THE ROLE OF PROTEIN PHOSPHATASE 2A AS A TUMOUR SUPPRESSOR IN BREAST CANCER

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B. Biomed Sci (Hons)

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ABSTRACT

Breast cancer is a worldwide health issue, and while many advances have been made in recent years, continued understanding of the development and progression of breast cancer is required to produce novel therapies to improve patient survival. Breast cancer is characterised by disruption in signalling pathways that control key cellular processes such as growth, proliferation and survival. Protein Phosphatase 2A (PP2A) is a key cellular signalling molecule that regulates numerous signalling pathways involved in breast cancer. PP2A is a trimeric protein complex, consisting of a structural subunit (PP2A-A), to which a catalytic subunit (PP2A-C) and a regulatory B subunit bind. PP2A is a proposed tumour suppressor, yet the role of PP2A in breast cancer has not been examined in detail to date. This thesis firstly examines PP2A expression in breast cancer cell lines and human breast cancer tissue. Dramatic reductions in expression of the PP2A-A and also a number of regulatory B subunits were observed in a panel of breast cancer cell lines compared to normal human mammary epithelial cells. In addition, a significant reduction in PP2A-A expression was identified in human breast tumours compared to normal mammary tissue. These results suggest that PP2A is important for the development or progression of breast cancer. In order to determine the functional role of PP2A in breast cancer, PP2A subunit expression was altered in a mammary breast epithelial cell line, MCF10A. A number of MCF10A cell lines were generated by transduction of shRNA directed to the PP2A-A or regulatory B subunits, or by expression of cancer-associated PP2A-A mutant genes. Functional analyses showed that shRNA knockdown or PP2A-A mutant expression had very little effect on MCF10A cells when grown using traditional two-dimensional cell culture techniques. However, in a more physiologically relevant three-dimensional culture method that maintains cellular polarisation and signalling with the basement membrane, a number of phenotypes indicative of cellular transformation were observed. MCF10A cells with reduced expression of regulatory B subunits, or PP2A-A mutations unable to bind regulatory B subunits, demonstrated increased cellular proliferation, MCF10A PP2A-A mutants that cannot interact with either the catalytic or regulatory B subunits displayed invasive properties. The results presented in this thesis provide clear evidence that PP2A is involved in breast cancer and presents a number of avenues for future investigation and potential novel therapies.
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Watt, LF. Protein phosphatase 2A in breast cancer. Hunter Medical Research Cancer Research Program seminar day, Newcastle.

Poster Conference Presentations:


Cottrell LF, Roselli S, Verrills NM (2010) Alterations in Protein Phosphatase 2A expression suggest a tumour suppressive role in breast cancer. Lowry Cancer symposium, Sydney, NSW.

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2007  Honourable mention for the Campion Ma Playhoust award (Best oral presentation or poster by a student or any member under 30 year of age at Australian Society for Medical Research National Scientific Conference, Katoomba, NSW)

2010  Winner Faculty of Health 10 of the Best Research Showcase, University of Newcastle.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>2D</td>
<td>Two dimensional</td>
</tr>
<tr>
<td>3D</td>
<td>Three dimensional</td>
</tr>
<tr>
<td>5-FU</td>
<td>Fluorouracil</td>
</tr>
<tr>
<td>ADH</td>
<td>Atypical ductal hyperplasia</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl2-agonist of death</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BME</td>
<td>Basement membrane extract</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAMKII</td>
<td>Calcium/calmodulin-dependent protein kinase II</td>
</tr>
<tr>
<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CIP2A</td>
<td>Cancerous inhibitor of PP2A</td>
</tr>
<tr>
<td>CSF-1R</td>
<td>Colony-stimulating factor receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6 Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCIS</td>
<td>Ductal carcinoma in situ</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DUSP</td>
<td>Dual specificity phosphatase</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE</td>
<td>Estrogen response element</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GSK-3β</td>
<td>Glycogen synthase kinase-3β</td>
</tr>
<tr>
<td>HEAT (repeat)</td>
<td>Huntington-elongation-PP2A-A subunit-TOR</td>
</tr>
<tr>
<td>HEK-TER</td>
<td>Human embryonic kidney cells immortalised by addition of hTERT, SV40 LT and active Ras</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human mammary epithelial cell</td>
</tr>
<tr>
<td>HRT</td>
<td>Hormone replacement therapy</td>
</tr>
<tr>
<td>hTERT</td>
<td>Human catalytic subunit of telomerase</td>
</tr>
<tr>
<td>IEX-1</td>
<td>Immediate early response gene X-1</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KSR1</td>
<td>Kinase suppressor of Ras</td>
</tr>
<tr>
<td>LCIS</td>
<td>Lobular carcinoma in situ</td>
</tr>
<tr>
<td>LCMT-1</td>
<td>Leucine Carboxyl Methyltransferase</td>
</tr>
<tr>
<td>LT</td>
<td>SV40 Large T antigen</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCF10A ecoR</td>
<td>MCF10A cell line expressing the mouse ecotropic retroviral receptor</td>
</tr>
<tr>
<td>Mdm-2</td>
<td>Mdouble minute homologue 2</td>
</tr>
<tr>
<td>M-Leu309</td>
<td>Methylated PP2A-C at Leucine 309</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>M-PP2A-C</td>
<td>Methylated PP2A-C</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>Mut3</td>
<td>SV40 Small T mutant unable to bind PP2A</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor of κB</td>
</tr>
<tr>
<td>NHMRC</td>
<td>Nation health and medical research council</td>
</tr>
<tr>
<td>OA</td>
<td>Okadaic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Plecstrin homology (domain)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol (4,5)P&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol (3,4,5)P&lt;sub&gt;3&lt;/sub&gt;</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKR</td>
<td>Protein kinase R</td>
</tr>
<tr>
<td>PME-1</td>
<td>Phosphatase methylesterase (specific for PP2A)</td>
</tr>
<tr>
<td>PP</td>
<td>Ser/Thr protein phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase 2A</td>
</tr>
<tr>
<td>PP2A-A</td>
<td>Structural subunit of PP2A</td>
</tr>
<tr>
<td>PP2A-C</td>
<td>Catalytic subunit of PP2A</td>
</tr>
<tr>
<td>P-PP2A-C</td>
<td>Phosphorylated PP2A-C</td>
</tr>
<tr>
<td>PPM</td>
<td>Metallo-protein dependent phosphatase</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PTP</td>
<td>Protein Tyrosine Phosphatase</td>
</tr>
<tr>
<td>PTPA</td>
<td>Phosphotyrosyl phosphatase activator</td>
</tr>
<tr>
<td>PyMT</td>
<td>Polyoma virus middle T antigen</td>
</tr>
<tr>
<td>PyST</td>
<td>Polyoma virus small T antigen</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute media</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp Alkaline Phosphatase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMP</td>
<td>Skim milk powder</td>
</tr>
<tr>
<td>ST</td>
<td>SV40 Small T antigen</td>
</tr>
<tr>
<td>SV40</td>
<td>Simian virus 40</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris buffered saline-Tween 20 buffer</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UICC</td>
<td>Union for International Cancer Control</td>
</tr>
<tr>
<td>UTD</td>
<td>Untrasduced (MCF10A) cells</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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</table>
1. INTRODUCTION

1.1. Breast Cancer

1.1.1. Epidemiology

Breast cancer is one of the most common cancers in the world with an age standardised rate of 39.0 per 100,000, which equates to 1.38 million women newly diagnosed each year (Ferlay et al. 2010; Jemal et al. 2011). The most recent global prevalence statistics estimated that in 2002, a total of 4.4 million women were currently living with the disease, having been diagnosed in the previous 5 years (Hortobagyi et al. 2005). Breast cancer is also the leading cause of cancer-related mortality for females. The estimated number of deaths for breast cancer worldwide in 2008 was 458,400; which is approximately 14% of all cancer related deaths in women (Ferlay et al. 2010; Jemal et al. 2011).

The global burden of breast cancer is not evenly distributed, as the incidence of breast cancer is greatest in the industrialised world, while mortality rates are highest in the developing world (Parkin 2001; Hortobagyi et al. 2005; Ferlay et al. 2010; Jemal et al. 2011). Despite the highest incidence rates, mortality rates are declining in the industrialised world. Early detection screening programs are diagnosing more women earlier, allowing more effective treatment with the latest treatment regimes. Over three quarters of breast cancer deaths occur in the developing world due to lack of early detection screening, poor access to health care and substandard treatment regimes (Hortobagyi et al. 2005; Jemal et al. 2011).

In 2006 the number Australian women newly diagnosed with breast cancer was 12,614 (AIHW 2009). This equates to 35 women diagnosed every day, and an age-standardised rate of 112 per 100,000; one of the highest incidence rates in the world. While the mortality rate is declining, 2,618 Australian women died from breast cancer in 2004. The estimated 2004-05 breast cancer total expenditure was $331 million – including screening programs, in and out of hospital care and pharmaceuticals (AIHW 2009). Breast cancer is clearly both a
national and global health issue and research aimed at both prevention and cure is imperative.

1.1.2. Breast cancer development and progression

Breast cancer is the abnormal proliferation of epithelial cells lining the breast ducts or lobes, termed ductal and lobular carcinoma respectively (Figure 1.1). Tumours that remain within the basement membrane are classified as ‘in situ’ or non-invasive (Figure 1.1A). Invasive breast cancer occurs when the tumour cells penetrate the basement membrane and invade the surrounding adjacent tissue (Sainsbury et al. 2000). Patient prognosis is largely dependent on metastasis of breast cancer cells to distant sites, which is the main cause of death, rather than the primary tumour (Weigelt et al. 2005). The most common sites of metastasis are lymph nodes, contra-lateral breast, lungs, bones, liver, adrenal glands, gastrointestinal tract, brain and skin (Baselga et al. 2002; Weigelt et al. 2005).

Breast tumours are commonly classified according to the stage of disease based on morphological features, in order to determine patient prognosis and establish a treatment regime (Elston et al. 1999). The TNM classification system assesses tumour size (T), number of lymph nodes with metastasis (N) and evidence of metastasis to distant sites (M). The Union for International Cancer Control (UICC) grading system converts the TNM score to a stage I-IV. Increasing tumour size, number of lymph nodes infiltrated and metastasis to distant sites are indicators of poorer prognosis (Sainsbury et al. 2000). In addition, a number of molecular markers are routinely screened for, including estrogen, progesterone and HER2/ErbB2 receptor status (NHMRC 2001b; Baselga et al. 2002). These are used to determine effective treatment regimes and also prognosis. Breast cancer is a highly heterogeneous disease, at both the clinical and molecular level (Polyak 2007). Highly varied patterns of gene alterations, including mutations and altered gene expression, lead to a variety of clinical presentations of the disease. This makes diagnosis, prognosis and treatment difficult to determine. For this reason, genetic and molecular profiling are becoming increasingly utilised to further understand the heterogeneous nature of breast cancer, with the
Figure 1.1 Breast cancer.

A) Anatomy of a human breast with lobular carcinoma in situ. Anatomy of the breast labels are:
   A. Ducts
   B. Lobes
   C. Dilated section of duct to hold milk
   D. Nipple
   E. Fat
   F. Pectoralis major muscle
   G. Chest wall/rib cage
   Lobular carcinoma in situ (lower section) labels:
   A. Normal lobular cells
   B. Lobular cancer cells
   C. Basement membrane

B) Progression of ductal carcinoma.

ultimate aim of optimising patient treatment regimes (Baselga et al. 2002; Polyak 2007).

Breast cancer is a progressive disease, and it is the accumulation of genetic alterations conferring new cellular properties that drives disease progression (Figure 1.1B, 1.2) (Singletary 2002; Kenemans et al. 2004; Karakosta et al. 2005; Polyak 2007). Each progressive stage of the disease is associated with increased risk of developing metastatic breast cancer (Singletary 2002). Initially, the breast epithelial cells abnormally proliferate, termed ductal hyperplasia, with altered cellular shape and chromatin patterns (Kenemans et al. 2004). Ductal hyperplasia is diagnosed if the cells exhibit a heterogenic phenotype, an indicator that multiple cells have increased proliferation. The progression to atypical ductal hyperplasia (ADH) is determined by a homogenous cell type, indicative of clonal expansion of a particular cell (Pinder et al. 2003). Following ADH, ductal carcinoma in situ (DCIS) is the next stage of the disease. The differences between ADH and DCIS are based on the architectural pattern, cytology, and disease extent. DCIS is defined as a proliferation of malignant epithelial cells, yet still contained within the basement membrane. Similarly, lobular carcinoma in situ (LCIS) also progresses from atypical hyper-proliferation of epithelial cells in the breast lobes (Pinder et al. 2003; Kenemans et al. 2004). As tumour cells acquire more genetic alterations, they are able to detach from, and invade through, the basement membrane into the surrounding tissue (Kenemans et al. 2004). Invasion of breast tumour cells into the surrounding breast tissue, lymphatic or vascular vessels, is associated with more aggressive disease (Sainsbury et al. 2000). The majority (85-95%) of invasive carcinomas are of ductal origin, with only approximately 10% being of lobular origin (Kenemans et al. 2004). As mentioned previously, metastasis to distant sites is a marker of poor prognosis, and the most common cause of breast cancer related death (Sainsbury et al. 2000; Weigelt et al. 2005).

The progressive nature of breast cancer is related to accumulation of advantageous alterations in intracellular signalling pathways that regulate
Figure 1.2: Progression of breast cancer requires multiple mutations.
Breast cancer is a progressive disease, from atypical hyperplasia, through carcinoma in situ and then invasive and metastatic disease. The driving force for this progression is accumulation of genomic and proteomic alterations. Only some potential alterations are depicted here, with many other alterations at different stages possible considering the heterogenic nature of breast cancer. Adapted from (Debnath et al. 2005; Singletary 2002; Kenemans et al. 2004; Karakosta et al. 2005). Images were obtained from Dr Jeremy Thomas, Breast Pathology on the Web available at: http://www.breastpathology.info/index.html accessed on 12/04/2012. Note images are not to scale and are intended to demonstrate features only, far right shows breast tumour within lymph node tissue (LN).
normal cellular processes including proliferation, apoptosis, survival and adhesion (Figure 1.2) (Singletary 2002; Kenemans et al. 2004; Karakosta et al. 2005). These signalling pathways may be disrupted by multiple means including genetic mutation, amplification or suppression of gene or protein expression as well as post-translational modification of proteins. Activated or amplified oncogenes drive tumour formation, while the inactivation or loss of tumour suppressor genes removes normal cellular regulatory mechanisms, thus allowing tumour formation (Karakosta et al. 2005). The causes of these genetic and proteomic alterations that disrupt signalling pathways and lead to the development of breast tumours are still under investigation. Breast cancer is considered a multi-factorial disease with a combination of genetic, environmental and lifestyle factors contributing to the disease (Brewster et al. 2001). A family history of breast cancer is one of the most significant risk factors for developing the disease, although it is estimated that only 5-10% of breast cancers are hereditary cases (Lux et al. 2006). Women with a first degree relative diagnosed with breast cancer have twice the risk of developing the disease (Jeng et al. 2000; Antoniou et al. 2006; Stratton et al. 2008). In familial breast cancer, mutations that lead to genomic instability are inherited. BRCA1 and BRCA2 are the most commonly identified predisposing genes in familial breast cancer. The BRCA1/2 genes encode DNA repair proteins (Kenemans et al. 2004), and hence, without proper repair mechanisms, further genetic alterations accumulate when these proteins are impaired (Karakosta et al. 2005). Many theories exist about potential environmental and lifestyle risk factors that increase the risk of gaining genetic alterations that lead to the development of sporadic breast cancer. Some of these include physical activity, reproduction, radiation and toxic chemical exposure, use of exogenous reproductive hormones and diet, however most still remain inconclusive (Brewster et al. 2001; Coyle 2004; Ferlay et al. 2010).

