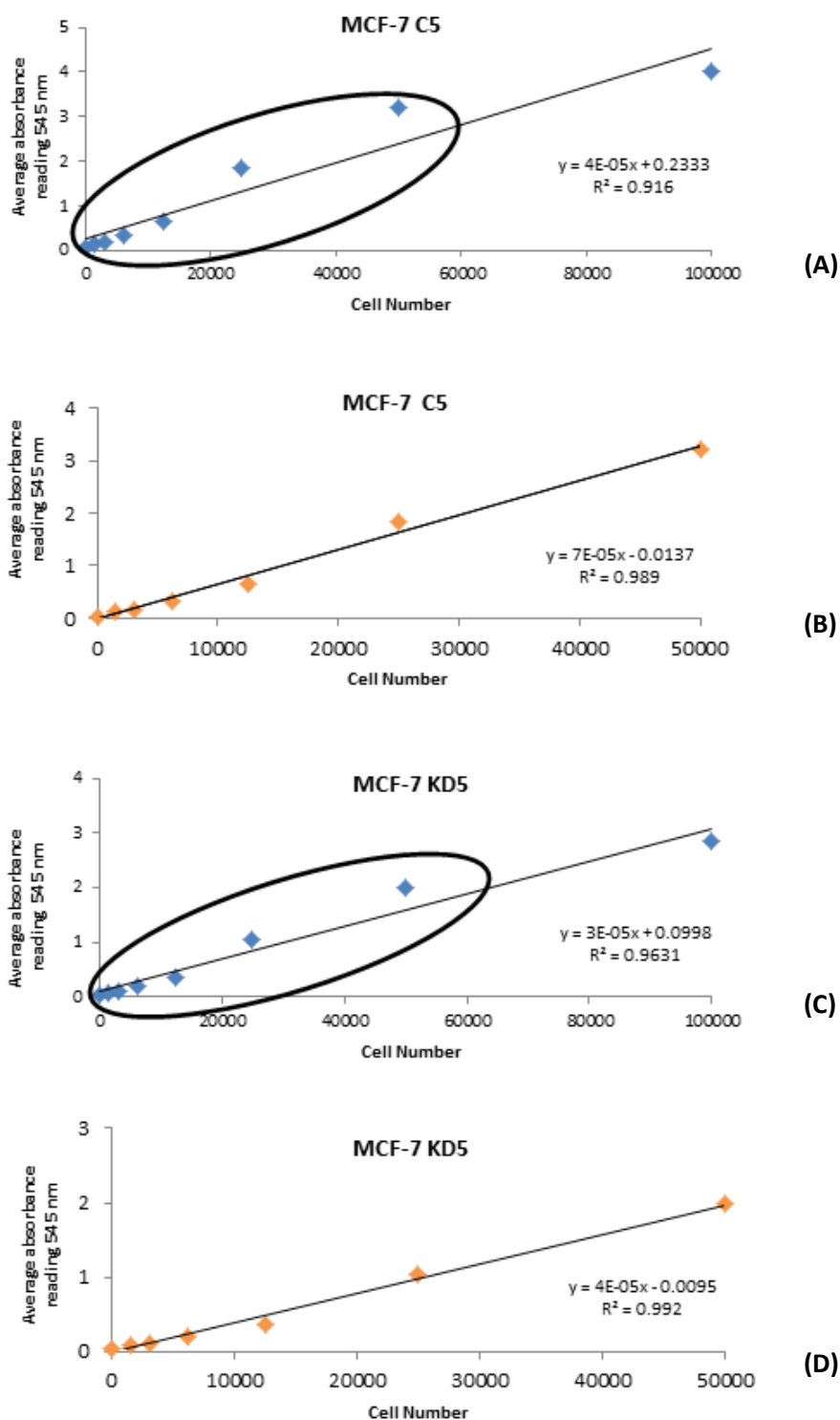
**Figure 3.1: Standard Curves for MCF-7 control (C5) and knockdown (KD5) cells.** Absorbance readings at 545 nm were plotted against cell number for each cell line in order to determine the linear range where cell number is proportional to absorbance reading. Each standard curve was then re-potted and is displayed below its original standard curve where the linear range reflects cell number to be proportional to absorbance.

3.3 MCF-7 PRH control cells are more sensitive to dasatinib than MCF-7 PRH knockdown cells

To determine the concentration of dasatinib which results in decreased cell number in the MCF-7 control and knockdown cells, cell viability assays were performed in the presence of increasing concentrations of dasatinib: 0.05 µm, 0.1 µm, 0.5 µm and 1 µm. Cells were seeded at 5000 cells/well in a 96 well plate and allowed to adhere for 24 hours. They were then incubated with the equivalent volume of DMSO or with dasatinib for 72 hours. Cell viability assays were performed in quadruplicate for each dasatinib concentration. DMSO was used as the solvent to dissolve dasatinib and therefore an equal volume of DMSO was used as a control in all cell viability assays. It is well documented that DMSO has a cytotoxic effect upon the viability of some cell lines (Violante, *et al*, 2002). Therefore absorbance values were expressed as a percentage of the DMSO treated cells to control for any effects of DMSO on cell viability in these assays. Absorbance values for dasatinib treated samples were expressed as a percentage of the DMSO control, after the DMSO control was compared to the untreated cells. All experiments were carried out three independent times.

A one-way ANOVA statistical test was used to show that statistically there is a significant reduction in cell viability following dasatinib treatment in both MCF-7 control and MCF-7 PRH knockdown cells. An un-paired students' t-test was then able to identify the concentration at which cell viability-was statistically significantly reduced for each cell line. Figure 3.2 shows that the viability of MCF-7 control cells is significantly reduced following treatment with 0.1 µm dasatinib whereas MCF-7 PRH knockdown cells are more resistant showing significantly reduced viability following treatment with 1 µm dasatinib. Therefore a higher concentration of dasatinib is required to reduce the viability MCF-7 PRH knockdown cells.