Whatever the initiating factors for the development of breast cancer, many of the genetic alterations identified in breast tumours cluster into key signalling pathways that can confer growth and invasion advantages to the cancer cells
Initially cells must increase proliferation and survival pathways while avoiding protective mechanisms. Proliferation pathways include those downstream of the receptor tyrosine kinases, epidermal growth factor receptor (EGFR) and ErbB2/Her2, both of which commonly demonstrate up-regulated expression in breast cancer (Singletary 2002). In addition, many breast tumours are driven by steroid receptors, estrogen and progesterone, which also contribute to cellular proliferation (Kenemans et al. 2004). Activating mutations in any of the signalling molecules driving cellular proliferation are considered oncogenes, for example, Ras. In order for cells to proceed through the cell cycle, especially once they begin to accumulate mutations, cell cycle checkpoints must be disabled. Over-expression of Cyclin D1 and Cyclin E are a common way breast tumours disrupt the cell cycle (Singletary 2002; Kenemans et al. 2004). Another protective mechanism tumour cells must overcome is cellular senescence through the shortening of chromosomal telomeres. Activation of telomerase in human mammary epithelial cells contributes to their immortalisation (Singletary 2002). In addition, DNA damage and cellular stress initiate pro-apoptotic factors such as p53 and Bcl2. Inactivating mutations in both p53 and Bcl2 are observed in breast cancer (Singletary 2002; Kenemans et al. 2004). Many tumours also demonstrate a loss of cellular polarity. This was initially thought to be a consequence of rapid proliferation, but it has been suggested that lack of normal cellular signalling that controls cellular polarity may in fact drive increased proliferation (Bissell et al. 2001). Signalling pathways controlling cellular polarity are particularly regulated by cellular adhesion, to both neighbouring cells and also the extracellular basement membrane proteins. In order for breast tumour cells to invade into the surrounding tissues, cells must become detached from their normal extracellular matrix supports and also invade through the basement membrane. Invasive breast cancer cells demonstrate increased expression of matrix metalloproteinase (MMP) molecules, which are capable of degrading extracellular matrix proteins, hence permitting basement membrane invasion (Chabottaux et al. 2006; Rizki et al. 2008). In order to infiltrate surrounding tissues, cancer cells must also develop a migratory capacity. This involves
re-polarisation of the cell body, extension of projections out into the surrounding tissue, formation of new adhesion sites at the ends of the projection and then re-arranging cellular cytoskeletal proteins to move the remainder of the cell to the new adhesion site (Friedl et al. 2003). E-cadherin and integrins are adhesion molecules that are altered in the metastatic progression of breast cancer (Wang et al. 1998a; Bissell et al. 2001; Wang et al. 2002; Liu et al. 2004a; Karakosta et al. 2005).

Importantly, many of the tumourigenic features discussed here including cellular proliferation, survival and apoptosis are controlled by a few key signalling pathways disrupted in breast cancer (Polyak 2007). The determination of which cellular signalling pathways are disrupted in breast cancers and the particular proteins involved aims to identify key genes that can be targeted to prevent the progression of breast cancer (Singletary 2002; Karakosta et al. 2005).
1.2. Signalling pathways in breast cancer

Cellular processes, including ones that drive cancer, such as proliferation, apoptosis, survival and adhesion are all controlled by intracellular signalling pathways. Extracellular information from sources such as soluble factors and adhesion molecules are transmitted into the cell via membrane receptors. Most cellular signalling pathways constitute an entire cascade of signalling proteins, each activating the next protein in the chain, until a physiological response is generated, for example, gene transcription. The cascade of proteins in each signalling pathway allows for signal amplification and also multiple levels of regulation (Pearson et al. 2001). Appropriate regulation of these pathways provides exquisite control and cross-talk between pathways culminating in appropriate cellular responses to the current environment (Schlessinger 2000). Many of the regulatory enzymes in these pathways are protein kinases and protein phosphatases. Protein kinases add phosphate groups from ATP to proteins, where the large negative charge changes the conformational shape of the protein and consequently its biological properties (Cohen 1989). Conversely, protein phosphatases remove phosphate groups from proteins. In signalling pathways, protein phosphorylation by kinases generally activates the next protein in the cascade, whereas de-phosphorylation by phosphatases inactives signalling proteins, often controlling the rate and duration of the signalling pathways, but this is not always the case. Tyrosine kinases specifically phosphorylate tyrosine residues, while serine/threonine (Ser/Thr) kinases phosphorylate either serine or threonine residues (Blume-Jensen et al. 2001; Vlahovic et al. 2003). Some kinases do have dual specificity (Dhanasekaran et al. 1998). Similarly, phosphatases can have tyrosine, Ser/Thr or dual specificity (Denu et al. 1996; Patterson et al. 2009). Extensive investigations into the role of kinases in cancer have been undertaken (Vlahovic et al. 2003) and have resulted in the development of specific kinase inhibitors as breast cancer therapies, providing therapeutic benefit to many patients (Moulder et al. 2008). In contrast, investigations into the role of phosphatases, which equally regulate signalling pathways, is still required. Novel therapeutics aimed at protein phosphatases may provide wider treatment options for drug resistant patients or
provide greater therapeutic benefit when used in combination with kinase inhibitors.

1.2.1. Estrogen receptor

The estrogen receptor (ER) is a steroid hormone receptor with a wide range of physiological functions. Apart from controlling development, reproduction and metabolism, it also has a role in regulating cellular growth and differentiation (Nicholson et al. 2005). The ER has 3 functional domains (Figure 1.3A); an N-terminal hormone independent transcriptional activation function 1 (AF1) domain, a central DNA binding domain and a C-terminal hormone dependent activation function 2 (AF2) domain (Ribeiro et al. 1995; Nicholson et al. 2005). The ER can be activated by three different mechanisms (Figure 1.3B). Firstly, in estrogen dependent activation, estrogen binding activates the AF2 domain, leading to receptor dimerisation, increased nuclear targeting, ER binding to estrogen response elements (EREs) on target genes and subsequently gene transcription (Nicholson et al. 2005; Howell 2006). Prior to activation, the ER may be present in either the cytoplasm or weakly bound to EREs in the nucleus (Howell 2006). The ER may also be activated in the absence of estrogen by a second, estrogen independent mechanism. Growth factor signalling, such as epidermal growth factor (EGF) binding to the epidermal growth factor receptor (EGFR), activates a signalling cascade and downstream kinases that can phosphorylate ER, activating the AF1 domain. This activation can lead to either ER dimerisation and activation of EREs in a similar manner to estrogen binding, or alternatively recruitment of other co-activators and transcription factors, leading to non-ERE gene transcription (Lannigan 2003; Nicholson et al. 2005; Howell 2006; Mendelsohn et al. 2010). A number of different sites can be phosphorylated on the ER by different signalling pathways, leading to activation and gene transcription (Lannigan 2003). Two isoforms of ER have been identified, ERα and ERβ, which are highly homologous in their DNA binding domains, but variant in their N-terminal domains. As this is the site of hormone independent activation, it has been suggested that these two isoforms may be differentially regulated by signalling pathways depending on the cellular
Figure 1.3 Estrogen receptor signalling.
A) The estrogen receptor contains 3 functional domains; AF1, DNA binding domain, and AF2 domain.
B) Three mechanisms of estrogen receptor activation.
conditions (Howell 2006). Finally, some actions of estrogen activation are too rapid to be explained by transcriptional activation, such as vasodilatation of coronary arteries (Nicholson et al. 2005). This mechanism of action is less well studied, but may be the result of interaction of ER with other receptors, particularly cell membrane G-protein coupled receptors (Mendelsohn et al. 2010). Currently this research is focused on cardiovascular disease, and activation of this pathway in the MCF7 breast cancer cell line did not affect cellular proliferation or migration (Chambliss et al. 2010). Any further roles for this pathway in breast cancer remain to be elucidated.

The link between breast cancer and exogenous estrogen use in either oral contraception or hormone replacement therapy (HRT) has been controversial. Women using oral contraceptives before 1975, when formulations used much higher estrogen concentrations, had a significantly increased risk of developing breast cancer (Brewster et al. 2001). However, current oral contraceptives with lower estrogen concentrations appear to only slightly increase the risk of breast cancer, and this risk is reduced following cessation of use (Rosenberg et al. 2009; Hunter et al. 2010). Interestingly, this increased risk does not correlate with estrogen receptor status of the tumour (Rosenberg et al. 2009). Similarly, use of HRT appears to increase the risk of developing cancer during treatment, but this risk declines following discontinuation of treatment (Chlebowski et al. 2009). The duration of therapy may also affect risk, as HRT use for only 2-5 years may be associated with very low risk, however with longer term use the risk of developing breast cancer increases (Brewster et al. 2001; Opatrny et al. 2008; Chlebowski et al. 2009).

1.2.2. Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) act as signal transducers, receiving extracellular stimuli via ligand binding to their external domains, which then activates internal signalling pathways responsible for regulating cellular transcription and other processes. The epidermal growth factor receptor (EGFR; ErbB1) is a RTK that is amplified, mutated or aberrantly activated by auto-
phosphorylation in many cancers, including 15-37% of breast cancers (Vlahovic et al. 2003; Agrawal et al. 2005). EGFR initiates multiple signalling pathways (Figure 1.4), including the PI3K/Akt and Ras/ERK pathways, which are involved in cellular processes that affect tumour progression such as proliferation, apoptosis, migration and invasion (Schlessinger 2000). ErbB2 (Her2/Neu), another RTK in the same family as EGFR, is rarely mutated in cancer, but is amplified at the genomic and/or protein level in 20-30% of breast cancers, and is associated with poorer prognosis and an aggressive phenotype (Slamon et al. 1987; Harari et al. 2000). Like EGFR, ErbB2 signals through the survival and proliferation pathways PI3K/Akt and Ras/ERK. The Ras/ERK and PI3K/Akt pathways are described in Sections 1.2.3 and 1.2.4 respectively.

1.2.3. Ras/ERK pathway

The Ras/ERK pathway is one of four mitogen activated protein kinase (MAPK) pathways, each named after the final kinase in the pathway; ERK1/2, p38, JNK, and ERK5. These pathways regulate development, cellular differentiation, growth, proliferation, adaptation to environmental stress and apoptosis (Widmann et al. 1999; Kolch 2000; Junttila et al. 2008). An extremely diverse range of stimuli including cytokines, growth factors, cellular stress, hormones and cellular adherence are able to stimulate the MAPK pathways. The ERK1/2 pathway (referred to from here on as ERK) is generally activated by surface RTKs, and is key for controlling cellular proliferation and survival. Other MAPK family members are activated by cellular stressors, cytokines and growth factors (Roberts et al. 2007). Aberrant activation of the ERK pathway at multiple points is frequently observed in breast cancer (Maemura et al. 1999; Malaney et al. 2001; Santen et al. 2002), and hence is the focus of this section.

ERK is a Ser/Thr kinase that phosphorylates a number of substrates including transcription factors, membrane proteins and cytosolic proteins, such as other kinases (Pearson et al. 2001; Roberts et al. 2007). The MAPK families are characterised by a chain of 3 kinases; a MAPK kinase kinase (MAPKKK) phosphorylates a MAPK Kinase (MAPKK), a dual specificity kinase, which
Figure 1.4 Epidermal growth factor pathway.
The Ras/ERK and PI3K/Akt pathways are downstream of EGFR. Dashed lines indicate other proteins are present in this cascade. Akt inhibits p53 through activation of Mdm-2, and hence signals cellular survival rather than apoptosis. Akt and ERK both activate c-Myc to induce cellular proliferation. In addition, ERK also phosphorylates the ER to up-regulate cellular proliferation. Note Akt and ERK activate other proteins and p53, c-Myc and ER are also activated by other mechanisms, but these have not been included for simplicity.
activates the MAPK by phosphorylation of both a tyrosine and threonine site (Widmann et al. 1999; Koleh 2000; Pearson et al. 2001; Junttila et al. 2008). In the ERK pathway (Figure 1.5), growth factor activation of RTKs activates Ras, a GTP binding protein, that recruits a number of scaffolding and binding proteins to the cellular membrane, including the adaptor protein Shc. The protein complex then activates Raf-1, a MAPKKK. Unusually, Raf-1 is activated by de-phosphorylation (Adams et al. 2005). In resting cells, Raf-1 is held in the cytoplasm by a signalling protein 14-3-3 which binds to phosphorylated serine residues on Raf-1. De-phosphorylation of these serine residues by protein phosphatase 2A (PP2A) is mediated by Ras and results in Raf-1 translocation to the cell membrane. Raf-1 complexes at the cell membrane with a scaffolding protein, kinase suppressor of Ras (KSR1), which is recruited by an identical mechanism to Raf-1 (Ory et al. 2003). Once bound to KSR1, Raf-1 phosphorylates the MAPKK, MEK, which in turn is able to activate ERK by dual phosphorylation. The majority of ERK targets are nuclear proteins and transcription factors that ultimately lead to altered gene expression. These include c-Myc, Elk1, Ets1, AFT-2, Fos and STAT3. Non-nuclear targets of ERK include the cytoplasmic kinases Syk and RSK1 (p90\textsuperscript{sk}), as well as cytoskeletal proteins such as paxillin (Widmann et al. 1999; Pearson et al. 2001; Ishibe et al. 2003; Webb et al. 2004; Roberts et al. 2007; Junttila et al. 2008). Raf-1 is also a target for ERK, and thus as phosphorylation inactivates Raf-1, this is a negative feedback mechanism that prevents excessive signalling through the pathway (Dougherty et al. 2005). As the Ras/ERK pathway is a chain of phosphorylation events, protein phosphatases are required to regulate this pathway at all levels and hence also regulate proliferation and survival processes which are aberrantly activated in breast cancer.

The role of the Ras/ERK pathway in breast cancer was first investigated over 10 years ago, in a panel of normal breast, fibrotic disease and breast cancer tissues. This study found a 5-10 fold increase in ERK activity in breast cancer samples that was due to a 5-20 fold increase in overall ERK expression, accompanied by an increase in ERK phosphorylation (Sivaraman et al. 1997). In a panel of 20
In response to growth factor signalling, the RTK recruits a complex of proteins to the cell membrane including the GTP binding protein Ras and adaptor protein Shc. Ras then recruits both Raf-1 and KSR-1 to the cell membrane by mediating release of both proteins from the scaffolding protein 14-3-3. Once at the cell membrane, Raf-1 phosphorylates MEK, which in turn phosphorylates ERK. The molecular targets activated by ERK phosphorylation are numerous and include cytoskeletal proteins such as paxillin, nuclear transcription factors and also cytosolic kinases. Other substrates of ERK exist, but only selected ones are shown for simplicity. Dashed lines indicate translocation of the protein.
breast carcinomas, over-expression of both Raf-1 and ERK was observed in all cancerous tissues compared to normal mammary gland tissue (Maemura et al. 1999). In a study using breast tumours compared to normal adjacent tissue, 5/11 tumours exhibited increased ERK activity (Salh et al. 1999). Further upstream, Ras mutations causing constitutive activation of the Ras protein transforms human mammary epithelial cells (Martinez-Lacaci et al. 2000; Rangarajan et al. 2004). Interestingly, this led to increased phosphorylation of not only ERK, but also EGFR. The mechanism by which activated Ras might increase phosphorylation of EGFR upstream of itself was not determined (Martinez-Lacaci et al. 2000). Although Ras is mutated in many types of cancer, mutations in Ras have been identified in less than 5% of breast cancers (Malaney et al. 2001). However, amplified expression of Ras at both the mRNA and protein level has been demonstrated in many breast cancer samples (Malaney et al. 2001), due in part to increased expression of RTKs (von Lintig et al. 2000).