MCF-7 Control

MCF-7 PRH Knockdown
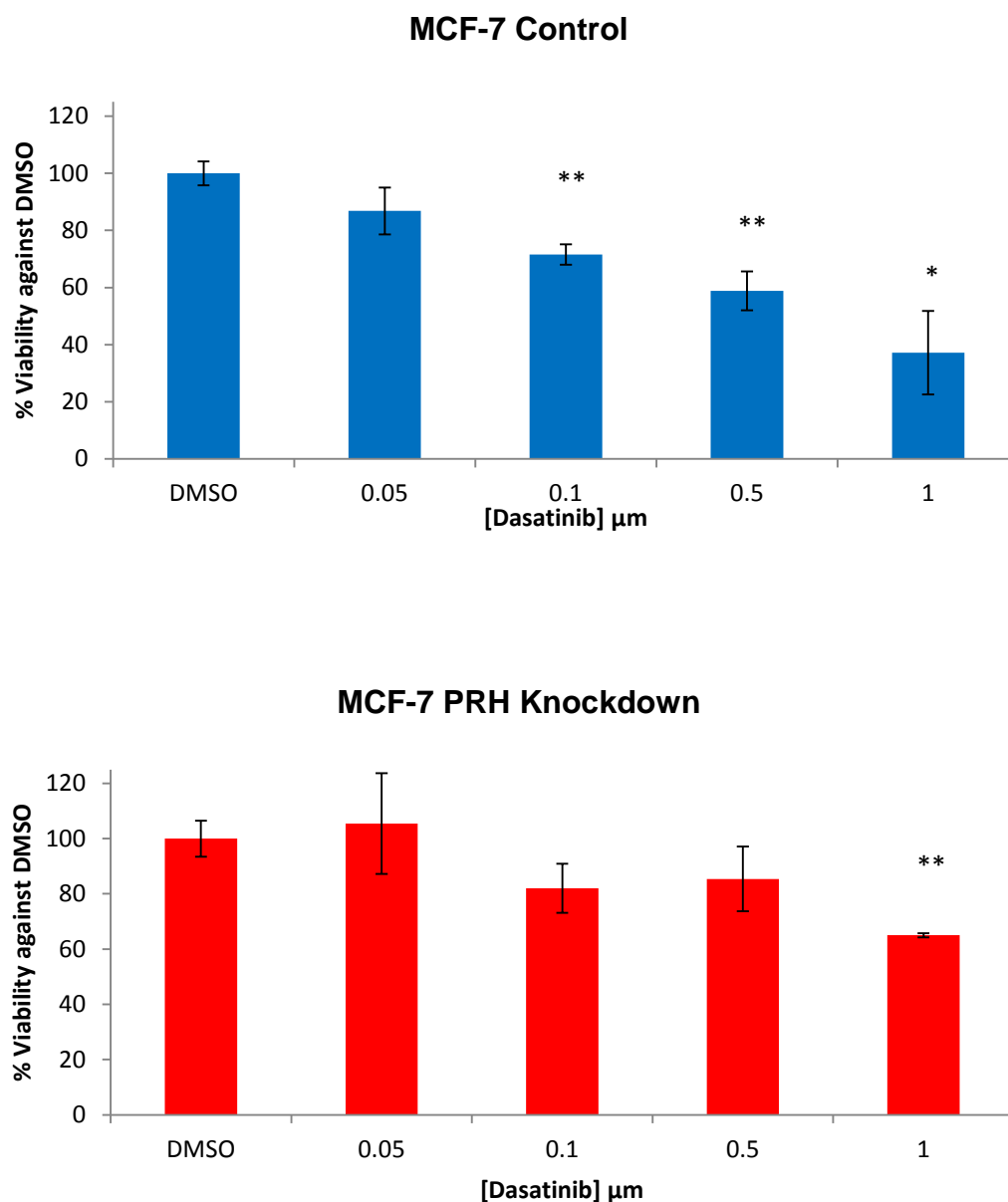
Figure 3.2: Cell viability graphs for MCF-7 control and MCF-7 knockdown cells treated with dasatinib. MCF-7 control cells show a significant decrease in cell viability following treatment with 0.1 µm (p<0.01) dasatinib, whereas the MCF-7 knockdown cells show a significant decrease in viability following treatment with dasatinib at higher concentration of 1 µm (p<0.01).

*= p<0.05 **=p<0.01

3.4   Microarray Analysis

Cell viability assays show that there is a difference between the MCF-7 control and MCF-7 PRH knockdown cells with dasatinib treatment. So, in order to determine the molecular basis of the dasatinib response in MCF-7 control and MCF-7 PRH knockdown cells, data from the MCF-7 microarray was used to identify known dasatinib target genes that have significantly altered gene expression in the MCF-7 PRH knockdown cells. This is shown below in table 3.1. The MCF-7 microarray was generated and analysed by Dr Rachael Kershaw (Kershaw *et al*, submitted). We found that expression levels of the SFKs such as Fyn, Lyn and Yes are significantly up-regulated in the MCF-7 PRH knockdown cells. Interestingly, increased expression of EGFR and FGFR1 were also found but no increase in expression levels of c-KIT and PDGFR were observed.

Table 3.1: Summary of gene expression of various dasatinib targets MCF-7 control and PRH knockdown cells. Targets of dasatinib which are significantly up-regulated when PRH is silenced in MCF-7 cells, shown by fold change. The microarray data was generated and validated by Dr Rachael Kershaw.

Gene	Fold Change KD/C
Src Family Kinases (SKF): Fyn, Lyn, Yes	3.66
Fibroblast Growth Factor Receptor 1 (FGFR1)	2.37
Epidermal Growth Factor Receptor (EGFR)	7.49
EPH receptor A2 (EPHA2)	4.78

3.5   MCF-7 cells sensitivity to dasatinib is partly mediated through PRH

An analysis of the microarray data showed that several dasatinib targets are up-regulated in MCF-7 PRH knockdown cells, suggesting that signalling through these targets underlies the difference in the effect of dasatinib on MCF-7 cell viability. We focused primarily on two questions, firstly, whether dasatinib inhibits EGFR and Src kinase signalling and downstream effector proteins such as pAKT, in the MCF-7 control and knockdown cells. Secondly, whether there is a difference in signalling through EGFR and Src kinases between MCF-7 control and MCF-7 PRH knockdown cells.

MCF-7 control and MCF-7 PRH knockdown cells were incubated with DMSO or 10 µm dasatinib for 4 hours. Whole cell extracts were produced and 20 µg of protein was loaded on an SDS-PAGE gel for protein separation. Western blotting experiments were carried out where phosphoEGFR (pEGFR), phosphoSrc (pSrc), and phosphoAKT (pAKT) and Lamin A/C were probed for using their corresponding antibody. Lamin expression serves as a loading control and was used for all experimental work in this project. The western blots for the dasatinib treated MCF-7 control and PRH knockdown cells were representative of three independent biological treatments whereas the pAKT blot was representative of two independent biological treatments.

Figure 3.3A shows that levels of pSrc and pEGFR are elevated in the MCF-7 PRH knockdown cells compared to the MCF-7 control cells. This strongly suggests that increased Src and EGFR signalling occurs in cells expressing low amounts of the active PRH protein. However, levels of the unphosphorylated Src protein were not reproducibly elevated in the MCF-7 PRH knockdown cells. Indeed, the microarray data does not indicate an increase in the Src transcript in these cells, but does show elevated levels of other SFKs in the microarray see table 3.1. Interestingly, with dasatinib treatment levels of pEGFR expression was reduced in the MCF-7 PRH knockdown cells but pEGFR was not detected in the MCF-7 control cells, so no alteration in pEGFR expression could not be deduced in MCF-7 control cells. We believe this is because EGFR is not expressed at high levels in MCF-7 control cells.