1.2.4. Phosphoinositide 3-kinase pathways

Activation of RTKs not only activates the Ras/ERK pathway, but also recruits Phosphoinositide 3-kinase (PI3K) to the plasma membrane (Figure 1.6). The scaffolding p85 subunit of PI3K directly binds to phosphorylated tyrosine residues on the RTKs via its SH2 domains (Vivanco et al. 2002), often with the aid of adaptor proteins such as Grb2 and Sos (Cantley 2002). The PI3K p110 subunit is a lipid kinase that phosphorylates a lipid substrate in the plasma membrane, phosphatidylinositol (4,5)P$_2$ (PIP$_2$), at the D3 position to generate phosphatidylinositol (3,4,5)P$_3$ (PIP$_3$). Phosphorylation of PIP$_3$ at D3 binds pleckstrin homology (PH) domains in cellular proteins (Vivanco et al. 2002; Hennessy et al. 2005). Two Ser/Thr protein kinases, Akt (Protein kinase B) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) bind via their PH domains to PIP$_3$ at the plasma membrane. PDK1 phosphorylates Akt on Thr308, which is sufficient for Akt activation, but additional phosphorylation on Ser473 fully activates Akt by further opening the catalytic site (Vivanco et al. 2002; Franke 2008). The kinase that phosphorylates Akt at Ser473 was unidentified for a number of years, and is often referred to as PDK2. More recent evidence has
Figure 1.6 PI3K/Akt signalling pathway.
RTKs activated by growth factor binding recruits adaptor proteins, Ras and PI3K to the plasma membrane. PI3K phosphorylation converts PIP2 to PIP3 which then facilitates PDK1 phosphorylation of Akt. Full activation of Akt requires further phosphorylation by PDK2. Active Akt phosphorylates a number of substrates that control cellular proliferation, growth and survival via inhibition of apoptosis. Dashed lines indicate non-direct mechanisms of activation.
suggested a few different previously identified kinases are actually PDK2, with the mTORC2 complex the most likely candidate (Sarbassov et al. 2005; Franke 2008). Akt phosphorylates a number of downstream signalling proteins that control cellular proliferation, growth and also survival by the inhibition of apoptosis (Vivanco et al. 2002; Hennessy et al. 2005). Akt phosphorylation of glycogen synthase kinase-3β (GSK-3β) inactivates this constitutively active protein. GSK-3β phosphorylation of c-Myc and cyclin D1 normally mark them for ubiquitinated degradation; thus Akt-mediated inhibition of GSK-3β results in accumulation of these proteins which advance cells through the cell cycle, and hence increases proliferation (Cantley 2002; Vivanco et al. 2002). Conversely, Akt phosphorylation of mouse double minute homologue 2 (Mdm-2, sometimes called human homologue Hdmt-2) leads to increased ubiquitin mediated degradation of p53, thus inhibiting its control of cell cycle arrest and also apoptosis. In addition, Akt directly inactivates a number of pro-apoptotic proteins including the nuclear factor of κB (NF-κB), forkhead transcription factors (FKHR) and Bcl-2 family member Bcl2-agonist of death (BAD) (Vivanco et al. 2002; Hennessy et al. 2005; Franke 2008). This results in increased cellular survival, including in tumours where DNA damage should induce apoptosis. Finally, activation of the mammalian target of rapamycin (mTOR) controls cellular glucose metabolism and also increases cellular size due to enhanced protein synthesis. This process is required by tumour cells to generate the large number of factors required for increased cellular proliferation (Vivanco et al. 2002; Hennessy et al. 2005; Franke 2008). In normally functioning cells, phosphatase and tensin homologue (PTEN) negatively regulates this pathway to prevent excessive cellular survival and proliferation. PTEN is an interesting dual specificity phosphatase, as it can de-phosphorylate both proteins and lipids. In the PI3K/Akt pathway (Figure 1.6), PTEN de-phosphorylates PIP3 back to PIP2, preventing further activation of Akt (Cantley 2002; Vivanco et al. 2002).

In an Akt independent pathway, the small GTP binding protein Rac1 is also activated by PI3K, and regulates cellular polarity and migration through
cytoskeletal proteins (Figure 1.6) (Cantley 2002; Vivanco et al. 2002). Loss of cellular polarity is associated with loss of cell-cell adhesion, is commonly observed in tumours, and correlates with invasion and metastasis. Cell-cell junctions between epithelial cells maintain separation between apical and basolateral cellular domains, which express different proteins and must be maintained for normal cellular function (Hirohashi et al. 2003; Wodarz et al. 2007). Over-expressed wild-type Rac1, or a constitutively active Rac1 mutant, prevented polarisation of breast cells and increased their motility across, as well as invasion through, basement membrane structures. Interestingly, these effects were inhibited by an α2 integrin blocking antibody, but not addition of growth factors, suggesting that integrin mediated activation of PI3K is important for cellular polarity and migration (Keely et al. 1997). Conversely, addition of PI3K inhibitors led to the re-polarisation of invasive breast cancer cells and also prevented anchorage independent growth. Expression of activated Rac1 abrogated the re-polarising effect of the PI3K inhibitor, suggesting that Rac1 induces a loss of polarity in breast tumour cells (Liu et al. 2004a).

Mutations in the catalytic subunit of PI3K have been identified in multiple cancers, including 18-40% of breast cancer samples. They are most frequent in ErbB2 amplified, hormone receptor positive breast cancers, and also correlate with good prognosis (Bachman et al. 2004; Samuels et al. 2004; Hennessy et al. 2005; Isakoff et al. 2005; Kalinsky et al. 2009). Loss of function of PTEN is common in breast cancer, with around 50% of breast cancers having either an inactivating somatic mutation or reduced protein expression of PTEN. Akt was also found to be activated in 19/50 (38%) breast cancers (Sun et al. 2001). Intriguingly, in this study, further investigation into the mechanism of Akt activation revealed elevated PI3K activity in only 7/19 (37%) of the tumours with active Akt, and none of them had any alterations in PTEN. This suggests an additional mechanism of Akt activation, other than upstream pathway activation or PTEN inhibition. Whether inactivation of other negative regulators of Akt, such as PP2A, contributes to the increased Akt activity has not been investigated. Mutations in Akt are rare in breast cancer, although one study
identified a mutation in the lipid binding domain that activated Akt by increasing its affinity for the cell membrane in 5/61 (8%) of breast tumours (Carpten et al. 2007). All together approximately 70% of breast cancers exhibit activation of the PI3K/Akt pathways (Serra et al. 2011).

Expression of PI3K mutants (Isakoff et al. 2005) or over-expression of downstream proteins Akt and Rac1 (Zhao et al. 2003) in human mammary epithelial cell lines induced multiple phenotypic characteristics of breast cancer cells. These included growth factor independent proliferation, anchorage independent growth and resistance to chemotherapies. Different phenotypes appear to be regulated by two independent pathways downstream of PI3K; with Akt regulating cellular proliferation, while Rac1 controls cellular polarity (Liu et al. 2004a).

1.2.5. Cross talk between signalling pathways and implications for breast cancer therapies

A number of breast cancer therapies aimed at inhibiting signalling pathways have demonstrated resistance that is not due to lack of inhibition of the target signalling molecule (see Section 1.3 below), but rather a compensatory increase in other signalling pathways. RTKs are a common point of activation of the PI3K/Akt, Ras/ERK and also ER signalling pathways (Figure 1.7). This is actually beneficial for breast cancer therapies, as a RTK inhibitor could potentially inhibit signalling though all these signalling pathways. Both ERK and Akt can activate the hormone independent AF1 domain of the ER by phosphorylation, and hence, breast tumours with increased activation of either pathway are resistant to anti-oestrogen therapies that target the hormone dependent AF2 domain (Nicholson et al. 2005).

Ras activates Raf-1 (Widmann et al. 1999; Pearson et al. 2001) as well as the p110 catalytic subunit of PI3K (Vivanco et al. 2002; Hennessy et al. 2005). Raf-1 is also directly inactivated by Akt phosphorylation (Zimmermann et al. 1999; Moelling et al. 2002). This at first appears contradictory, as both the Akt and
Figure 1.7 Cross talk between signalling pathways with a role in breast cancer.

The Ras/ERK and PI3K/Akt pathways are both downstream of RTKs, and both can activate the AF1 site on ER by phosphorylation. Ras is most commonly associated with ERK pathway activation, but can also activate the catalytic subunit of PI3K, and hence can regulate both the Akt and Rac1 pathways. Akt can inactivate Raf-1, potentially preventing cell cycle arrest via p53 accumulation. Dashed lines indicate non-direct mechanisms of activation.
ERK pathways contribute to cellular proliferation and survival. However, high expression of oncogenic Ras or Raf-1 induces growth arrest in mammary cells though accumulation of p53 (Olsen et al. 2002; Sarkisian et al. 2007). As ERK phosphorylation of p53 protects it from ubiquitin mediated degradation (Persons et al. 2000), Akt inhibition of Raf-1 may mediate cellular proliferation by preventing continued ERK signalling.
1.3. Breast cancer therapies targeting specific signalling pathways

Inhibition of signalling pathways aberrantly activated in breast cancer cells, but not adjacent normal cells, is a key goal for novel breast cancer therapies. Numerous agents that target specific receptors and cytosolic kinases have been developed with varying levels of clinical benefit to patients (Nicholson et al. 2005; Roberts et al. 2007; Engelman 2009). Drug resistance continues to be a problem for these therapies, with compensatory increases in other signalling pathways as the key mechanism of resistance. This compensation may be due to cross talk between pathways as described in Section 1.2.5, or by other as yet unknown mechanisms of signalling pathway regulation.

1.3.1. Current breast cancer therapies

The declining mortality rate for breast cancer is due in part to advances in breast cancer therapies, in particular, more targeted treatments based on molecular profiling of tumours. As the first line of localised treatment, the current Australian guidelines recommend surgery with the aim of eradicating the primary tumour. This may take the form of complete local excision, which is defined as removal with clear histological margins and a rim of normal breast tissue completely surrounding the tumour, together with axillary lymph node dissection (NHMRC 2001b). For tumours with ill defined margins, total mastectomy with axillary lymph node dissection is considered appropriate treatment. In cases deemed to have high risk of axillary lymph node recurrence, axillary lymph node irradiation may also be considered (NHMRC 2001b). Radiotherapy after complete local excision, or following mastectomy in women with a high risk of relapse is also standard practice (NHMRC 2001b).

Systemic adjuvant therapy is administered in conjunction with localised therapy and aims to treat any undetectable remaining cancer, in order to reduce the risk of local recurrence and also metastatic disease. In general, several months of a combination treatment that includes an anthracycline, usually adriamycin, is currently recommended. Other cytotoxic drugs used in combination chemotherapies include cyclophosphamide, doxorubicin, methotrexate and
Fluorouracil (5-FU). All of these agents target DNA synthesis, thus preventing cellular division and proliferation. The combination of drugs is usually chosen based on particular features of the cancer and also the patients’ ability to tolerate side effects (NHMRC 2001b). For hormone receptor positive tumours, drugs specifically targeting these receptors are standard treatment. Tamoxifen is an anti-estrogen receptor drug and Trastuzumab is an anti-ErbB2(Her2) targeted therapy (discussed further in Sections 1.3.2 and 1.3.3 below) (NHMRC 2001b). Breast tumours that do not over-express the estrogen, progesterone or ErbB2 (Her2) receptors are classified as triple negative breast cancer (TNBC), and are associated with poorer patient prognosis. TNBC is particularly difficult to treat as anti-hormonal therapies will not be effective. While Australia currently has no specific clinical guidelines for treating TNBC (NHMRC 2001b; NHMRC 2001a), recent studies have found that combinations of surgery, radiotherapy and chemotherapies are the most effective ways to treat TNBC (Oakman et al. 2010; Yagata et al. 2011). The most effective chemotherapies to treat TNBC include cisplatinum, paclitaxel, cyclophosphamide, PARP inhibitors and ixabepilone (a microtubule inhibitor) may be of benefit to TNBC patients (Podo et al. 2010).

In advanced breast cancer, the treatment aims are to improve length and quality of life (NHMRC 2001a). Some treatments used for early diagnosed breast cancer, such as surgery, radiotherapy or chemotherapy may be appropriate and are considered on a case-by-case basis. Other classes of chemotherapies are also used, such as taxanes, that prevent cell division through targeting the microtubules rather than the DNA. These are currently only used in Australia after standard treatments have failed (NHMRC 2001a). Women with advanced breast cancer may benefit from enrolling in clinical trials for novel therapies when no other treatment options are appropriate. In the final stages of advanced breast cancer, palliative care to maintain quality of life by managing symptoms is the focus of treatment (NHMRC 2001a).

As many of the standard chemotherapeutic agents target rapidly dividing cells, numerous side effects are associated with these treatment options. Therefore,
more targeted therapies are required to improve the effectiveness of breast cancer therapies for both early and advanced breast cancer, while minimising toxic side effects.

1.3.2. Anti-estrogen therapies
Approximately 80% of breast tumours are estrogen receptor positive (ER+) at the time of diagnosis, which is associated with positive prognosis (Osborne et al. 1980; Anderson et al. 2002; Bentzon et al. 2008; Setiawan et al. 2009; Morgan et al. 2011). Favourable prognosis is due to both less invasive tumour characteristics and also response to anti-estrogen therapies (Nicholson et al. 2005). The first anti-estrogen therapy, tamoxifen (Nolvadex®), was introduced as a breast cancer therapy over 20 years ago. This selective estrogen receptor modulator (SERM) has led to significant reduction in both breast cancer recurrence and also overall mortality due to breast cancer (Nicholson et al. 2005; Howell 2006). Tamoxifen acts as an ER antagonist in the breast, blocking signalling pathways that control cellular proliferation. However, in the uterus and bone, tamoxifen acts as an ER agonist and consequently treatment with tamoxifen increases the risk of endometrial cancer 2-4 fold (Dutertre et al. 2000). A number of other SERMs have been generated to avoid these effects, such as raloxifene (Evista®), but these have not been as effective as tamoxifen in treating breast cancer (Dutertre et al. 2000; Nicholson et al. 2005). A more recently developed pure estrogen receptor antagonist, fulvestrant (Faslodex®), which prevents ER dimerisation, nuclear targeting, and subsequent gene transcription, has also been very successful in treating breast cancer patients (Dutertre et al. 2000; Osborne et al. 2004; Howell 2006). In addition, fulvestrant reduces the expression of ER in breast tumours (Osborne et al. 2004; Howell 2006) and has provided clinical benefit in patients who developed tamoxifen resistance (Howell 2006). In post-menopausal women, estrogen is no longer produced by the ovaries, but rather is synthesised from androgens by an aromatase enzyme. Aromatase inhibitors such as anastrozole (Arimidex®) are highly specific, reduce estrogen expression by approximately 90%, and have provided therapeutic benefit to many patients. However, these agents do not
directly target the ER, and therefore non-hormonal activation of the AF1 domain may still drive breast cancer progression. For this reason, aromatase inhibitors are most effective in combination therapies (Nicholson et al. 2005).

Approximately 20-30% of ER+ cells have *de novo* resistance to tamoxifen (Osborne et al. 1980; Shen et al. 2003) and in addition, breast cancers that initially respond to tamoxifen treatment may develop resistance during treatment with anti-estrogen therapies (Knowlden et al. 2003). A proposed mechanism of resistance is alternate intracellular signalling, especially through the EGFR and the Ras/ERK pathway (Jeng et al. 2000; Nicholson et al. 2005). Tumours with Ras/ERK pathway activation have been associated with a loss of clinical benefit from tamoxifen treatment, but not other chemotherapies (McGlynn et al. 2009). In addition, 20% of breast tumours do not express the ER at diagnosis (Bentzon et al. 2008). ER negative (ER-) tumours are associated with poor prognosis and have a more aggressive nature (Anderson et al. 2002). For patients with endocrine therapy resistance and ER- tumours, investigation into other signalling pathways aberrantly activated in breast cancer is required to improve targeted treatment options.

1.3.3. Receptor tyrosine kinase inhibitors

RTKs are an attractive target for anti-cancer therapies, as inhibition of these upstream receptors can affect a number of cellular processes through inactivation of multiple signalling pathways (Figure 1.4). Getfitinib (Iressa®) is a small molecule EGFR inhibitor developed for the treatment of many cancers. While initial results were promising with some effect in anti-estrogen-resistant cancers (Agrawal et al. 2005), later clinical trials demonstrated no therapeutic effect due to the development of drug resistance. The mechanism of resistance was not due to lack of receptor inhibition (Baselga et al. 2005; Normanno et al. 2005), but because other signalling mechanisms, namely the Ras/ERK pathway, were up-regulated (Normanno et al. 2006). Greater success has been achieved with the small molecule inhibitor lapatinib (Tykerb/Tyverb®) that targets both EGFR and also ErbB2. A small (6%) response rate was observed when used as a
single agent, but in combination therapy with other chemotherapies, lapatinib improved the response rate compared to chemotherapy alone (Moulder et al. 2008; Blackwell et al. 2010).

Trastuzumab (Herceptin®) is a humanised monoclonal antibody against ErbB2 (Park et al. 2007) which acts by internalising the receptor (Harari et al. 2000). Clinical response to Trastuzumab as a first line treatment was observed in 25-40% of patients with ErbB2 expressing cancers (Vogel et al. 2002; Moulder et al. 2008). Activation of PI3K by loss of the tumour suppressor gene PTEN is strongly suggested as a mechanism for Trastuzumab resistance (Badache et al. 2006; Berns et al. 2007; Park et al. 2007). More recently, use of Trastuzumab in combination therapies has also been successful, with better tumour response and overall survival observed in patients treated with the combination therapy compared to standard chemotherapy alone (Moulder et al. 2008; Blackwell et al. 2010).

1.3.4. Ras/ERK pathway inhibitors

Treatments targeting the Ras/ERK pathway for use in breast cancer have mainly focused on MEK inhibition, as this is the most specific signalling molecule in the pathway, known only to phosphorylate ERK (Roberts et al. 2007). One MEK inhibitor, CI-1040, was well tolerated in patients with advanced lung, breast, colon and pancreatic cancers, but as no patient had a complete or even partial response, trials with this inhibitor were abandoned due to lack of anti-tumour activity (Rinehart et al. 2004; Lorusso et al. 2005). Investigation into the mechanism of action of CI-1040 and another MEK inhibitor, U0126, in breast cancer cell lines revealed alternate signalling through the PI3K/Akt pathway as a potential mechanism for the observed tumour resistance (Mirzoeva et al. 2009). Another MEK inhibitor PD-0325901 was in phase II clinical trials for patients with advanced melanoma, colon and breast cancer between 2004-2007. While this treatment reduced phosphorylated ERK and tumour cell proliferation in melanoma patients, these results were not observed in breast cancer patients; although this may have been due to small sample size (LoRusso
et al. 2010). Despite confirmed complete response in one patient with melanoma, this trial was terminated due to unacceptable ocular and neurological toxicity (Boasberg et al. 2011). Another MEK inhibitor AZD6244 is currently in Phase I clinical trials to assess the safety of this newer compound. These results suggest that other regulators of the Ras/ERK pathways may need to be considered as drug targets.

### 1.3.5. PI3K/AKT pathway inhibitors

Due to the activation of the PI3K/Akt pathway in many cancers, and also the resistance to ErbB2 therapies mediated by up-regulation of this pathway (Section 1.3.3), therapies aimed at inhibiting the PI3K/Akt pathway at multiple levels have been the focus of much research in recent years (Serra et al. 2011). Initial Akt pathway inhibitors have demonstrated limited clinical efficacy, most likely due to enhanced ERK activation (Engelman 2009; Serra et al. 2011). Indeed, treatment of an ErbB2 over-expressing breast cancer cell line with the dual PI3K/mTOR inhibitor BEZ235, effectively inhibited Akt activity, but also resulted in a compensatory increase in ERK signalling (Serra et al. 2011). Combined administration of the MEK inhibitor AZD6244 with BEZ235 successfully reduced cellular proliferation and tumour volume, suggesting Akt pathway targeted inhibitors may be most effective when used in combination with inhibitors of the Ras/ERK pathway due to cross activation between these pathways (Engelman 2009; Serra et al. 2011). Clinical trials to assess BEZ253 as a combination therapy for breast cancer are currently recruiting. The most advanced specific Akt inhibitor, Perifosine, demonstrated no therapeutic response in patients with metastatic breast cancer that had 2 prior lines of chemotherapy (Leighl et al. 2008), however this drug is also in current clinical trials as a combination therapy.

The generation of many therapeutic compounds that target kinases in signalling pathways demonstrates the importance of kinases in breast cancer progression and current clinical therapies, however, investigation into the role of their reciprocal regulators, protein phosphatases, has been lacking. As described in
this section, combination therapies targeting multiple signalling pathways demonstrate the greatest therapeutic benefit to patients, and thus novel treatments targeting phosphatases may provide the next ammunition in the arsenal for the fight against breast cancer.

A wide range of drugs targeting other specific proteins in signalling pathways such as these, including the Bcl-2 family of apoptosis inhibitors (Kang et al. 2009) and DNA damage replair pathways (eg. Poly(adenosine diphosphate [ADP]-ribose) polymerases (PARP) inhibitors) (Rios et al. 2011), to name a few, are also being investigated in breast cancer and other solid tumours either alone or in combination with standatd chemotherapies (www.clinicaltrials.gov).
1.4. Protein phosphatases

Reversible protein phosphorylation is one of the most important mechanisms of regulating protein function in eukaryotic cells. Coordination of protein kinases and phosphatases controls the intracellular signalling response to changing physiological demands within cells. Protein phosphatases hydrolyse phosphatase ester bonds between phosphate groups and proteins. The majority of protein phosphorylation occurs on serine (Ser) residues (86.4%), followed by threonine (Thr) residues (11.8%), with the lowest amount on tyrosine (Tyr) residues (1.8%) (Olsen et al. 2006). Protein phosphatases are distinguished firstly by their substrate specificity, being either Ser/Thr protein phosphatases (PPs), protein tyrosine phosphatases (PTPs), or dual specificity phosphatases (DUSPs), which are considered a sub-family of the PTPs (Denu et al. 1996). It is interesting that approximately equal numbers of protein Tyr kinases (90) and protein Tyr phosphatases (107) have been putatively identified in the human genome; but a huge difference in the numbers of Ser/Thr kinases (428) and Ser/Thr phosphatases (~30) has been demonstrated (Shi 2009). While Tyr kinases and phosphatases as well as Ser/Thr kinases demonstrate very specific substrate targeting, the catalytic sites of Ser/Thr phosphatases are much more undiscerning. Research over the past two decades has shown that substrate specificity of Ser/Thr phosphatases is conferred by binding of regulatory subunits to the quaternary structure of the phosphatase (Shi 2009).

Ser/Thr PPs were originally distinguished based on their substrate specificities and sensitivity to two small protein inhibitors of phosphatases termed inhibitor-1 (I-1) and inhibitor-2 (I-2). Type 1 PPs, of which PP1 is the only identified member, de-phosphorylate the β subunit of phosphorylase kinase and are inhibited by both I-1 and I-2. Type 2 PPs on the other hand, de-phosphorylate the α subunit of phosphorylase kinase and are not inhibited by either inhibitor. Three classes of Type 2 PPs were further distinguished by their requirement for divalent cations into PP2A, PP2B (Calcineurin) and PP2C (Cohen 1989). Later sequence analysis revealed PP1, PP2A and PP2B are all members of the same gene family by sequence homology of their catalytic subunits, whereas PP2C is
a distinct gene family (Denu et al. 1996; Shi 2009). PP2C has now been classified as a metallo-protein dependent phosphatase (PPM). More recently identified members of the PP family are PP4-PP7. The catalytic subunits of PP family members PP1, PP2A, PP2B and PP5 have very similar crystal structures (Xing et al. 2006; Cho et al. 2007a). Protein phosphatase 2A (PP2A) is the most abundant member of this family and is involved in the regulation of many intracellular signalling pathways responsible for controlling cellular metabolism, development, proliferation and apoptosis.

1.4.1. Protein phosphatase 2A (PP2A)

About one third of the total PP2A in cells exists as a core dimer (Figure 1.8A) consisting of a structural subunit (PP2A-A), and a catalytic subunit (PP2A-C) (Kremmer et al. 1997). The remainder of PP2A is a trimeric holoenzyme with a regulatory B subunit (PP2A-B) subunit bound to the core dimer (Table 1.1 and Figure 1.8B,C). Regulatory B subunit binding alters the activity of the core dimer towards different substrates. For example, the holoenzyme has much greater activity for substrates phosphorylated by cyclin dependent kinases than the core dimer (Ruediger et al. 1997).

PP2A-A is a 65 kDa U-shaped protein with 15 HEAT (huntington-elongation-PP2A-A subunit-TOR) repeats (Groves et al. 1999). Two isoforms of PP2A-A, α and β, are 87% homologous (Hemmings et al. 1990) and are differentially expressed in tissues (Zhou et al. 2003). While PP2A-Aα is highly expressed in all tissues, PP2A-Aβ is highly expressed in the testis; moderately expressed in brain, heart and liver; but only weakly expressed in colon, lung, kidney and ovary, suggesting the two isoforms have different functions (Zhou et al. 2003). Indeed in cell based experiments where either isoform is suppressed in order to assess function, exogenous expression of the other isoform is unable to compensate for the altered cellular phenotype (Chen et al. 2005a; Sablina et al. 2007). Recently, a PP2A-Aα knockout mouse was generated, but embryonic death of these mice showed PP2A-Aα is essential for development (Ruediger et al. 2011). Further, reduced expression of PP2A-Aα to one third of original levels
Figure 1.8 Crystal Structure of PP2A.

A) PP2A core dimer consisting of the structural PP2A-A and catalytic PP2A-C subunits. Note that the C-terminal tail of the PP2A-C subunit is truncated in this structure, but demonstrates that PP2A-C binds to one side of the PP2A-A subunit and that the ends of PP2A-A make a wide $\cup$ with only the catalytic subunit bound.

B) PP2A heterotrimer with the regulatory PP2A-B$\alpha$ subunit bound. Microcystin-LR in orange is bound to the catalytic subunit active site. The C-terminal tail of PP2A-C is also truncated in this structure.

C) PP2A heterotrimer with the regulatory PP2A-B$'$γ subunit bound. Microcystin-LR in orange is bound to the catalytic subunit active site. Note the way that the PP2A-A subunit bends into a tighter $\cup$ shape when the PP2A-B$'$γ subunit is bound and that the C-terminal tail of PP2A-C extends out behind the PP2A-B$'$γ subunit.

These figures were generated with PyMOL (http://pymol.sourceforge.net) with coordinates from PDB entries A) 2IE4 (Xing et al. 2006) B) 3DW8 (Xu et al. 2008) and C) 2IAE (Cho et al. 2007).
### Table 1.1 PP2A Subunits.

<table>
<thead>
<tr>
<th>Subunit Type</th>
<th>Protein Names</th>
<th>Gene Name</th>
<th>Isoform</th>
<th>Splicing and variants</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural</td>
<td>PP2A-A or PR65</td>
<td>PPP2R1A</td>
<td>α</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R1B</td>
<td>β</td>
<td>2 variants: WT and exon 15 deleted but extra exons 16-17</td>
<td>(Hemmings et al. 1990)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalytic</td>
<td>PP2A-C</td>
<td>PPP2CA</td>
<td>α</td>
<td></td>
<td>(Stone et al. 1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2CB</td>
<td>β</td>
<td></td>
<td>(Stone et al. 1987)</td>
</tr>
<tr>
<td>Regulatory</td>
<td>PP2A-B or B55 or PR55</td>
<td>PPP2R2A</td>
<td>α</td>
<td></td>
<td>(Mayer et al. 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R2B</td>
<td>β</td>
<td>Brain enriched 4 variants that differ at N-terminus</td>
<td>(Mayer et al. 1991)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R2C</td>
<td>γ</td>
<td>Brain enriched 2 variants: WT and shorter 5’ UTR</td>
<td>(Zolnierowicz et al. 1994)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R2D</td>
<td>δ</td>
<td>2 variants: WT and exons 1-6 deleted</td>
<td>(Strack et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>PP2A-B’ or B56 or PR56</td>
<td>PPP2R5A</td>
<td>α</td>
<td>2 variants: WT and shorter 5’ UTR</td>
<td>(McCright et al. 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R5B</td>
<td>β</td>
<td></td>
<td>(McCright and Virshup 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R5C</td>
<td>γ</td>
<td>3 variants: WT, exon 13 and exons 13-14 deleted</td>
<td>(McCright and Virshup 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R5D</td>
<td>δ</td>
<td>3 variants: WT, exons 3-4 and 2-3 deleted</td>
<td>(McCright et al. 1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R5E</td>
<td>ε</td>
<td>2 variants: WT and deleted N-terminus</td>
<td>(McCright et al. 1996; Yang et al. 2010)</td>
</tr>
<tr>
<td></td>
<td>PP2A-B” or PR</td>
<td>PPP2R3A</td>
<td>PR130 or B”α2</td>
<td></td>
<td>(Hendrix et al. 1993; Jin et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R3A</td>
<td>PR72 or B”α1</td>
<td>Alternative splicing of PPP2R3A</td>
<td>(Hendrix et al. 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R3B</td>
<td>PR70 or B”β</td>
<td></td>
<td>(Stevens et al. 2003)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPP2R3B</td>
<td>PR48 or B”β</td>
<td>Likely an experimental truncation of PR70. Exons 1-10 deleted</td>
<td>(Yan et al. 2000; Stevens et al. 2003)</td>
</tr>
</tbody>
</table>
in an inducible PP2A-Aα knockout mouse, resulted in disease and death due to liver cell apoptosis; thus indicating that PP2A-Aα expression is also essential for normal cellular function (Ruediger et al. 2011).

Binding of PP2A-C to HEAT repeats 11-15 induces a conformational change in PP2A-A, where the two arms are brought closer together via folding at a hinge point between repeats 11-12 and 12-13 bringing the two arms closer together (Xing et al. 2006; Cho et al. 2007b; Xu et al. 2008). PP2A-C is a 3.7 kDa protein, also with α and β isoforms that are ubiquitously expressed, however the PP2A-Cα isoform is approximately 10 times more abundant than the PP2A-Cβ isoform, partly due to the strength of different promoters (Khew-Goodall et al. 1988; Khew-Goodall et al. 1991). PP2A-Cα and -Cβ isoforms are highly conserved (Janssens et al. 2001) and 98% homologous, but are not redundant as the PP2A-Cβ isoform was not able to rescue PP2A-Cα knockout mice which die in utero (Gotz et al. 1998). This again demonstrates the essential requirement for PP2A in normal cellular development.

Regulatory PP2A-B subunits bind to HEAT repeats 1-10 of PP2A-A and are grouped into 3 families: B (B55 or PR55), B’(B56 or PR65) and B”(PR), with each family having multiple isoforms (Sontag 2001; Janssens et al. 2008) (Table 1.1). Isoforms within individual families are highly homologous (Hendrix et al. 1993; McCright et al. 1995; Cho et al. 2007a), however each individual family encodes completely different protein structures (Figure 1.8 B,C). The close proximity of the regulatory B subunits to the catalytic site of PP2A-C controls substrate specificity by providing a docking site for substrates and also preventing non-specific substrates from accessing the catalytic site (Figure 1.8 B,C) (Janssens et al. 2008).

The PP2A-B subunit family encodes a WD40 repeat β-propeller structure (Fig 1.8B) and has α, β, γ and δ isoforms, all of which are encoded by different genes (Table 1.1) (Sontag 2001; Strack et al. 2002; Xu et al. 2008). The individual isoforms, as well as splice variants of some isoforms, vary greatest at the
N-terminal 20-30 amino acids and these are predicted to mediate isoform specific functions (Strack et al. 2002). Mutational analysis of PP2A-Bα suggests that the substrate binding site for these subunits is an acidic region in the centre of the β-propeller, adjacent to the catalytic site of PP2A-C (Figure 1.9) (Xu et al. 2008). A β-hairpin arm between WD40 repeats 2 and 3 reaches out and binds to the structural A subunit along HEAT repeats 1-2 (Xu et al. 2008).

In contrast, the PP2A-B’ family encodes an elongated, banana shaped structure composed of 8 pseudo HEAT repeats that are similar to, but not the same as, the structural PP2A-A subunit HEAT repeats (Fig. 1.8C) (Xu et al. 2006; Cho et al. 2007a). The PP2A-B’ family isoforms are also named α, β, γ, δ and ε, and these gene products can also have alternatively spliced variants (Table 1.1). For example, PP2A-B’γ1, -B’γ2 and-B’γ3 (Muneer et al. 2002). The different isoforms and splice variants of PP2A-B’ subunits are highly homologous in the pseudo heat repeat regions that contain the binding sites for both PP2A-A and PP2A-C, but are divergent at both the N- and C-termini (Cho et al. 2007b; Yang et al. 2010). These regions are predicted to be important for determining substrate specificity of the holoenzyme. The crystal structure of the PP2A holoenzyme with PP2A-B’γ bound reveals that the convex surface interacts with the structural PP2A-A subunit, while the highly acidic concave surface is pointed towards the catalytic PP2A-C subunit (Figure 1.10). This is the proposed binding site for PP2A substrates (Xu et al. 2006). Although the PP2A-A subunit and the PP2A-B’ subunit have large areas of adjacent surface, a relatively small number of amino acids make crucial binding sites between these subunits (Xu et al. 2006). Indeed, single point mutations in the PP2A-A subunits are sufficient to drastically reduce PP2A-B’ subunit binding (Ruediger et al. 2001b; Ruediger et al. 2001a).

The PP2A-B’’ family currently has two identified genes, which are alternatively spliced (Table 1.1). The PPP2R3A gene encodes two products, PR130 (B’’α1) and PR72 (B’’α2), which are transcribed from different promoters (Hendrix et al. 1993). The first gene product of PPP2R3B to be identified was a 60kDa protein
Figure 1.9 Structure of the PP2A-Bα holoenzyme.

PP2A heterotrimer with the regulatory PP2A-Bα subunit bound. Microcystin-LR in orange is bound to the catalytic subunit active site. The two red areas are E27 and D197 residues which when mutated prevented binding of the neurofilament protein Tau, suggesting this area (black circle) is the substrate binding site for PP2A-Bα (Xu et al. 2008). This figure was generated with PyMOL (http://pymol.sourceforge.net) using coordinates from PDB entry 3DW8 (Xu et al. 2008).
Figure 1.10 Structure of the PP2A-B′γ holoenzyme.