AKT is an indirect downstream effector of Src and EGFR and is activated via the activation of PI3K and PDK1. The MCF-7 PRH knockdown microarray showed AKT to be highly up-regulated in MCF-7 PRH knockdown cells. Therefore, we decided to investigate levels of the active form of AKT, phosphorylated AKT (pAKT) by immunoblotting with an antibody against pAKT. Figure 3.3A shows that pAKT levels are not increased in the PRH knockdown cells compared to the MCF-7 control cells, suggesting that Src/EGFR signalling is not strongly influencing pAKT levels. Interestingly, dasatinib treatment resulting in reduced pAKT expression in the MCF-7 control cells than in the MCF-7 PRH knockdown cells. This suggests that dasatinib can have some downstream effect on the active form of AKT. However as this is not seen in the MCF-7 PRH knockdown cells this is not the mechanism of dasatinib action in MCf-7 PRH knockdown cells that accounts for their resistance to dasatinib treatment.

Surprisingly, we found elevated expression of pPRH in the MCF-7 PRH knockdown cells compared to the MCF-7 control cells. This is unexpected as the cells are known to have decreased expression of hypo-phosphorylated PRH detected by the M6 antibody. Interestingly, with dasatinib treatment pPRH levels are decreased in the MCF-7 PRH knockdown cells but there was no alteration of pPRH expression in MCF-7 control cells. We infer that the reduction in pPRH in the MCF-7 PRH knockdown cells upon treatment occurs because CK2 dependent phosphorylation of PRH is decreased. .

As neither PI3/PDK1 nor CK2 are directly bound by dasatinib, we infer that pPRH is a downstream consequence of the inhibition of Src/EGFR signalling. Src and EGFR have been shown to interact directly with CK2 and influence its activity in many cell lines (Bliesath, *et al*, 2012). Findings from 3.3A led to the formation of the model shown in figure 3.3B. Here, dasatinib resistance of MCF-7 PRH knockdown cells occurs due to the reduction in level of PRH protein, which results in increased expression of multiple genes that are involved in tumour survival, including EGFR. Consequent effects on downstream signalling proteins such as Src and CK2, results in the elevated expression of transcriptionally inactive PRH protein and decreased growth inhibitory hypo-phosphorylated PRH protein. This leads to increased tumour survival. Therefore, higher activity and expression of dasatinib target proteins like EGFR and higher activity of pSrc, lead to the requirement of increased dasatinib

concentration for the inhibition of breast tumour cell survival. Ultimately, dasatinib resistance of MCF-7 PRH knockdown is a consequence of decreased PRH activity in these cells, resulting in increased tumour survival signalling.

(A)
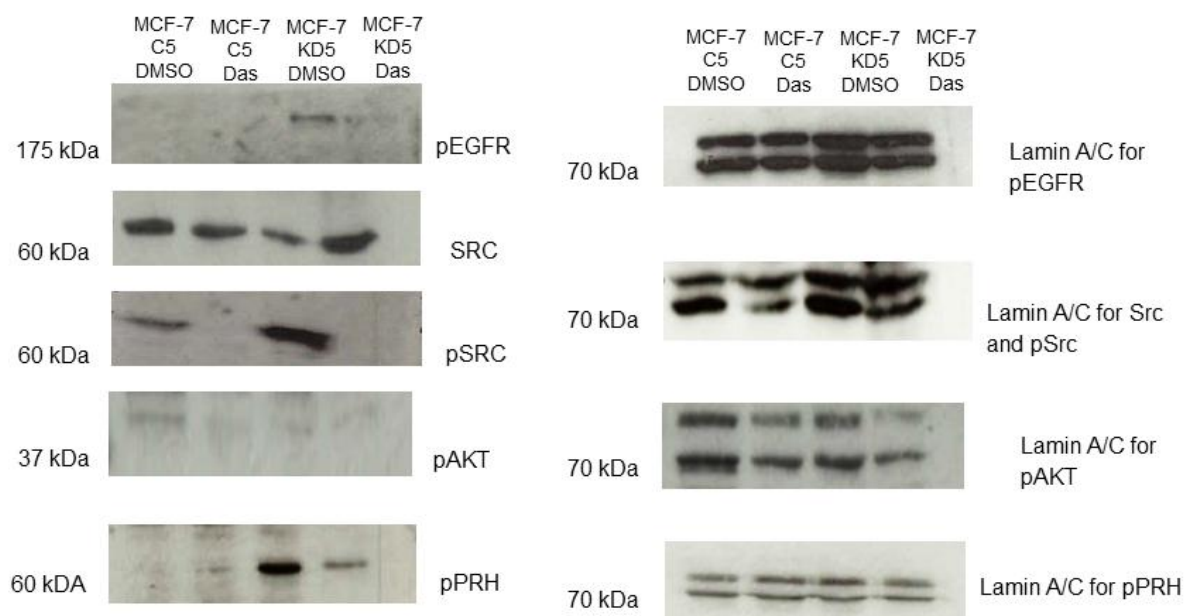
(B)
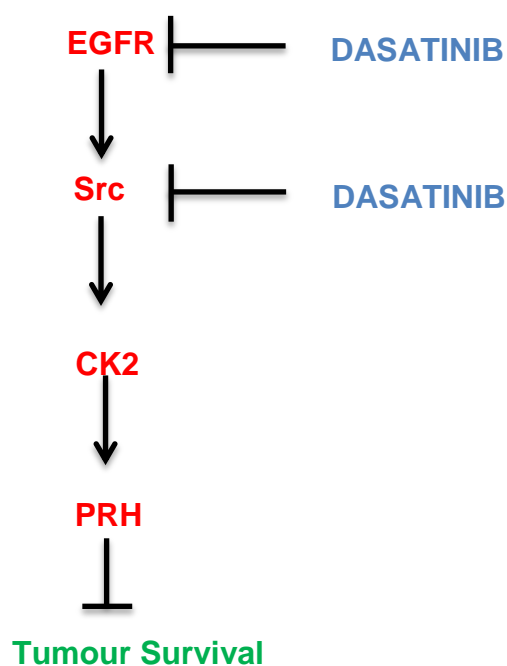
Figure 3.3: (A) Western blotting to determine the protein levels of pEGFR, Src, pSrc, PRH and pPRH. (B) Model depicting the inhibition of EGFR and Src kinase activity which would lead to reduced CK2 activity and a reduction in the amount of phosphorylated PRH, reducing breast tumour cell survival. Src is a major regulator for the PI3K signalling pathway which is needed for the activation of AKT. As dasatinib is a Src inhibitor,by inhibiting Src kinase activity this would also lead to a reduction in tumour cell survival via the PI3K pathway.

3.6   Cell Number proportional to absorbance, following MTT incubation for MDA-MB-231 control and knockdown cells.

As dasatinib sensitivity is PRH dependent in the tumorigenic MCF-7 control cells, we decided to further investigate whether dasatinib sensitivity would be mediated through PRH in the highly metastatic MDA-MB-231 control and MDA-MB-231 PRH knockdown cells. Again to establish the relationship of cell number against absorbance in the MDA-MB-231 control and MDA-MB-231 PRH knockdown cells cell viability assays were carried out. Following the same procedure for the MCF-7 standard curves, standard curves were plotted for the MDA-MB-231 control and MDA-MB-231 PRH knockdown cells. Figure 3.4 shows standard curves for the MDA-MB-231 cells where the absorbance was not proportional to cell number above 20,000 cells. Therefore standard curves were re-plotted encompassing only the linear range where cell number was proportional to absorbance, shown in figure 3.4 B and 3.4 D. Cell densities for the cell viability assays were selected so that subsequent to the 72 hour dasatinib treatment, cell number was within the linear range for the MDA-MB-231 control and MDA-MB-231 PRH knockdown cells.
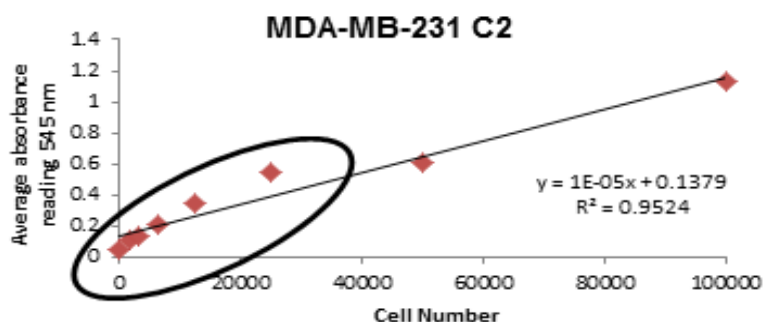
(A)
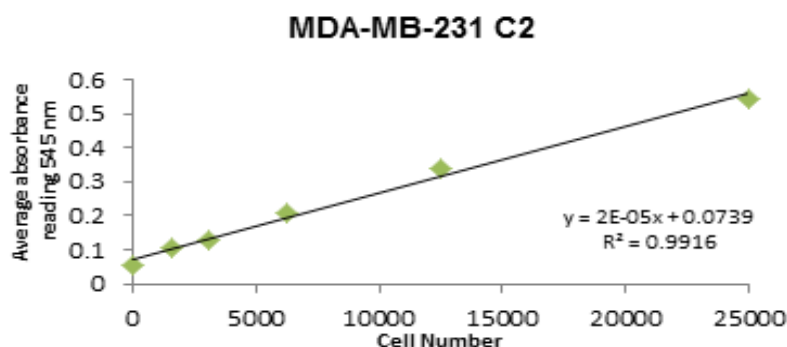
(B)
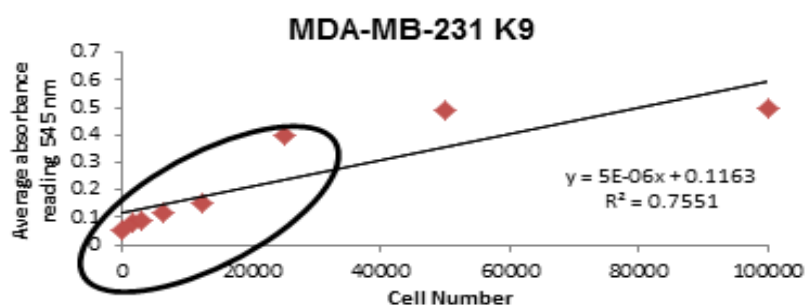
(C)

(D)

Figure 3.4: Cell Viability graphs for dasatinib treated MDA-MB-231 control (C2) and PRH knockdown (KD9) cells. MDA-MB-231 control cells were less sensitive to dasatinib compared to the MDA-MB-231 PRH knockdown cells. However, both cell lines are sensitive to dasatinib at the same dasatinib concentration 0.05 µm, but the PRH knockdown cells are more statistically significant at this concentration, suggesting that they are more sensitive to the treatment.

3.7   Dasatinib sensitivity in the MDA-MB-231 cells is not PRH dependent.

PRH knockdown in MDA-MB-231 cells results in a decrease in cell proliferation whereas PRH MCF-7 cells result in increased proliferation (Roberts, 2014). Thus, the role of PRH in the MDA-MB-231 cells appears to be very different to that in the MCF-7 cells. Cell viability assays were carried out for the MDA-MB-231 control and knockdown cells, as described in section 2.2.3. These experiments were performed on three independent occasions. Figure 3.5 shows no difference in the response of dasatinib between the MDA-MB-231 control and MDA-MB-231 PRH knockdown cells. In support of this finding an analysis of MDA-MB-231 control and PRH knockdown microarray, did not reveal any significant change in gene expression levels of known dasatinib targets following PRH knockdown.