A) PP2A heterotrimer with the regulatory PP2A-B′γ subunit bound. PP2A-B′γ is a banana shaped structure with its convex side interacting with PP2A-A.

B) Microcystin-LR in orange is bound to the catalytic subunit active site. The suggested substrate binding site in the acidic convex side of PP2A-B′γ is circled with a black ring (Xu et al. 2006).

These figures were generated with PyMOL (http://pymol.sourceforge.net) with coordinates from PDB entry 2IAE (Cho et al. 2007).
named PR48 (Yan et al. 2000). However, further analysis suggests this was an artificial experimental artefact, and PPP2R3B actually encodes a 70kDa gene product termed PR70 (Stevens et al. 2003). The crystal structure of the PP2A-B” family has not been determined, but sequence analysis of PR72 and mutational studies suggest this family encodes two calcium binding EF-Hand motifs. Calcium ions induce a conformational change in PR72, and mutation of the calcium binding region of the second EF motif prevents binding to the PP2A-A subunit, suggesting that calcium ions are required to induce a conformational change prior to incorporation into a PP2A holoenzyme (Janssens et al. 2003).

Other proteins are capable of binding to the PP2A-A and also the PP2A-C subunits (described in Section 1.5.3), however these are not considered regulatory B subunits because they have other functions within the cell and are stable when free from the PP2A holoenzyme. In contrast, regulatory B subunits are unstable when released from the PP2A-A subunit, and are rapidly degraded through ubiquitin mediated proteasomal degradation (Strack et al. 2002; Strack et al. 2004). This is demonstrated by knockdown of the PP2A-A or PP2A-C subunits resulting in a concurrent reduction in regulatory B subunit expression (Li et al. 2002; Strack et al. 2002; Strack et al. 2004; Chen et al. 2005a; Sablina et al. 2007; Sablina et al. 2010).
1.5. PP2A regulation

1.5.1. Regulatory B subunit binding

Binding of a regulatory B subunit to the core PP2A-A/C dimer has been recognised as a key method of PP2A regulation for over a decade (McCright et al. 1996; Strack et al. 1998). As described in Section 1.4.1, the regulatory B subunit families encode very different protein structures and this naturally suggests different binding abilities with various protein substrates based on physical interactions. In addition, isoforms of regulatory B subunit families have divergent N- and C-termini, which are likely to be important for determining substrate specificity. Some substrates are specific for only one regulatory B subunit isoform, whereas others may be targeted by a particular family (Table 1.2). For example, the PP2A-Bα subunit is able to directly de-phosphorylate Ser/Thr sites that are immediately upstream of a proline site, which are the targeting sequences for proline directed kinases such as cyclin dependent kinases or ERK. PP2A-B’ subunits are also capable of de-phosphorylating these sites, but require the peptidyl prolyl isomerase Pin1 to isomerise the proline residue into the correct conformation prior to de-phosphorylation (Janssens et al. 2008). Other substrates can only be targeted by a specific regulatory B subunit isoform. For example, PP2A-B’α is the only isoform able to target the PP2A holoenzyme activity to the transcription factor c-Myc, where de-phosphorylation marks c-Myc for ubiquination-mediated degradation (Arnold et al. 2006). The mechanism of this targeting is through binding to the scaffolding protein Axin1, which supports and entire c-Myc degradation complex (Arnold et al. 2009).

Within cells, regulatory B subunits are localised in different subcellular locations. This is due to the presence of either particular localisation signals encoded in their proteins, or binding to adaptor proteins within defined cellular compartments. For example, a splice variant PP2A-Bβ2 differs from alternatively spliced PP2A-Bβ1 only at the N-terminus. Despite the similarities, only PP2A-Bβ2 co-localises with mitochondria in neuronal PC6-3 cells, and further its N-terminal sequence is sufficient to target a green fluorescent fusion protein to mitochondria (Dagda et al. 2003). Over-expression of PP2A-Bβ2
Table 1.2 Substrate specificity of regulatory B subunits important for cancer signalling.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Substrate</th>
<th>Role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2A-Bα</td>
<td>β-catenin</td>
<td>Wnt signalling</td>
<td>(Zhang et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>PKC</td>
<td>Tight junction formation</td>
<td>(Nunbhakdi-Craig et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>Microtubules</td>
<td>Cytoskeleton</td>
<td>(Sontag et al. 1995)</td>
</tr>
<tr>
<td></td>
<td>Raf-1</td>
<td>Ser/Thr kinase</td>
<td>(Ory et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>KSR</td>
<td>Raf-1 binding protein</td>
<td>(Ory et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>MEK</td>
<td>Dual specificity kinase</td>
<td>(Sontag et al. 1993)</td>
</tr>
<tr>
<td></td>
<td>ERK</td>
<td>Ser/Thr kinase</td>
<td>(Sontag et al. 1993)</td>
</tr>
<tr>
<td></td>
<td>Akt</td>
<td>Ser/Thr kinase</td>
<td>(Kuo et al. 2008)</td>
</tr>
<tr>
<td></td>
<td>P107, p130</td>
<td>Cell Cycle</td>
<td>(Jayadeva et al. 2010)</td>
</tr>
<tr>
<td>PP2A-Bβ</td>
<td>Cdc27</td>
<td>Cell Cycle</td>
<td>(Torres et al. 2010)</td>
</tr>
<tr>
<td>PP2A-Bδ</td>
<td>ERK</td>
<td>Ser/Thr kinase</td>
<td>(Sontag et al. 1993)</td>
</tr>
<tr>
<td>All PP2A-B’ family subunits</td>
<td>ERK</td>
<td>Ser/Thr kinase</td>
<td>(Letourneux et al. 2006)</td>
</tr>
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<td></td>
<td>Akt</td>
<td>Ser/Thr kinase</td>
<td>(Van Kanegan et al. 2005)</td>
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<td>APC</td>
<td>Wnt signalling</td>
<td>(Eichhorn et al. 2008)</td>
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<tr>
<td></td>
<td>Chk2</td>
<td>DNA damage</td>
<td>(Dozier et al. 2004)</td>
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<td>PP2A-B’α</td>
<td>c-Myc</td>
<td>Transcription factor</td>
<td>(Arnold et al. 2006)</td>
</tr>
<tr>
<td></td>
<td>β-catenin</td>
<td>Transcription factor</td>
<td>(Seeling et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>Bcl2</td>
<td>Anti-apoptotic</td>
<td>(Ruvolo et al. 2002)</td>
</tr>
<tr>
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<td>Pim1</td>
<td>Ser/Thr kinase</td>
<td>(Ma et al. 2007)</td>
</tr>
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<td>PP2A-B’γ</td>
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<td>(Li et al. 2007)</td>
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<td></td>
<td>Paxillin</td>
<td>Cytoskeleton</td>
<td>(Ito et al. 2000)</td>
</tr>
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<td></td>
<td>Mdm2</td>
<td>E3 ubiquitin ligase</td>
<td>(Okamoto et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>p300</td>
<td>Transcription co-activator</td>
<td>(Chen et al. 2005)</td>
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<td></td>
<td>β-catenin</td>
<td>Transcription factor</td>
<td>(Sablina et al. 2010)</td>
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<tr>
<td>PP2A-B’δ</td>
<td>cdc25</td>
<td>Cell Cycle</td>
<td>(Margolis et al. 2006)</td>
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<tr>
<td>PR130/B”α2</td>
<td>c-Myc</td>
<td>Transcription factor</td>
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<td>PR72/B”α1</td>
<td>c-Myc</td>
<td>Transcription factor</td>
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<td>PR70/B”β</td>
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<tr>
<td>PR48/B”β</td>
<td>Cdc6</td>
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This table was produced with the aid of (Arnold et al. 2008; Eichhorn et al. 2008).
results in cellular apoptosis, suggesting this localisation is also functional. PP2A-B’α was the only subunit identified to co-localise with the adaptor protein Ankyrin-B in cardiomyocytes, and deletion of 13 amino acids in the C-terminal tail, unique to this isoform, abolished the observed co-localisation (Bhasin et al. 2007). This suggests that a targeting sequence for Ankyrin-B is present in the C-terminal tail of PP2A-B’α. The PP2A-B’γ3 variant contains a bipartite nuclear targeting sequence and is highly expressed in the nucleus (McCright et al. 1996). However, this isoform has also been identified in focal adhesion complexes bound to the actin cross-linking protein, IQGAP1 (Suzuki et al. 2005; Takahashi et al. 2006a), suggesting that individual subunits may be regulated by multiple mechanisms depending on the cellular requirements at the time. The other PP2A-B’ subunit isotypes, PP2A-B’α, -B’β, -B’δ and -B’ε, are predominantly cytoplasmic, however these localisations are by no means permanent as PP2A-B’δ translocates to the nucleus during cell division (McCright et al. 1996). Interestingly, the two isoforms of PP2A-A differ in their ability to bind regulatory B subunits despite their similar sequence homology and structure. While PP2A-Aα binds all known regulatory B subunits, PP2A-Aβ does not bind Bα and has reduced ability to bind the PP2A-B’α and B’’α subunit family isoforms (Zhou et al. 2003).

Regulatory B subunits demonstrate varied expression during development and also in different tissue types. The PP2A-Bα protein is highly expressed in brain, lung, ovaries, and placenta; moderately expressed in skeletal muscle, spleen and thymus; but little expression is observed in kidney, liver, pancreas or heart in rat tissues. In contrast, PP2A-Bβ and -Bγ were only identified in brain tissue (Strack et al. 1998). While PP2A-Bα expression levels remained constant in the rat brain during development, the PP2A-Bβ subunit reduced 2-3 fold during later embryonic development and also following birth, whereas PP2A-Bγ levels increased sharply after birth (Strack et al. 1998). A similar pattern of expression for PP2A-Bα was identified in mouse tissues, with highest expression identified in the brain and lung while moderate expression was observed in the liver, kidney and heart. In contrast, PP2A-B’α was expressed at lower levels in the
brain and heart, with higher expression in the liver, kidney and lung (Ruediger et al. 2011). Interestingly, another PP2A-B’ family member, PP2A-B’δ, demonstrated high expression in brain and lung, but not liver, heart or kidney, in direct contrast to its family member PP2A-B’α (Ruediger et al. 2011).

An elegant example of substrate specificity and spatial regulation by a regulatory B subunit is the interaction of PP2A-B’β with tyrosine hydroxylase (TH), the rate limiting enzyme in catecholamine synthesis. Exogenous expression of PP2A-B’β, but not -B’α, -B’γ or -B’δ, resulted in a dramatic reduction in TH phosphorylation, suggesting that PP2A-B’β was specific for the TH substrate (Saraf et al. 2007). In addition, mutation of the PP2A-B’β residue Glu153, situated adjacent to the catalytic site on the PP2A-C subunit, prevented PP2A mediated de-phosphorylation of TH. However, this mutation did not affect PP2A-B’β associating with the PP2A dimer, nor activity towards some other PP2A substrates, indicating a specific docking site for TH on PP2A-B’β (Saraf et al. 2010). Highly phosphorylated TH is required for catecholamine synthesis, and PP2A-B’β demonstrates high expression in the neuronal cell body which corresponds with lower levels of phosphorylated TH. In contrast, PP2A-B’β has a lower level of expression in neuronal terminals which corresponds with highly phosphorylated TH, thus allowing catecholamine synthesis (Saraf et al. 2007). The mechanism that directs PP2A-B’β to the different subcellular locations is as yet unknown, but this example demonstrates both cellular targeting and substrate specificity. TH regulation by PP2A-B’β also highlights tissue specific effects, as PP2A-B’β expression is greatly reduced in the adrenal gland, where TH is hyper-phosphorylated, thus allowing large volumes of the catecholamine adrenalin to be synthesised (Saraf et al. 2007).

1.5.2. Post-translational modifications
In addition to regulatory B subunit binding, PP2A is also regulated by post-translational modification of PP2A-C at the highly conserved final six residues of the C-terminus, \(^{304}\text{TPDYFL}^{309}\). Phosphorylation at Threonine 304 and Tyrosine 307, as well as methylation at Leucine 309, are all important for PP2A
regulation (Longin et al. 2007). The crystal structure of PP2A holoenzymes reveals that this C-terminal tail lies between the structural PP2A-A subunit and the PP2A-B regulatory subunits (Xing et al. 2006; Cho et al. 2007a). This tail is in prime position to influence binding of regulatory B subunits, but is on the opposite side to the catalytic binding site (Figure 1.11).

1.5.2.1. Methylation

Methylation at Leucine 309 (M-Leu\textsuperscript{309}) has been implicated in regulating both holoenzyme assembly and also PP2A activity, however the precise role has caused some controversy (Tolstykh et al. 2000; Wu et al. 2000; Yu et al. 2001; Longin et al. 2007). It seems most likely that addition of a methyl group to Leu\textsuperscript{309} by leucine carboxyl methyltransferase (LCMT-1) (Lee et al. 1993; De Baere et al. 1999) aids binding of all regulatory B subunit isoforms, but is most important for the formation of holoenzymes with the PP2A-B subunit family \textit{in vivo} (Tolstykh et al. 2000; Wu et al. 2000; Yu et al. 2001; Longin et al. 2007; Janssens et al. 2008). Methylation at leucine residues is reversible, and PP2A-C is de-methylated by a specific phosphatase methylesterase PME-1 (Lee et al. 1996). In cell extracts, 70-90\% of PP2A-C is estimated to be methylated (M-PP2A-C), with both PP2A-B subunits and also PP2A-B’ subunits in holoenzymes with M-PP2A-C (Tolstykh et al. 2000; Yu et al. 2001; Longin et al. 2007). Post-translational M-Leu\textsuperscript{309} neutralises charge repulsions between the carboxyl group of the PP2A-C tail and the PP2A-A subunit, allowing regulatory B subunits to bind more easily with the C-terminal tail nestled into the interface between PP2A-A and regulatory B subunits (Cho et al. 2007b; Janssens et al. 2008). \textit{In vitro} incubation of purified LCMT-1 with either the PP2A dimer or trimer with PP2A-B\textalpha, resulted in highly methylated PP2A-C, but no alteration in PP2A activity towards phosphorylase \alpha was observed (De Baere et al. 1999). Thus, while some have reported that methylation increases PP2A activity towards substrates (Favre et al. 1994), this is most likely due to alterations in regulatory B subunit binding (Tolstykh et al. 2000; Wu et al. 2000; Yu et al. 2001).
Figure 1.11 Crystal structure of PP2A showing locations of post translational modifications.

A) PP2A heterotrimer with the regulatory PP2A-B′γ subunit bound. The C-terminal tail of PP2A-C lies nestled between the PP2A-A subunit and the regulatory B subunit. Leucine 309, the site of PP2A methylation is shown in orange. The two sites of phosphorylation; Tyrosine 307 is shown in grey and Threonine 304 is shown in yellow.

B) Rotation of the holoenzyme in (A) to show that the catalytic site of PP2A-C is on the opposite side of the enzyme compared with the C-terminal tail. Microcystin-LR is shown as a cyan stick bound to the catalytic site.

C) Magnified view of C-terminal tail of PP2A-C, shown as sticks for clarity.

These figures were generated with PyMOL (http://pymol.sourceforge.net) with coordinates from PDB entry 2NPP (Xing et al. 2006).
The functional effects of reduced M-PP2A-C have been investigated by suppression of LCMT-1 expression, which showed that PP2A-B’ subunits will readily bind non-M-PP2A-C, whereas the PP2A-Bα subunit will preferentially bind to any remaining M-PP2A-C (Yu et al. 2001). Once the amount of M-PP2A-C falls below critical levels, PP2A-Bα expression is reduced (Yu et al. 2001), presumably by protein degradation (Longin et al. 2007). This loss of PP2A-Bα protein expression following reduced M-PP2A-C ultimately resulted in cellular apoptosis (Longin et al. 2007). Controversy arose when experiments using recombinant subunits demonstrated that PP2A-Bα can bind to non-M-PP2A-C in vitro (Ikehara et al. 2007; Xu et al. 2008), However, as no PP2A-Bα subunits have been found bound to un-M-PP2A-C in vivo, this is likely to be the result of forced interactions in vitro. Interestingly, PME-1 not only de-methylates PP2A-C at Leu^{309}, thus altering regulatory B subunit binding, but also inactivates PP2A by removing the manganese ions required for PP2A activity (Xing et al. 2008). This inactivated form of PP2A may be reactivated by PTPA (Section 1.5.3.4), but the mechanism of re-activation still remains to be determined (Longin et al. 2004; Xing et al. 2008).