A one-way ANOVA statistical test was used to show that statistically there is no significant reduction in cell viability following dasatinib treatment in both MCF-10A control and MCF-10A PRH knockdown cells. Thus, dasatinib sensitivity in the MDA-MB-231 cells is not PRH dependent.
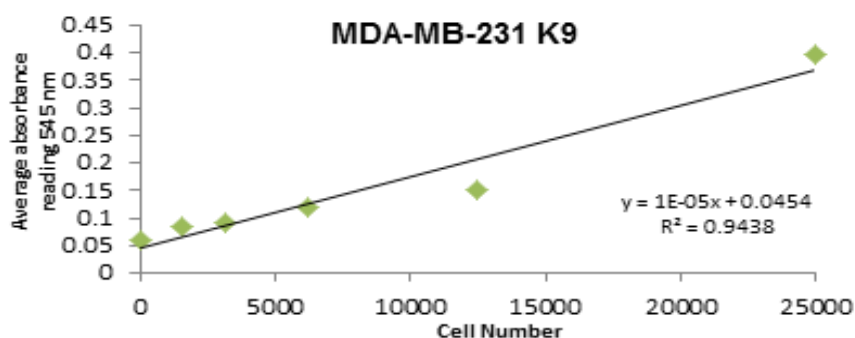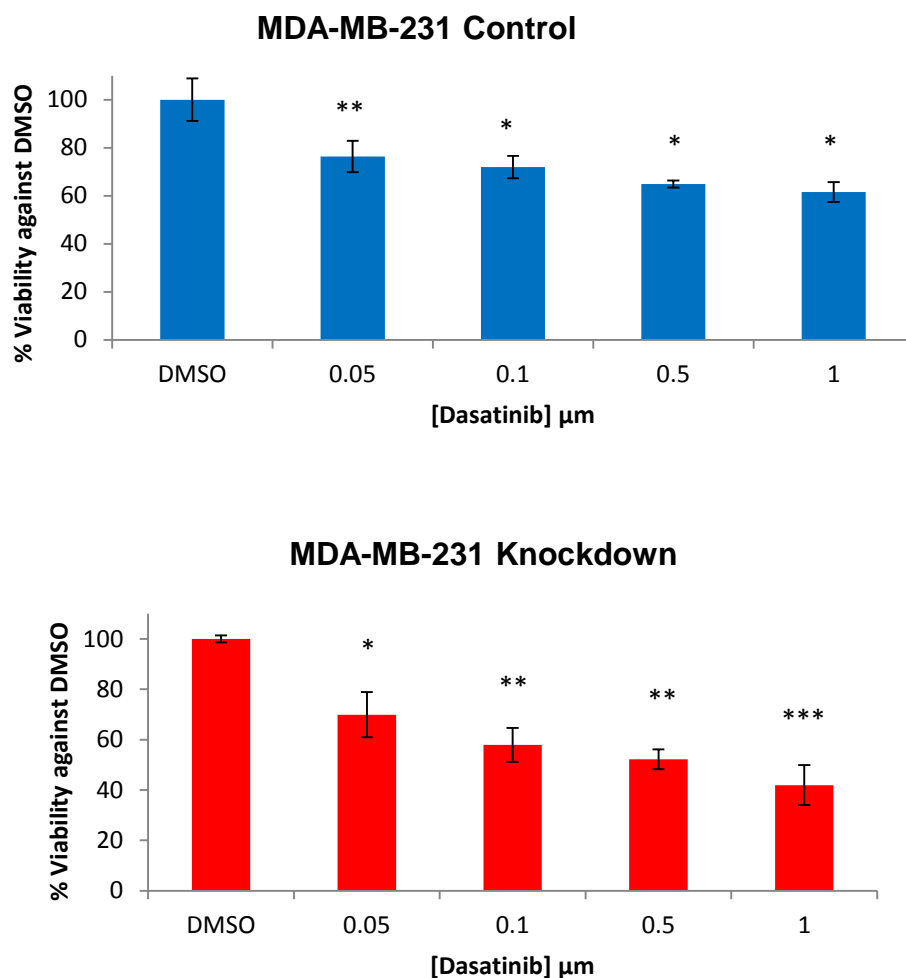
Figure 3.5: Cell Viability graphs for dasatinib treated MDA-MB-231 control and PRH knockdown cells. MDA-MB-231 control and knockdown cells showed no significant difference between the cell lines. This strongly suggests that their sensitivity to dasatinib is not mediated via PRH.

*= p<0.05 **=p<0.01 ***=p<0.001

3.8 Generation of MCF-10A control and knockdown cell lines with a stronger PRH knockdown

The current MCF-10A PRH knockdown cells have a low amount of PRH protein compared to the MCF-7 PRH knockdown cells (PSJ, communications). Therefore we attempted to generate a stronger MCF-10A PRH knockdown cell line. To do this we selected cells which could tolerate a greater puromycin concentration of 10 µg/ml, compared to the original puromycin concentration 0.5 µg/ml.

Puromycin is an aminonucleoside antibiotic which inhibits protein synthesis by causing premature chain termination, when the peptide transfer on the ribosomes is disrupted, during translation (Life Technologies, 2014). By increasing the puromycin resistance in both MCF-10A control and knockdown cell lines the aim was to produce a stronger knockdown cell line by selecting for cells where the lentivirus has randomly integrates into a region of open chromatin, thereby allowing a high level of expression of puromycin as well as other genes present in the same viral integration sites.

MCF-10A control and knockdown cells were seeded at 200,000 cell/well in a 6 well plate and were left to adhere for 24 hours. Puromycin titrations were carried out in order to find the highest concentration of puromycin at which both MCF-10A control and MCF-10A PRH knockdown cells would survive. Initially MCF-10A control and MCF-10A knockdown cells were infected with 0.5 µg/ml, 10 µg/ml and 50 µg/ml puromycin. Figure 3.6 shows the alteration of cell morphology at 50 µg/ml when cell death had occurred in both MCF-10A control and MCF-10A PRH knockdown cells. Hence, 50 µg/ml puromycin was deemed too high and an additional puromycin titration was set up with the following concentrations of puromycin: 10 µg/ml, 20 µg/ml and 30 µg/ml. After 5 days of incubation with the above puromycin concentrations, more cell death was observed following incubation with 30 µg/ml puromycin than with 20 µg/ml, shown in figure 3.6. 10 µg/ml was selected as the optimum puromycin concentration that the MCF-10A control and MCF-10A knockdown cells are able to tolerate. Western blotting was carried out to assess whether there was an increase in the MCF-10A PRH knockdown upon induction. However, PRH proteins in the MCF-10A control or MCF-10A PRH knockdown cells were not apparent in the western blotting experiments, therefore additional western

blotting   experiments are necessary   to determine whether we successfully generated a MCF-10A PRH knockdown cell with a more effective knockdown.
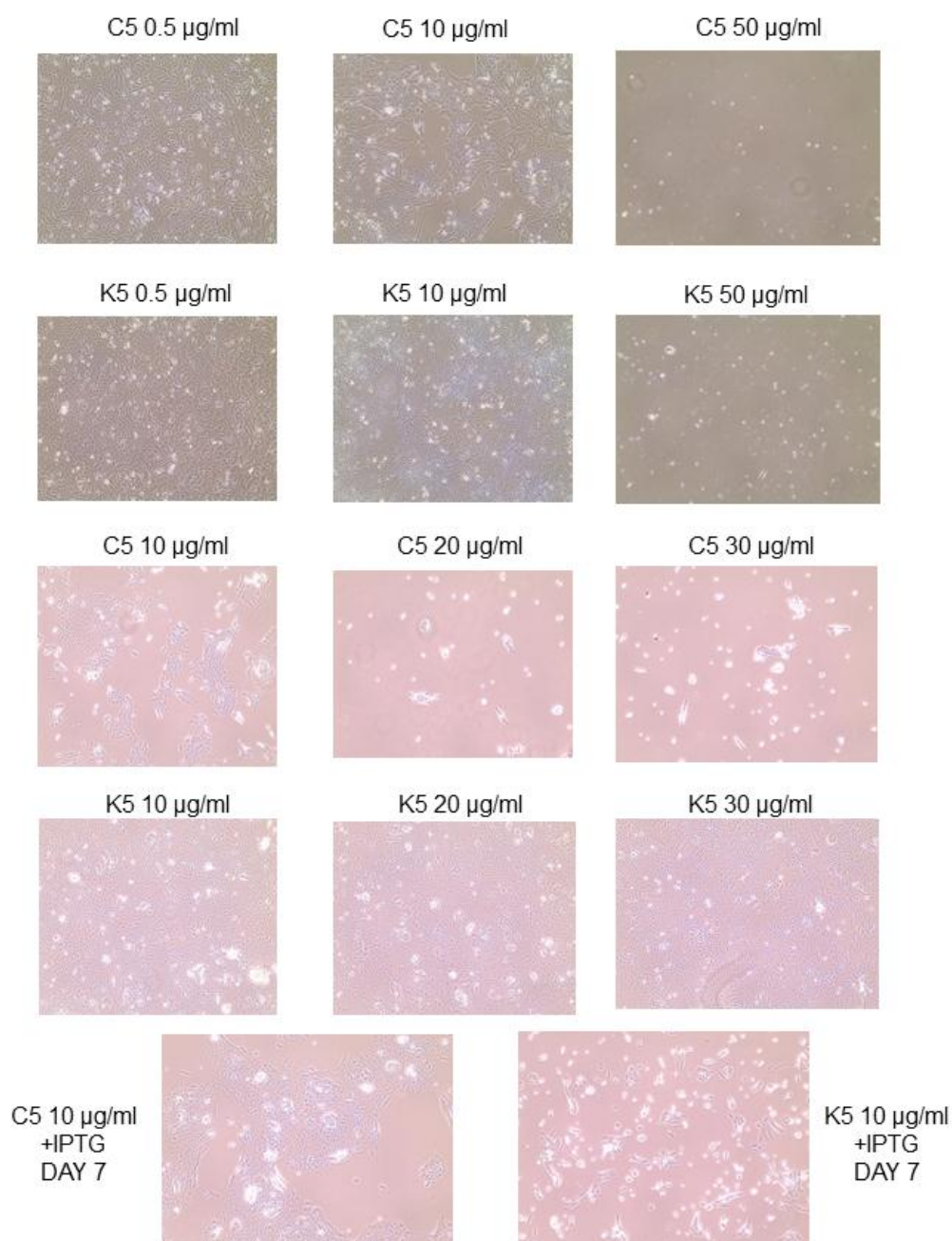
Figure 3.6: Images of MCF-10A 10 µg/ml puromycin selection. Initially, puromycin titrations were carried out but for control and knockdown cells at 0.5 µg/ml, 10 µg/ml and 50 µg/ml. Cell death was observed at 50 µg/ml and so this concentration was not used. Additional puromycin titrations were then set up using 10 µg/ml, 20 µg/ml and 30 µg/ml, cell death was observed at 30 µg/ml and so was not used and 10 µg/ml was selected as the optimum puromycin selection for the generation of a stronger PRH knockdown cell line. The cell line was grown and induced used for all experimental work.

3.9 MCF-10A control cells are more sensitive to dasatinib than MCF-10A PRH knockdown cells

Although the MCF-10A cells were not found to be further knocked down for PRH, the parental MCF-10A PRH knockdown cells are known to be altered in comparison to the MCF-10A control cells. The difference being that MCF-10A PRH knockdown cells are more migratory (Kershaw*, et al*, 2013). Furthermore wound healing assays and transwell assays have shown the MCF-10A PRH knockdown cells to be more migratory than the MCF-10A control cells (Kershaw*, et al*, 2013). Thus, the high puromycin selected cells were used for cell viability assays to determine if a reduction in the level of PRH influences dasatinib sensitivity in this non-transformed cell line.

A one-way ANOVA statistical test was used to show that statistically there is a significant reduction in cell viability following dasatinib treatment in both MCF-10A control and MCF-10A PRH knockdown cells. An un-paired students' t-test was then able to identify the concentration at which cell viability-was statistically significantly reduced for each cell line. Figure 3.7 shows that the viability of MCF-10A control cells is significantly reduced following treatment with 0.1 µm dasatinib whereas MCF-10A PRH knockdown cells are more resistant, showing significantly reduced viability following treatment with 1 µm dasatinib. Therefore a higher concentration of dasatinib is required to reduce viability in MCF-10A PRH knockdown cells. Thus, the MCF-10A PRH knockdown cells are resistant to dasatinib sensitivity similar to the MCF-7 PRH knockdown cells.
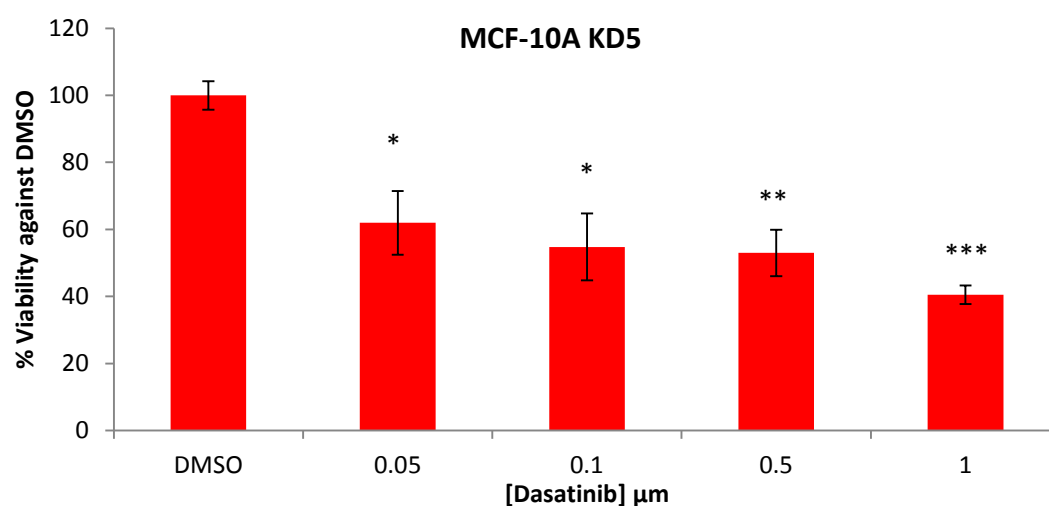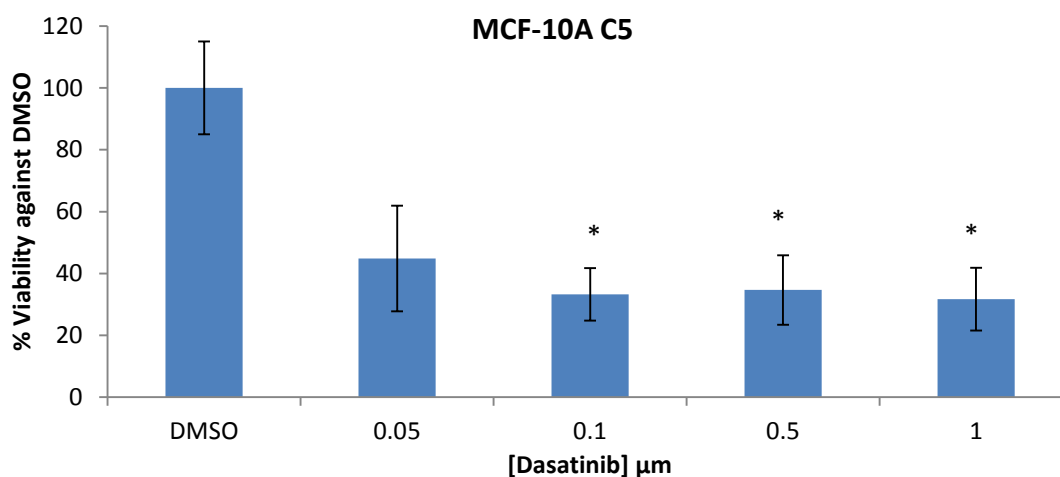
Figure 3.7: Cell Viability graphs for dasatinib treated puromycin selected MCF-10A control and knockdown cells. MCF10A control cells show a significant decrease in viability following treatment with 0.1 µm (p<0.01) dasatinib, whereas the MCF-10A knockdown cells show a significant decrease in viability following treatment with dasatinib at higher concentration of 1 µm (p<0.01).