1.5.2.2. Phosphorylation of PP2A-C

PP2A-C is also regulated by phosphorylation at Tyrosine 307 (P-Tyr^{307}), which potently inhibits PP2A activity (Chen et al. 1992). In one study, addition of the PP2A inhibitor okadaic acid enhanced P-Tyr^{307}, suggesting that PP2A may also be capable of auto-de-phosphorylation (Chen et al. 1992). This is not an unreasonable suggestion as although PP2A is primarily a Ser/Thr phosphatase, it has also been shown to have phospho-tyrosine activity under certain conditions (Cayla et al. 1993). Growth factor stimulation of cells in vivo causes a transient increase in phosphorylated PP2A-C (P-PP2A-C), suggesting that temporary inactivation of PP2A allows signalling from kinases to nuclear effectors to occur (Chen et al. 1994; Barisic et al. 2010). This is supported by multiple tyrosine kinases, including Lyn, Fyn, Jak2 and Src, being able to phosphorylate PP2A in vivo (Chen et al. 1994; Lechward et al. 2001; Barisic et al. 2010).
mechanism by which P-Tyr$^{307}$ inhibits PP2A activity still remains to be determined. P-Tyr$^{307}$ also affects regulatory B subunit binding as demonstrated by studies using phospho-mimic and non-phosphorylatable mutants of PP2A-C. A phospho-mimic Tyr$^{307}$ mutant (T307D), prevented binding of PP2A-B$\alpha$ and -B$\delta$ as well as all of the PP2A-B$'$ subunits except for PP2A-B$'$δ. Binding of the PP2A-B$''$ subunits were not affected by this mutant (Longin et al. 2007).

Control of regulatory B subunit binding by P-Tyr$^{307}$ appears to be linked with M-Leu$^{309}$ (Longin et al. 2007). The crystal structure of LCMT-1 reveals a deep well in which the C-terminal tail of PP2A-C must fit in order to be methylated, including a hydrophobic binding site for Tyr$^{307}$. This suggests that P-Tyr$^{307}$ prevents access of Leu$^{309}$ to the active site of LCMT-1 (Janssens et al. 2008; Stanevich et al. 2011) and without the neutralising effect of M-Leu309, the PP2A-C terminal tail may not readily nestle into the charged binding region between the PP2A-A and PP2A-B subunits.

Little is known about the effects of phosphorylation at Threonine 304, however phospho-mimic mutants prevented PP2A- B subunit binding without affecting -B$'$ or -B$''$ subunits (Longin et al. 2007), again suggesting a role for phosphorylation regulating B subunit binding.

1.5.2.3. Phosphorylation of PP2A-B$'$ subunits

PP2A-B$'$ subunits are also modified post translation, as metabolic labelling with $^{32}$P revealed all isoforms could be phosphorylated on serine residues (McCright et al. 1996). With regards to kinases that can phosphorylate PP2A-B$'$ subunits, PP2A-B$'$α is phosphorylated by protein kinase R (PKR) on Ser$^{28}$, which increases in vitro PP2A activity towards myelin basic protein and the transcription factor eIF2α (Xu et al. 2000). This study showed that okadaic acid increased the phosphorylation of PP2A-B$'$α by PKR, suggesting the PP2A-C catalytic subunit may be able to de-phosphorylate its own regulatory subunit (Xu et al. 2000). PP2A-B$'$β and -B$'$γ are phosphorylated on Ser$^{327}$ (PP2A-B$'$γ
numbering) by ERK2 in the presence of Immediate early response gene X-1 (IEX-1, ier3) in growth factor activated cells. Phosphorylation of PP2A-B’γ causes dissociation of the PP2A-A/C dimer from ERK, preventing de-phosphorylation of ERK by PP2A (Letourneux et al. 2006) (See also Section 1.6.2). PP2A-B’γ can also be phosphorylated by the checkpoint kinase Chk2, at an unidentified residue, which increases the in vitro activity of PP2A towards myelin basic protein and also Chk2 itself (Dozier et al. 2004). This study investigated the phosphorylation of only the PP2A-B’γ3 subunit by Chk2, but found that that Chk2 binds to all PP2A-B’ family isoforms. Therefore it remains to be determined whether Chk2 can phosphorylate all PP2A-B’ family members. PP2A-B’δ contains 4 consensus sites for phosphorylation by protein kinase A (PKA): Ser^{53}, Ser^{68}, Ser^{81} and Ser^{566}. Mutation of all these sites concurrently reduced PP2A mediated de-phosphorylation of DARPP-32, a component of the dopamine signalling pathway in neurons. When more selective mutant combinations were employed, the Ser^{566} site was found to be the critical site for modulating DARPP-32 phosphorylation (Ahn et al. 2007).

Interestingly, the PP2A-B’γ Ser^{327} site phosphorylated by ERK (Letourneux et al. 2006) is conserved across all PP2A-B’ subunit isoforms, suggesting this may be a general mechanism for attenuating PP2A de-phosphorylation of ERK mediated by PP2A-B’ subunits. However, the PP2A-B’α Ser^{28} site, phosphorylated by PKR (Xu et al. 2000) and the PP2AB’δ Ser^{566} site, phosphorylated by PKA (Ahn et al. 2007), are in the variant N- and C-terminal regions respectively, unique to these particular isoforms. This suggests that phosphorylation of serine sites unique to individual PP2A-B’ isoforms may be a newly identified mechanism by which these subunits perform their isoform specific substrate targeting.

1.5.3. PP2A binding proteins

PP2A binding proteins are distinguished from PP2A regulatory B subunits if they are stable without the necessity of binding to PP2A and have other functions in cells. In contrast, the PP2A regulatory B subunits, and also the
catalytic PP2A-C subunit, are degraded by the proteasome if they are no longer able to bind to the PP2A-A structural subunit (Strack et al. 2002; Strack et al. 2004; Ruediger et al. 2011).

1.5.3.1. SET

The nuclear oncoprotein SET (I2PP2A), an endogenous protein inhibitor of PP2A, binds to the PP2A-C catalytic subunit, but the mechanism of inhibition is still not known (Li et al. 1996; Switzer et al. 2011). SET also inhibits the metastasis suppressor nm23-H1 and Rac1, hence its classification as a binding protein rather than a regulatory B subunit. SET inhibition with a novel SET interacting peptide, COG112, dissociates SET from PP2A-C, with a corresponding increase in PP2A activity in the breast cancer cell line MDA-MB-231 (Switzer et al. 2011). This study also found that the COG112 induced increase in PP2A activity coincided with inhibition of Akt activity and its downstream substrates mTOR, GSK-3β and c-Myc. These effects were reversed by a potent inhibitor of PP2A, okadaic acid, suggesting that the inhibition of Akt and its substrates were specifically the result of increased PP2A activity by COG112 (Switzer et al. 2011). Similarly, ceramide, a sphingosine lipid signalling molecule, also promotes the dissociation of PP2A and a truncated version of SET, I2PP2A (Mukhopadhyay et al. 2009). One study found that ceramide treatment prevented lung tumour growth in immune-deficient mice injected with a lung cancer cell line, and over-expression of I2PP2A in the lung cancer cells induced resistance to ceramide treatment (Mukhopadhyay et al. 2009). Up-regulation of SET, with a corresponding decrease in PP2A activity, is also a mechanism by which the BCR/ABL kinase drives disease progression in chronic myeloid leukaemia (Neviani et al. 2005; Perrotti et al. 2006).

1.5.3.2. α4

The α4 protein is ubiquitously expressed and binds monomeric PP2A-C, as well as the catalytic subunits of the related PP4 and PP6 phosphatases, but not the
PP2A-A/C dimer (Murata et al. 1997; Chen et al. 1998). Indeed the binding sites for PP2A-A and α4 on PP2A-C overlap (Prickett et al. 2004). The binding of α4 to PP2A-C subunits renders it catalytically inactive (Kong et al. 2007; Chen et al. 2011). Despite only a small fraction (2-5%) of PP2A-C associating with α4 in cells (Murata et al. 1997), the physiological effects of reduced α4 include impaired cell spreading and migration (Kong et al. 2007) and ultimately apoptosis (Kong et al. 2004; Chen et al. 2011). Conversely, over-expression of α4 transforms both immortalised human embryonic kidney cells, and also hepatocytes, as evidenced by anchorage independent growth and tumour formation in mice (Chen et al. 2011). Furthermore, increased expression of α4 has been observed in primary hepatocellular, lung and also breast cancers (Chen et al. 2011).

A potential role for α4 in mediating the proteasomal degradation of PP2A-C is still incompletely understood. The α4 protein acts as a scaffold, binding both PP2A-C and also Mid1, an E3 ubiquitin ligase that marks PP2A-C for degradation by the proteasome (Liu et al. 2001; Trockenbacher et al. 2001). However, a further level of regulation must be involved, because over-expression of α4 does not result in reduced PP2A-C expression (Chen et al. 2011). With regards to experimental knockdown of α4, one group found suppression of α4 caused no change in PP2A-C expression levels, but rather slightly increased cellular PP2A-A expression (Chen et al. 2011). In contrast, another study found that suppression of α4 in mice resulted in reduced expression of both PP2A-A and -C, and also that proteasomal inhibition in cells with reduced α4 caused an accumulation of ubiquitinated PP2A-C (Kong et al. 2009). The authors suggested that α4 may protect PP2A-C from ubiquitination, but how Mid1 then accesses PP2A-C remains to be determined.

1.5.3.3. CIP2A

CIP2A is a 90 kDa protein that was discovered in the protein complex of PP2A-A immunoprecipitates from cancerous cells, and hence was termed
Cancerous Inhibitor of PP2A (Junttila et al. 2007). Suppression of CIP2A expression by small interfering RNA (siRNA) reduced cellular proliferation and anchorage independent growth in a number of cancer cell lines, and also inhibited tumour formation in mice (Junttila et al. 2007; Li et al. 2008; Come et al. 2009). Interestingly, one group has found that CIP2A siRNA reduced c-Myc protein expression and increased PP2A activity in c-Myc but not MDM-2 protein complexes, suggesting CIP2A may specifically regulate PP2A complexes that de-phosphorylate c-Myc (Junttila et al. 2007; Come et al. 2009). However, in another recent study, suppression of CIP2A in lung cancer cells resulted in a reduction of Akt phosphorylation, suggesting CIP2A may also regulate other signalling molecules (Ma et al. 2011).

1.5.3.4. PTPA

PTPA was originally named Phosphotyrosyl phosphatase activator (PTPA) for its ability to induce PP2A mediated de-phosphorylation of tyrosine residues, but only in the presence of Mg²⁺ ions and ATP (Cayla et al. 1990; Cayla et al. 1993; Chao et al. 2006). However, no physiologically relevant functions for PP2A as a tyrosine phosphatase have been identified (Chao et al. 2006). The name of PTPA was amended to the PP2A phosphatase activator when it was found that PTPA could also activate the Ser/Thr activity of an inactive pool of PP2A associated with the PP2A methyl esterase, PME-1 (Section 1.5.2.1). A proposed mechanism for PP2A activation by PTPA is that PTPA has peptidyl-prolyl cis/trans isomerase activity that changes the conformation of proline 190 in PP2A-C (Jordens et al. 2006). The crystal structure of the PP2A holoenzyme (Cho et al. 2007a) later revealed that the proline 190 residue lies directly adjacent to the catalytic site in PP2A-C, suggesting that altered conformation of this residue is a plausible explanation for the increased activity of PP2A.

1.5.3.5. Other Binding Proteins

I1PP2A is a longer form of SET (Section 1.5.3.1) that also binds PP2A (Li et al. 1996), but has not been shown to have any association with cancer. Striatin and
S/G2 nuclear autoantigen (SG2NA) are calmodulin binding proteins that are sometimes referred to as B''' subunits (Lechward et al. 2001). As these proteins are part of much larger protein complexes, they may represent scaffolding proteins for recruitment of PP2A signalling complexes (Moreno et al. 2000). They are also calcium dependent proteins, and therefore may regulate PP2A function specifically in the context of calcium mediated signalling (Moreno et al. 2000; Lechward et al. 2001). Recently, α-endosulfine and adenosine monophosphate-regulated protein 19 (ARPP19) have been shown to bind PP2A during mitosis, with α-endosulfine binding specifically to complexes containing PP2A-Bδ (Gharbi-Ayachi et al. 2010; Mochida et al. 2010).

The exquisite regulation of PP2A activity provides information about how PP2A can be involved in the regulation of numerous signalling pathways, many of which are perturbed in the development of cancer. A number of lines of evidence point to PP2A as a tumour suppressor, however the role PP2A plays in any particular pathway in any given cancer has been, and continues to be, difficult to distinguish. This is due mainly to the large number of pathways PP2A is involved in, the number of different regulatory B subunits available to form holoenzymes and also taking into consideration cell type specific regulation of PP2A.
1.6. **PP2A in cellular signalling**

PP2A regulates multiple signalling pathways implicated in tumourigenesis. PP2A mutations have been identified in a number of cancers and altered PP2A subunit expression is also predicted to perturb many of the signalling pathways contributing to cancer progression.

1.6.1. **Estrogen receptor**

The initial link between PP2A and estrogen receptor (ER) status was suggested on the basis of 3 ER negative (ER-) breast cancer cell lines having much lower PP2A activity than three ER positive (ER+) cell lines (Gopalakrishna *et al.* 1999). In addition, estrogen deprivation reduced PP2A activity in ER+ MCF7 cells, which then increased over a period of 72 hours with re-introduction of estrogen (Gopalakrishna *et al.* 1999). Another group observed that treatment of MCF7 cells with a pharmacological inhibitor of PP2A, okadaic acid (OA), resulted in reduced ER mRNA and protein expression, and also correlated with decreased ER activity. ER promoter activity was not found to be affected by okadaic acid using a Luciferase assay, suggesting that PP2A does not directly inhibit ER expression (Keen *et al.* 2005). In contrast to ER+ MCF7 cells, when ER- MDA-MB-231 cells expressing exogenous ER were treated with OA, a further increase in ER protein expression was observed (Keen *et al.* 2005). However, as the exogenously expressed ER in the MDA-MB-231 cells lacked a 3’-UTR sequence when it was experimentally cloned from the MCF7 cells, it was hypothesised that this region may be important for PP2A regulation of ER. These experiments were repeated in MDA-MB-231 cells following introduction of an exogenous ER that did contain the 3’UTR. When these cells were treated with OA, ER mRNA expression was decreased, in a similar manner to what was observed for the ER+ MCF7 cells (Keen *et al.* 2005). Further, the OA induced reduction in ER was reversed by addition of a proteasome inhibitor. The authors proposed that PP2A mediates stabilisation of ER mRNA by another as yet unidentified protein binding factor, but this unknown factor is degraded by the proteasome in the absence of PP2A (Keen *et al.* 2005). PP2A has also recently been shown to physically associate with ERα in MCF7 cells, but a
mechanism of regulation of the ER protein by PP2A was not investigated (Bocca et al. 2010). These results suggest PP2A regulates ER expression, at least by altering mRNA expression, and possibly also protein stability. Whether ER can reciprocally affect PP2A is yet to be determined. In an investigation of epigenetic mechanisms in breast cancer, treatment of MDA-MB-231 cells with a methylation inhibitor increased both ER mRNA expression and also PP2A-Bβ mRNA expression (Keen et al. 2004). This regulatory B subunit isoform is predominantly expressed in the brain (Mayer et al. 1991), but has recently been implicated in breast cancer. The PPP2R2B gene which encodes PP2A-B’β is significantly methylated in breast tumours compared to normal tissue (Muggerud et al. 2010) and a single nucleotide polymorphism in this gene is associated with a better response to chemotherapy (Vazquez et al. 2011), however no link to ER status was observed in either of these studies. Further investigation into whether these mRNA and genetic alterations translate into altered protein function is required before any conclusions about regulation of PP2A by ER are made.

1.6.2. Receptor tyrosine kinases

Increased expression of ErbB2 is observed in 20-30% of breast cancers, and is associated with poorer prognosis and aggressive phenotype (Slamon et al. 1987; Harari et al. 2000). An unbiased phospho-proteomic screen of a breast cancer cell line with active ErbB2 identified 80 proteins that were tyrosine phosphorylated in response to ErbB2 activation, including PP2A-C (Wong et al. 2009). Further analysis revealed phosphorylation of PP2A-C at Tyr307 was significantly increased in ErbB2 positive tumours 15/25 (60%) compared with 14/44 (32%) ErbB2 negative tumours, and that P-Tyr307 correlated with cancer progression (Wong et al. 2009). As P-Tyr307 inhibits PP2A activity (Chen et al. 1992), this suggests that reduced PP2A activity is associated with breast cancer progression.
1.6.3. Ras/ERK pathway

The role of PP2A in the ERK pathways has long been controversial and complex to understand. PP2A has been reported to both negatively (Sontag et al. 1993; Alessi et al. 1995; Ugi et al. 2002; Adams et al. 2005) and positively (Abraham et al. 2000; Jaumot et al. 2001; Kubicek et al. 2002; Ory et al. 2003; Dougherty et al. 2005) regulate the MEK/ERK pathway in vivo. These discrepancies may be explained by PP2A regulating the pathway at multiple levels and also being involved in a complicated negative feedback loop (Figure 1.12) (Adams et al. 2005).