*= p<0.05 **=p<0.01 ***=p<0.001

DISCUSSION

4.1   Introduction

The single-agent dasatinib is an inhibitor of multiple oncogenic tyrosine kinases such as Src, c-kit and EGFR and has shown modest activity as monotherapy against breast tumour cells (Mayer and Krop, 2010, Mayer, *et al*, 2011 and Herold, *et al,* 2011). Here, we investigated whether dasatinib-induced inhibition is partly through PRH in various breast tumour cells lines MCF-7, MDA-MB-231 and MCF-10A.

4.2   Molecular mechanism of dasatinib action differs in K562 leukemic cells to MCF-7 breast tumour cells

Previous experimental work from the Jayaraman laboratory has established that dasatinib treatment inhibits K562 leukemic cell survival, but a higher concentration of dasatinib is required to inhibit survival in the absence of PRH (Noy, *et al,* 2012). Figure 4.1 shows that the dual Src/ABL inhibitor reduces activity of the inactive form of PRH (pPRH) and elevates levels of hypo-phosphorylated PRH to re-establish the repression of the VSP genes. Thus, the effect of dasatinib is mediated via PRH to inhibit leukemic cell survival (Noy, *et al,* 2012).

Although western blotting experiments show that K562 control cells have high pPRH levels in comparison to the K562 PRH knockdown cells. This was a contrast to the findings from the present study in MCF-7 cells. Here, we show that dasatinib decreases pPRH levels in the MCF-PRH knockdown cells but that there is no visible alteration in pPRH with dasatinib treatment in the MCF-7 control cells. This suggests that the basis for MCF-7 PRH knockdown resistance to dasatinib may differ from that observed in the K562 leukemic cells.
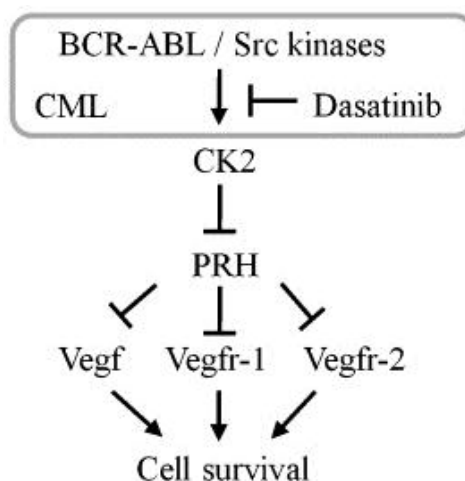
Figure 4.1: Model depicting the effect of dasatinib on PRH activity in chronic myeloid leukaemia (CML). Dasatinib affects the interaction between CK2 and BCR-ABL causing CK2 activity to be reduced, resulting in reduced p-PRH. Consequently, this re-establishes the repression of the VSP genes that lead to inhibition of leukaemic cell survival (Noy *et al.,* 2012).

4.3   MCF-7 dasatinib sensitivity

We showed that MCF-7 control cells have a significant decrease in cell viability following treatment with 0.1 μm (p<0.01) dasatinib, in comparison to the MCF-7 PRH knockdown cells which are significantly more resistant to dasatinib at 1 μm (p<0.01). MCF-7 PRH knockdown cells are also more migratory and proliferative in comparison to MCF-7 control cells (Roberts, 2014).

EGFR is a critical regulator and signal transducer in the inhibition of breast tumour growth (Kuraya, *et al*, 2004 and reviewed by Masuda, *et al*, 2012). EGFR is able to undergo auto-phosphorylation where ligand binding to the extracellular domain prompts dimerisation. This is caused by a conformational change in the EGFR monomers, stimulating EGFR kinase activity, leading to trans-auto-phosphorylation. This results in the phosphorylated tyrosine residue to act as a docking site for downstream protein signalling (Schulze, *et al*, 2005). We have shown that dasatinib decreases pEGFR levels in MCF-7 control cells and infer that dasatinib inhibits the phosphorylation of EGFR. This is also the site for trans-phosphorylation of Src (Nautiyal, *et al*, 2009). Auto-phosphorylation of Src is a type of post-translational modification whereby the kinase phosphorylates itself to sustain its active site. Src auto-phosphorylation results in greater levels of active Src and increased

expression and/or of active downstream dasatinib targets/proteins. We have shown that dasatinib reduces the level of pSrc in MCF-7 control and MCF-7 PRH knockdown cells and infer that dasatinib reduces the amount of Src auto-phosphorylation in order to decrease the amount of active Src. Thus, by reducing the activation of downstream signalling pathways which are regulated by Src, cell survival can be inhibited through the down-regulation of various downstream pathways such as the PI3K pathway.

The PI3K pathway is transduced by AKT which effects cellular proliferation, programmed cell death and cell migration by the phosphorylation of substrates (Vogt, *et al*, 1999 and reviewed by Shtilbans, *et al*, 2007). Ligand-dependent binding of AKT to EGFR activates the receptor via auto-phosphorylation. Phosphatidyl inositol-3 kinase (PI3K) is activated by EGFR and converts phosphatidyl inositol-4,5 bisphosphate (PIP2) at the 3' position of the inositol ring to PIP3. PIP3 is important for the activation of AKT via AKT phosphorylation. AKT phosphorylation is mediated through phosphatidyl inositol-3-phosphate dependent kinase (PDK1), which phosphorylates AKT, leading to the activation of AKT (Vogt, *et al*, 1999 and reviewed by Shtilbans, *et al*, 2007). Here we have shown that levels of pAKT are only reduced in the MCF-7 control cells with dasatinib treatment and infer that dasatinib is not causing inhibition of pAKT in MCF-7 PRH knockdown cells and that this is not the reason for dasatinib resistance of the MCF-7 PRH knockdown cells.

4.4   MDA-MB-231 dasatinib sensitivity

MDA-MB-231 cells are known to be the most invasive, proliferative and mesenchymal breast cancer cell line. High expression of such genes that control migration and proliferation result in the up-regulation of various pathways that are associated with increased cell survival. Thus, a higher concentration of dasatinib would be expected to be required to down-regulate pathways to reduce tumour cell survival. No significant difference in response was found between the dasatinib treated MDA-MB-231 control and PRH knockdown cells in our cell viability assays. Findings from the MDA-MB-231 cell viability assays are in agreement with the microarray data, as no significant alteration in expression levels of known dasatinib

targets were found in the MDA-MB-231 PRH knockdown cells. Thus, in MDA-MB-231 control cells dasatinib sensitivity is PRH dependent.

4.5   MCF-10A dasatinib sensitivity

The immortalised MCF-10 control cells exhibit a greater reduction in cell viability in comparison to MCF-10A PRH knockdown cells, which are relatively dasatinib resistant. We speculate that the mechanism by which the MCF-10A exhibits its dasatinib sensitivity is similar to the tumorigenic MCF-7 control and MCF-7 PRH knockdown cells. Further experiments are needed to test this hypothesis.

4.6   MDA-MB-231 control cells more sensitive to dasatinib than MCF-7

We have shown by cell viability assays that MCF-7 control cells have a greater reduction in cell viability compared to the MCF-7 and MDA-MB-231 PRH knockdown cells. This strongly suggests that the mechanism by which dasatinib is functioning is partly through PRH in the MCF-7 control cells. Interestingly, MDA-MB-231 control cells are more sensitive to dasatinib at a lower concentration of dasatinib 0.05 µm (p<0.01) see figure 3.5. Whereas the MCF-7 control cells are significantly sensitive to dasatinib at a higher dasatinib concentration 0.1 µm (p<0.01), see figure 3.2. This finding is supported by other investigations that have found the MDA-MB-231 cell line to be more sensitive to dasatinib in comparison to other cell lines (Finn *et al*, 2006, Huang *et al*, 2007 and Park, *et al*, 2012).

One study reported that the mesenchymal MDA-MB-231 cells have an altered cytoskeletal nature with dasatinib treatment (Pichot, *et al,* 200). Prior to treatment the MDA-MB-231 cells are spindle-shaped and grow as flattened cells, but with dasatinib treatment they are denser with compact cell bodies, with disrupted actin and tubulin structures. MDA-MB-231 dasatinib treated cells were observed to be round with less actin branching, reducing their mesenchymal nature (Pichot, *et al,* 2009). Thus, dasatinib treatment is able to inhibit MDA-MB-231 cell growth (Pichot, *et al,* 2009). As such dramatic changes were not observed with the MCF-7 cells in this study; these findings offer some explanation as to why our findings have shown that the highly PRH expressing MDA-MB-231 cells are more sensitive at a lower dose of dasatinib compared to the low PRH expressing MCF-7 cells. Significantly,

MDA-MB-231 cells that are known to express high amounts of the PRH protein (Roberts, 2014) are more sensitive to dasatinib treatment than MCF-7 cells that express low amounts of the PRH protein (Roberts, 2014). This difference supports the hypothesis that breast cells with low PRH are more resistant to dasatinib treatment. However, further investigations are needed to confirm this notion.

4.7   Transcriptional Factors as biomarkers in Breast Cancer Therapy

Many transcription factors are currently used in breast cancer management, however we have shown that dasatinib sensitivity is PRH dependent in MCF-7 and MCF-10A cells.

Estrogen receptor (ER) expression is currently used to determine the sensitivity of patients to endocrine therapy, where high levels of ER are correlated with a better therapeutic response to endocrine therapy (reviewed by Marion, *et al*, 2010). *HER2* amplification is also currently used a biomarker where higher levels of the *HER2* oncogene are correlated with the increased chance of relapse and reduced survival (Pergram, *et al*, 2000 and reviewed by Marion, *et al*, 2010). Emerging biomarkers such as FOXA1, GATA3 and circulating microRNAs have also shown great potential in the improvement of breast cancer management. FOXA1 is a transcription factor essential for the development of mammary gland (Carroll and Brown, 2006 and reviewed by Qing, *et al*, 2014). One study has shown the potential of FOXA1 as a prognostic biomarker that is correlated with favourable clinical outcome in breast cancer (Mehta, *et al*, 2012 and reviewed by Qing, et al, 2014). GATA3 overexpression inhibits metastasis of breast cells, revealing its potential as a biomarker for breast cancer metastasis (Yan, *et al*, 2010). Furthermore, circulating microRNAs are emerging novel breast cancer biomarkers prior to the perioperative period. One study showed that cancer-specific miRNAs are altered in circulating breast tumour patients, where specifically *mi*R-195 levels were increased (Heneghan, *et al,* 2010). Following curative resection *mi*R-195 levels were significantly reduced in breast tumour patients, strongly indicating the use of miRNAs as non-invasive breast cancer biomarkers (Heneghan, *et al,* 2010). With the advancement in the production of biomarkers for breast cancer patients, this

study has shown the potential of the transcription factor PRH to be a biomarker to dasatinib treatment in breast cancer patients.

4.8   Future Work

In order to establish that dasatinib sensitivity is truly PRH dependent, additional cell viability assays should be carried out with additional control and PRH knockdown cell lines in the MCF-7, MCF-10A and MDA-MB-231 cell lines. Additional cell viability repeats are necessary for the MCF-10A control and MCF-10A PRH knockdown cells, as the reproducibility of the current cell viability assays is somewhat questionable. As our findings suggests that MCF-10A cells are PRH dependent in response to dasatinib treatment, it would also be interesting to further understand the molecular mechanism by western blotting for signalling proteins involved in tumour cell survival in MCF-10A cells. Furthermore, blocking EGFR signalling with a specific EGFR inhibitor and then experimenting the effects of dasatinib treatment on control and PRH knockdown in these breast tumour cells cell lines. This would enhance our understanding into the mechanism by which the direct dasatinib target EGFR, can reduce breast tumour survival through various signalling pathways.

CONCLUSION

The immortalised MCF-10A control cells are more sensitive to dasatinib treatment than the MCF-10A PRH knockdown cells. Similarly, the tumorigenic MCF-7 control cells are more sensitive to dasatinib treatment than the MCF-7 PRH knockdown cells. Interestingly, the highly aggressive MDA-MB-231 control cells showed no significant difference to dasatinib treatment in comparison to the MDA-MB-231 PRH knockdown cells. Interestingly, the MDA-MB-231 control cells appear to be more sensitive to dasatinib treatment than the MCF-7 control cells. This is in agreement with current literature. The MCF-7 microarray data was used to identify the alteration in gene expression levels of known dasatinib target genes, in the MCF-7 PRH knockdown cells. On identifying that EGFR and SFKs are highly up-regulated in the MCF-7 PRH knockdown cells, we used western blotting to gain an in depth understanding into the mechanism by which reduces breast tumour survival, through the active forms of EGFR (pEGFR) and Src (pSrc) with dasatinib treatment. We found that dasatinib inhibits EGFR and Src activity and infer that CK2 activity is inhibited as its substrate the inactive form of PRH (pPRH) is reduced with dasatinib treatment. Thus, our findings provide preliminary evidence that the transcription factor PRH has the potential in being a novel biomarker in breast cancer patients.

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