During the resting state PP2A associates with Shc, an adaptor protein that is part of the Shc/Grb2 complex, which couples to the RTK via the Sos complex at the start of the Ras/ERK pathway (Ugi et al. 2002). In response to growth factor signalling, PP2A is phosphorylated at Tyr307, resulting in reduced activity and also physical dissociation from Shc (Chen et al. 1994; Ugi et al. 2002). When PP2A activity is experimentally inhibited by the SV40 small T antigen, the entire Ras/ERK pathway demonstrates increased activation, including increased recruitment of Shc (Ugi et al. 2002). Thus PP2A acts as negative regulator of the ERK pathway at the level of Shc.

Multiple groups have also found that PP2A associates with Raf-1, the MAPKKK for the ERK pathway downstream of Ras (Abraham et al. 2000; Ory et al. 2003; Adams et al. 2005; Dougherty et al. 2005). In contrast to other members of the ERK pathway, de-phosphorylation of Raf-1 activates the protein. Inhibition of PP2A by okadaic acid prior to growth factor stimulation results in increased phosphorylation and reduced activity of Raf-1 (Abraham et al. 2000). Conversely, exogenous expression of PP2A-Bα and PP2A-Bδ PP2A regulatory subunits caused activation of Raf-1 by de-phosphorylation, which in turn activated MEK and ERK (Adams et al. 2005). In resting cells, the PP2A-A/C dimer associates with both Raf-1 and Kinase suppressor of Ras (KSR1), but does not de-phosphorylate either protein. Both Raf-1 and KSR1 are
Figure 1.12 PP2A regulation of the Ras/ERK signalling pathway.

In resting cells, PP2A binds to Shc and prevents signalling through the entire Ras/ERK pathway. In response to growth factors, RTKs inhibit PP2A, to allow Ras/ERK signalling. At the level of Raf-1 PP2A acts as a positive regulator of this signalling pathway. PP2A is activated by Ras to de-phosphorylate both Raf-1 and KSR1, resulting in dissociation of 14-3-3 and translocation to the cell membrane. PP2A also removes the inhibitory phosphorylation of Raf-1 by the ERK negative feedback loop to re-activate the pathway. PP2A is a negative regulator of the pathway at both MEK and ERK, as de-phosphorylation of these kinases inactivates them. Some specific holoenzymes of PP2A that target MEK and ERK have been identified, but may be cell type specific.
held in the cytoplasm by a signalling protein named 14-3-3. Activated Ras recruits PP2A-Bα to the PP2A dimer, which is then able to de-phosphorylate residues on both Raf-1 and KSR1 causing dissociation of 14-3-3 from each protein. This allows Raf-1 and KSR1 to translocate to the cell membrane where their binding to membrane associated complexes allows Raf-1 to phosphorylate MEK (Ory et al. 2003). This elegantly highlights the role of regulatory B subunits in substrate targeting. In these in vivo studies, PP2A-Bα and -Bδ have been identified in Raf-1 complexes, however the role of other regulatory B subunits has not yet been ruled out.

To control the amount of cellular proliferation in normal cells, ERK phosphorylates Raf-1, rendering it inactive and thus turning the pathway off in a negative feedback loop. PP2A, with the aid of prolyl isomerise Pin1, de-phosphorylates these sites, returning the pathway into a signalling competent state (Dougherty et al. 2005). Intriguingly, again the PP2A-Bα subunit was found to be the regulatory B subunit involved in this reaction. Thus PP2A, specifically with the PP2A-Bα subunit, is involved in both the initial activation, and also re-activation of the ERK signalling pathway at the level of Raf-1. Thus, as Raf-1 is activated by de-phosphorylation, PP2A acts as a positive regulator of the Ras/ERK pathway specifically at the level of Raf-1. PP2A has also been shown to directly bind to and de-phosphorylate both MEK (Sontag et al. 1993) and ERK (Sontag et al. 1993; Zhou et al. 2002; Letourneux et al. 2006). As de-phosphorylation inactivates MEK and ERK, PP2A negatively regulates the pathway through these proteins, thus preventing excessive ERK signalling and cellular proliferation. PP2A-Bα may be most important for the de-phosphorylation of MEK (Sontag et al. 1993), while at least one study found that only PP2A-B’ and not PP2A-B subunits were able to de-phosphorylate ERK in Chinese hamster ovary (CHO) cells (Letourneux et al. 2006). However in direct contrast, PP2A-Bα was found to de-phosphorylate ERK in neuronal PC12 cells (Van Kanegan et al. 2005). These discrepancies might reflect cell type specificity of PP2A signalling.
An interesting study, investigating the role of Immediate early response gene X-1 (IEX-1), found that IEX-1 binding to both ERK and PP2A-B’γ can also affect PP2A mediated regulation of ERK (Figure 1.13) (Letourneux et al. 2006). IEX-1 expression is under tight regulation by a number of transcription factors including p53, Sp1 and c-Myc (Wu 2003). IEX-1 can bind to both ERK and PP2A-B’γ independently, facilitating ERK phosphorylation of PP2A-B’γ. This causes dissociation of the PP2A-A/C dimer from PP2A-B’γ and hence ERK, resulting in enhanced phosphorylation of ERK and prolonged ERK signalling (Letourneux et al. 2006). IEX-1 must not always be present in cells or PP2A could not attenuate ERK signalling. The tight control of IEX-1 signalling suggests this protein is induced when the cellular environment requires prolonged ERK signalling.

In numerous studies, global inhibition of PP2A by either pharmacological agents (Liu et al. 2004b; Van Kanegem et al. 2005; Letourneux et al. 2006), DNA tumour viruses (Sontag et al. 1993) or more specific inhibition of PP2A by suppressing the PP2A-Aα, or a number of regulatory B subunits (Van Kanegem et al. 2005; Letourneux et al. 2006) results in increased phosphorylation of MEK and ERK, subsequently increasing cellular proliferation and growth factor independence. Therefore the overall effect of PP2A is to inhibit this pathway, as expected for a tumour suppressor in a growth signalling pathway. The apparent confusion appears to arise with PP2A having a role in reactivating the pathway at Raf-1 under normal growth conditions. Clearly further investigation remains to identify which regulatory mechanisms of PP2A are able to so exquisitely target PP2A to a particular part of this pathway at the appropriate time, in order to more fully understand how alterations to PP2A could cause deregulation of this pathway in the context of breast cancer.

While most work has been done on the ERK pathway, it appears that PP2A can negatively regulate all MAPK pathways, again depending on the regulatory B subunits involved (Junttila et al. 2008). Indeed PP2A can act as a bridge between the p38 MAPK pathway and the ERK pathway. In cardiac myocytes,
Figure 1.13 IEX-1 alters PP2A regulation of ERK by facilitating PP2A-B’γ phosphorylation.

A) In the activated Ras/ERK pathway, MEK phosphorylates ERK, which in turn phosphorylates transcription factors, leading to cellular proliferation.

B) PP2A de-phosphorylates ERK via PP2A-B’γ subunit binding, attenuating ERK signalling and hence cellular proliferation.

C) IEX-1 binds to both PP2A-B’γ and ERK, facilitating ERK phosphorylation of PP2A-B’γ. The PP2A-A/C dimer dissociates from phosphorylated PP2A-B’γ, and hence ERK signalling is sustained.
PP2A mediates p38 induced inhibition of ERK (Liu et al. 2004b), and in addition, inhibition of p38 results in accumulation of both phosphorylated MEK and ERK (Junttila et al. 2008). The dynamic balance of these two pathways in response to cellular stress determines whether a cell will survive (ERK signalling) or die by apoptosis (p38 and PP2A activation) (Liu et al. 2004b). In this capacity, inhibition of PP2A prevents cells from dying by apoptosis, and in a cancer setting inhibition of PP2A could allow damaged cells to escape apoptosis and continue replicating.

1.6.4. PI3K/Akt pathway

In the PI3K pathway, Akt is a direct substrate of PP2A (Figure 1.14) (Millward et al. 1999; Ivaska et al. 2002; Kuo et al. 2008). Suppression of either the structural PP2A-Aα subunit or PP2A-B’γ regulatory subunit in immortalised human embryonic kidney (HEK-TER) cells activated Akt and subsequently induced cellular proliferation, anchorage independent growth and even tumour formation in mice (Chen et al. 2005b). Inhibition of PP2A by DNA tumour viruses (Yuan et al. 2002), pharmacological agents or a dominant negative mutant (Haendeler et al. 2003) increased telomerase activity via activation of Akt. This is a potential mechanism whereby tumour cells can avoid cell death and senescence. Over-expression of PP2A-B’β or -B’γ, but not -Bα were able to prevent phosphorylation of Akt in CHO cells at both the Thr308 and Ser473 residues (Rocher et al. 2007), suggesting that at least PP2A-B’β and -B’γ may target PP2A to Akt. In direct contrast, in pro-lymphoid cells suppression of PP2A-Bα resulted in enhanced Akt phosphorylation at Thr308 (Kuo et al. 2008). These results may indicate cell type specific effects, or perhaps even alternate regulatory B subunits differentially targeting Thr308 and Ser473 residues of Akt. PP2A-B’α may also target PP2A to Akt, as one study has found that if capalin, a calcium dependent cytosolic protease, is knocked out of fibroblasts then PP2A binds more tightly with Akt and induces apoptosis (Bertoli et al. 2009). In vitro, incubation of recombinant PP2A-B’α and -B’γ with capalin resulted in degradation of the regulatory B subunits by capelin. Thus in cells, capelins may degrade regulatory B subunits leading to weak binding of PP2A to Akt.
Figure 1.14 PP2A inhibits Akt signalling.

PP2A directly de-phosphorylates and inactivates Akt affecting numerous downstream signalling pathways including telomerase activity. The regulatory B subunits involved may depend on the cellular context. The calcium dependent protease capalins may have an anti-apoptotic role by degrading PP2A-B' subunits, allowing Akt mediated apoptosis. Inhibition of Rac1 in adhesion complexes is further described in Figure 1.19.
suggesting a possible mechanism by which cells may avoid apoptosis. Indeed capalins have been attributed an anti-apoptotic role in response to some death inducing stimuli by activation of the Akt pathway (Tan et al. 2006). PP2A also inhibits Rac1 downstream of PI3K to regulate cellular adhesion and migration, which is reviewed with PP2A regulation of other cellular adhesion complexes in Section 1.6.8.

An intriguing recent paper suggests that Akt can be switched from an anti-apoptotic protein in the absence of growth factors, to a pro-apoptotic protein in the presence of growth factors, and this switch is controlled by PP2A (Figure 1.15) (Andrabi et al. 2007). When NIH-3T3 fibroblasts are cultured in serum, Akt phosphorylation is balanced between phosphorylation at Thr\(^{308}\) and Ser\(^{473}\), signalling cellular survival. When the SV40 ST antigen is expressed in these cells, phosphorylation of the PP2A target Thr\(^{308}\) increases, causing an unbalanced phosphorylation state and cell death by apoptosis. In contrast, when these cells are serum starved, Akt phosphorylation levels at both sites are very low, again promoting apoptotic cell death. However, when ST is expressed in serum starved cells, both sites are hyper-phosphorylated, thus promoting cell survival. This indicates that PP2A must target both phosphorylation sites in the absence of growth factors (Liao et al. 2004a). These results reinforce not only the importance of understanding cellular signalling in a context dependent manner, but also the crucial role PP2A has in regulating normal cellular function.

A number of agents have recently been shown to prevent cancerous characteristics such as cellular proliferation and migration, or induce apoptosis in cancer cell lines by increasing PP2A activity towards de-phosphorylation of Akt. Treatment of leukaemia cells with the immunosuppressive agent FTY720 caused a time and concentration dependent reduction in Akt activity that induced caspase-3 mediated apoptosis (Matsuoka et al. 2003). The mechanism of FTY720 inhibition of Akt was found to be enhanced PP2A activity rather than
In the work by Andrabi et al. 2007 inhibition of PP2A by the SV40 small T antigen (ST) had different effects on Akt signalling depending on the cellular context. In the presence of serum (left), ST inhibited de-phosphorylation of Thr\textsuperscript{308} only, leading to cellular growth, but not apoptosis. In contrast, when cells were serum starved (right), ST inhibited PP2A mediated de-phosphorylation of both Thr\textsuperscript{308} and Ser\textsuperscript{473}, causing hyper-phosphorylation of both residues and subsequently induction of both cellular growth and survival.
upstream inhibition of PI3K. Similarly, treatment of the breast cancer cell line MDA-MB-231 with the adipose tissue cytokine, adiponectin, reduced Akt phosphorylation and subsequently cellular proliferation, invasion and migration (Kim et al. 2009). Examining the mechanism of adiponectin action found that it activates PP2A by reducing P-Tyr307, again demonstrating this post translational modification can have a role in inhibiting PP2A in cells. Further, knockdown of PP2A-B’γ or -B’δ, but not PP2A-B’α or -B’β rescued the de-phosphorylation of Akt, suggesting these particular subunits are involved (Kim et al. 2009). The dithiolethione compound ACS-1, known to have chemopreventative effects, was found to inhibit proliferation of both a lung and breast cancer cell line through increased PP2A activity and reduced Akt phosphorylation (Switzer et al. 2009). This compound demonstrated reduced Akt phosphorylation induced by either growth factor stimulation or integrin signalling pathways, demonstrating that PP2A activation may target multiple pathways disrupted in cancer. The adenoviral type 5 E1A protein sensitises breast cancer cells to apoptosis by chemotherapies, and the mechanism of E1A induced sensitivity was found to involve repression of Akt activity, which correlated with increased PP2A activity (Liao et al. 2004b). All of these studies show the importance of PP2A in Akt mediated survival of cancer cells. What remains to be seen is whether these promising pre-clinical studies will translate into novel PP2A activating therapeutics for haematological cancers and also solid tumours, including breast cancer.

1.6.5. p53
One of the substrates that Akt phosphorylates is Mdm-2, a ubiquitin ligase that targets p53 for proteasome mediated destruction (Ogawara et al. 2002). As p53 is a transcription factor that normally induces apoptosis in response to cellular stress and DNA damage, Akt enhances cellular survival by activating Mdm-2 and thus inhibiting p53 mediated apoptosis. Apart from direct de-phosphorylation of Akt, PP2A can also de-phosphorylate Mdm-2 (Okamoto et al. 2002), thus enhancing cellular apoptosis (Figure 1.16). The mechanism of
Figure 1.16 PP2A in p53 signalling.
PP2A regulates p53 signalling at multiple levels. PP2A directly de-phosphorylates both Akt and Mdm-2, upstream inhibitors of p53, thus PP2A positively regulates apoptosis upstream of p53. The PyMT oncogenic viral antigen activates the ARF/p53 pathway that protects cells from oncogenic viruses. For cellular transformation co-transcription of PyST is required, which inhibits downstream p53 activity via PP2A inhibition. At the level of p53, PP2A appears to have opposing functions. De-phosphorylation of Thr^{55} in response to DNA damage activates p53 and results in transcription of p21. In contrast, de-phosphorylation of Ser^{37} is predicted to destabilise p53, although the functional effects of PP2A at Ser^{37} have not been determined. In addition PP2A de-phosphorylation of p300, a co-activator of p53 transcription, led to its degradation and consequently prevented p53 transcriptional activities. PP2A also regulates proteins downstream of p53. PP2A de-phosphorylation of the anti-apoptotic protein Bcl2 both inactivates the protein and also mediates binding of Bcl2 to p53, a further mechanism of Bcl2 inhibition.
this interaction is via another protein, cyclin G, which binds to both PP2A-B’γ (Okamoto et al. 1996) and also Mdm-2 (Okamoto et al. 2002) (Note in these papers the regulatory B subunit is termed PP2A-B’α, however sequence analysis confirms that this is actually what is now recognised as PP2A-B’γ).

The ARF protein initiates a cellular protective mechanism in response to threats such as oncogenes and DNA tumour viruses by inhibiting Mdm-2, and consequently activating p53. The DNA tumour virus polyoma middle T (PyMT) antigen induces expression of the protective ARF protein, and so in order for cells to be transformed by this DNA tumour virus, co-expression of polyoma small T antigen (PyST) is required as it ultimately inhibits p53 downstream of ARF. The mechanism of PyST inhibition of p53 is via PP2A as PyST directly binds to PP2A-A and inhibits PP2A activity (Campbell et al. 1995). This mechanism of p53 inhibition by PyST was proven PP2A dependent as PyST unable to bind PP2A was unable to cause cellular transformation (Moule et al. 2004).

Further, PP2A also directly binds to p53 in response to DNA damage (Dohoney et al. 2004). Interestingly, while both PP2A-Bα and -B’γ, but not -B’α, bind to p53, only over-expression of the PP2A-B’γ regulatory subunit resulted in de-phosphorylation of p53 at Thr55 (Li et al. 2007). PP2A-B’γ de-phosphorylation of p53 at Thr55 is required for p53 mediated induction of p21 and subsequent apoptosis (Li et al. 2007). Indeed, DNA damage induces interaction of the PP2A-B’γ holoenzyme with p53 (Shouse et al. 2008). The DNA tumour viral SV40 small T antigen (ST) was able to prevent p53 de-phosphorylation in response to DNA damage (Figure 1.16), presumably by competitively displacing PP2A-B’γ (Li et al. 2007). This is also a potential mechanism for the PyST antigen, described above. Recently, the region of PP2A-B’γ involved in p53 binding was identified (Shouse et al. 2010). Searching of public databases revealed a single mutation in this region in a lung cancer sample, and subsequent biochemical analysis revealed that this F395C mutation is indeed deficient in p53 binding leading to enhanced p53 phosphorylation and decreased induction.
of p21. Interestingly, the F395C mutant could still bind other PP2A substrates ERK, Cyclin G and Shugosin. While ectopic expression of wild-type PP2A-B’γ in colon carcinoma cells drastically reduces colony formation and proliferation, the F395C mutant only had limited ability to reduce these cancerous features. Interestingly, over-expression of PP2A-B’γ in p53 null cells still showed some inhibition of colony formation and proliferation, and a similar effect was seen with the F395C mutant, suggesting an additional p53 independent mechanism of tumour suppression by PP2A-B’γ. Thus, inhibition of both p53 and PP2A-B’γ may act synergistically in tumour formation and progression (Shouse et al. 2010).

PP2A can also regulate proteins downstream of p53. Bcl2 is an anti-apoptotic protein that prevents cell death induced by various stress mechanisms, including chemotherapies. Binding of p53 to Bcl2 prevents its anti-apoptotic functions and enhances cellular death in response to stressors. PP2A de-phosphorylation of Bcl2 has a dual mechanism of inhibition, as Bcl2 is activated by phosphorylation, and phosphorylation also aids p53 binding to Bcl2 (Deng et al. 2009).

PP2A may also negatively regulate p53, as PP2A de-phosphorylates Ser³⁷ in vitro, a site that is predicted to stabilise the protein. However, the regulatory B subunits involved and the functional effects of this in vitro reaction were not investigated (Dohoney et al. 2004). Considering it has been shown that both PP2A-Bα and also -B’γ bind to p53, but only PP2A-B’γ de-phosphorylates Thr⁵⁵ (Li et al. 2007), it is possible that the Ser³⁷ residue is targeted by the PP2A-Bα subunit, or indeed other regulatory B subunits. In addition PP2A-B’γ binds directly to the transcriptional co-activator p300, and over-expression of this regulatory subunit leads to proteasome mediated p300 degradation, consequently repressing p300 dependent p53 transcriptional activation (Chen et al. 2005a).
1.6.6. c-Myc

c-Myc is a transcription factor that regulates multiple genes involved in cellular proliferation, differentiation and apoptosis. The c-Myc protein is regulated by both the ERK and Akt pathways downstream of Ras (Figure 1.17). ERK phosphorylation of c-Myc at Ser$^{62}$ stabilises the protein (Sears et al. 1999), whereas phosphorylation at Thr$^{58}$ by glycogen synthase kinase-3β (GSK-3β) marks c-Myc for ubiquination and degradation (Sears et al. 2000). Akt indirectly stabilises c-Myc by inhibiting GSK-3β. c-Myc must be tightly regulated for normal cellular function as excessive accumulation of c-Myc in cells blocks differentiation and induces neoplastic transformation (Dang et al. 1999).

Inhibition of PP2A by multiple mechanisms results in accumulation of c-Myc (Yeh et al. 2004). Further investigation reveals that PP2A, with the aid of prolyl isomerase Pin1, de-phosphorylates c-Myc only at Ser$^{62}$, thus marking it for degradation (Yeh et al. 2004). Out of all the PP2A-B’ family members and Bα, PP2A-B’α was the only PP2A holoenzyme found to de-phosphorylate c-Myc (Arnold et al. 2006), and inhibition of PP2A-B’α results in increased accumulation of c-Myc. In addition, the PP2A binding protein, Cancerous Inhibitor of PP2A (CIP2A), inhibits PP2A mediated de-phosphorylation of c-Myc (Junttila et al. 2007). The mechanism of PP2A inhibition by CIP2A is yet to be determined, but as CIP2A was found to bind directly to both c-Myc and PP2A, a direct structural inhibition was hypothesised (Junttila et al. 2007).

Importantly, c-Myc is over-expressed in melanoma cell lines compared to normal melanocytes, which correlates with reduced PP2A-B’α expression and increased CIP2A protein expression (Mannava et al. 2011). Further, reduced expression of PP2A-B’α in melanomas correlates with increased metastasis. The CIP2A protein is also over-expressed in breast tumours and depletion of CIP2A in breast cancer cell lines reduced c-Myc levels and also cellular proliferation (Come et al. 2009). A recent investigation found that c-Myc is stabilised in both breast cancer cell lines and also breast tumours, compared with normal controls, due to increased phosphorylation at Ser$^{62}$ compared with Thr$^{58}$.
In addition to upstream regulation of c-Myc by inhibiting both Akt and ERK, PP2A also directly de-phosphorylates c-Myc. PP2A is part of a c-Myc degradation complex that assembles on the scaffolding protein Axin1 and only de-phosphorylates Ser62, marking c-Myc for proteasome mediated degradation. The PP2A-B’α subunit determines the substrate specificity for PP2A mediated de-phosphorylation of c-Myc and the prolyl isomerase Pin1 is also required for PP2A to de-phosphorylate c-Myc. CIP2A inhibits PP2A mediated de-phosphorylation of c-Myc, thus contributing to cancer progression.
(Mannava et al. 2011). A role for the scaffolding protein Axin1 was implicated in regulation of Ser$^{62}$ phosphorylation as this protein is the scaffold for binding PP2A, GSK-3β and also other proteins involved in the degradation of c-Myc. Interestingly, in addition to overall reduction of Axin1 expression, increased expression of a splice variant of Axin1 which does not contain the PP2A binding domain was found to be involved in the stabilisation of c-Myc (Mannava et al. 2011), again suggesting the importance of PP2A in regulating c-Myc expression.

1.6.7. Wnt

Wnt signalling is another pathway that is both positively and negatively regulated by PP2A depending on the regulatory B subunits involved (Figure 1.18). While Wnt signalling has a crucial role in embryogenesis, up regulation of this pathway can also lead to enhanced cellular proliferation and tumourigenesis (Eichhorn et al. 2008). This pathway is commonly mutated in cancers, with β-catenin activation being the central driver of tumourigenesis. In the absence of Wnt ligand, β-catenin is phosphorylated by GSK-3β in a protein complex that also contains the adenomatous polyposis coli (APC) protein and Axin. Phosphorylation of β-catenin marks it for ubiquitination and destruction by the proteasome. Over-expression of PP2A-B’ family members reduces the expression of β-catenin, likely through direct binding of the PP2A-B’ subunit to APC, although the precise mechanism of regulation is still to be determined (Seeling et al. 1999). Another group has also found that PP2A-Bα is directly complexed with β-catenin and axin in cells, and that expression of this subunit directly alters β-catenin expression. That is, suppression of PP2A-Bα increases β-catenin phosphorylation and hence degradation, whereas over-expression of PP2A-Bα suppresses β-catenin phosphorylation and increases protein expression (Zhang et al. 2009).

When the Wnt ligand is present, the β-catenin destruction complex, consisting of GSK-3β, Axin, APC and β-catenin is uncoupled by activation of dishevelled (Dsh), which results in stabilisation of β-catenin protein expression and consequently gene transcription (Eichhorn et al. 2008). Naked cuticle (Naked)
Figure 1.18 PP2A regulation of the Wnt signalling pathway.

In the absence of Wnt ligand (left) GSK-3β phosphorylates β-catenin in a protein complex containing the adenomatous polyposis coli protein (APC) and Axin, which marks β-catenin for ubiquitination and destruction by the proteasome. PP2A negatively regulates β-catenin by enhancing phosphorylation, either directly thorough the PP2A-Bα subunit, or indirectly through PP2A-B’ subunit targeting of APC. In the presence of Wnt ligand (right) the Frizzled receptor activates dishevelled (Dsh), which uncouples the β-catenin destruction complex, stabilising β-catenin protein expression and allowing gene transcription to occur. PP2A regulates Dsh both by an unknown mechanism involving PP2A-B’e, and also through the Naked cuticle protein (Naked). In the absence of Wnt, the PR72 PP2A-B” subunit activates Naked, whereas in Wnt signalling, PR130 inhibits Naked, thus allowing activation of Dsh.
normally inhibits Dsh when no Wnt ligand is present, thus maintaining the β-catenin degradation complex. By an unknown mechanism, PP2A-B’’ subunit PR72 is required for the action of Naked (Creyghton et al. 2005). However, when Wnt ligand is present, PP2A-B’’ subunit PR130 inhibits Naked, allowing Dsh to uncouple the β-catenin degradation complex (Creyghton et al. 2006) and thus PP2A-B’’ PR130 acts as a positive regulator of Wnt signalling. In addition, PP2A-B’e is a positive regulator of Wnt signalling by regulating an unknown target upstream of Dsh (Eichhorn et al. 2008). Despite regulation at multiple levels, global inhibition of PP2A by the pharmacological inhibitor, okadaic acid, or suppression of PP2A-C expression, leads to stabilisation of β-catenin expression, suggesting that PP2A acts as an overall negative regulator of Wnt signalling (Seeling et al. 1999; Zhang et al. 2009).

1.6.8. Cellular adhesion and migration

Integrin containing focal adhesion complexes not only anchor cells to extracellular matrix proteins, but are also key components of cellular signalling, regulating proliferation, growth and migration (Figure 1.19) (Miranti et al. 2002; Wozniak et al. 2004; Sontag et al. 2006). It is proposed that integrins ‘sense’ the extracellular environment and regulate cellular processes according to what is appropriate for the cellular context (Miranti et al. 2002). Over 50 signalling proteins have been implicated in the focal adhesion protein complexes, including PP2A (Mulrooney et al. 2000; Zamir et al. 2001; Suzuki et al. 2005; Takahashi et al. 2006b). Cellular adhesion and polarity are required for the maintenance of tissue architecture, and changes to cellular adhesion structures is required for tumour invasion and metastasis (Hirohashi et al. 2003). Disruption of these protein complexes by PP2A inhibition has been linked to loss of cellular polarity, increased cellular motility and invasiveness (Sontag et al. 2006).

Integrin binding to extracellular matrix molecules induces recruitment and tyrosine phosphorylation of focal adhesion kinase (FAK), which in turn phosphorylates key proteins that regulate both the cytoskeleton and also signalling pathways (Wozniak et al. 2004). With regards to the cytoskeleton,
Figure 1.19 PP2A positively regulates focal adhesion formation and negatively regulates cellular migration.

In response to integrin binding to extracellular matrix molecules, focal adhesion kinase (FAK) is auto-phosphorylated, which in turn activates paxillin by tyrosine phosphorylation and the Rho family GTPases: Rho, Cdc42 and Rac1. PP2A recruits Rho family GTPase stabilising protein, IQGAP1, to the protein complex. These proteins promote F-actin polymerisation and cross-linking, thereby stabilising focal adhesions.

During cellular migration, focal adhesions are disassembled and similar protein complexes form focal contacts at the leading edge of the cell, mediated by Rac1. PP2A inhibits Rac1 mediated cellular migration, and thus for cellular migration to occur Rac1 recruits SET to inhibit PP2A at the migrating edge of the cell. In addition, PP2A de-phosphorylation of paxillin at serine residues is required to maintain focal adhesion complexes. Inhibition of PP2A causes increased serine phosphorylation of paxillin which is associated with increased cellular migration. FAK activation also regulates a number of cellular signalling pathways, including Ras/ERK and PI3K/AKT. Dashed lines indicate other proteins are present within these pathways. Note not all of the signalling proteins associated with focal adhesions are shown in this figure.
FAK activates a number of Rho family small GTPases: Rho, Rac1 and Cdc42, which in turn are required for cross linking of the F-actin cytoskeleton (Wozniak et al. 2004; Suzuki et al. 2005). In addition, the cytoskeletal adaptor protein paxillin is also directly phosphorylated by FAK, and is involved in cross-linking the actin filaments that are required for the structural integrity of the focal adhesion complex (Miranti et al. 2002; Wozniak et al. 2004). Focal adhesions also regulate cellular signalling pathways as the Ras/ERK pathway and the PI3K/AKT pathways can be activated by phosphorylated FAK. Indeed, ERK cannot be activated in suspended fibroblasts that lack extracellular contacts (Renshaw et al. 1997).

PP2A appears to have three main roles in maintaining focal adhesions. Firstly, PP2A is located in β1 integrin protein complexes and inhibition of PP2A with okadaic acid (OA) results in increased phosphorylation of β1 integrin. In addition, OA treatment corresponded with a loss of β1 integrin expression from focal adhesion sites, suggesting PP2A may aid targeting of β1 to focal adhesions (Mulrooney et al. 2000). Secondly, PP2A recruits IQGAP1 (Suzuki et al. 2005), a scaffolding protein that enhances actin polymerisation and aids the Rho GTPases in cross linking the actin filaments (Briggs et al. 2003). Thirdly, PP2A de-phosphorylates paxillin which is critical for regulating focal adhesion assembly and disassembly (Young et al. 2000; Jackson et al. 2002; Young et al. 2003).

When human mammary epithelial cells (HMECs) are growth arrested in the G1 phase of the cell cycle, the cells demonstrate adhesion to the cellular matrix, and β1 integrin is associated with PP2A subunits as well as actin (Suzuki et al. 2003b). However, when HMEC cells are stimulated to divide with EGF or are growth arrested during cell division in the G2/M phase of the cell cycle, cellular adhesion is reduced in order to allow cell division. Calcium/calmodulin-dependent protein kinase II (CAMKII) phosphorylates β1 integrin, resulting in dissociation of β1 integrin and Rac1 from PP2A, IQGAP1 and actin, thus breaking down the entire focal adhesion complex (Suzuki et al. 2003a;
Takahashi et al. 2006a). In addition, inhibition of PP2A by OA results in β1 integrin maintaining binding with Rac1, but dissociation of PP2A, IQGAP1 and actin from β1 integrin immunoprecipitations (Suzuki et al. 2005). As PP2A binds directly to IQGAP1, it has been suggested that PP2A anchors β1 integrin to F-actin via recruitment of IQGAP1 (Suzuki et al. 2005).

Cellular migration is controlled by disassembly and reassembly of focal adhesions. In particular the Rho GTPases, Rho, Rac1 and Cdc42 are responsible for these processes. In order for a cell to migrate, focal adhesions must first be disassembled at the front of the cell in order for the cell to extend projections in the direction of migration (Webb et al. 2002). Smaller focal adhesions, often called focal complexes, are then created in lamellipodia and filopodia at the leading edge of the cell. The generation of these smaller focal complexes are regulated by Rac1 and Cdc42. The cytoskeletal proteins such as actin then contract to pull the bulk of the cell in the direction of migration, which may be aided by formation of larger focal adhesions that are regulated by Rho. Cells then disassemble focal adhesions at the rear of the migrating cells resulting in retraction of the cell tail (Webb et al. 2002; Wozniak et al. 2004). PP2A negatively regulates Rac1 and Cdc42 mediated cellular migration, as inhibition of PP2A by SV40 small T increased Rac1 and Cdc42 expression and also membrane ruffling and lamellipodia formation (Nunbhakdi-Craig et al. 2003). Indeed, inhibition of PP2A by SET is a mechanism for permitting cellular migration to occur. SET translocates from the nucleus to the migrating edge of cells, and co-localises with Rac1. PP2A is also associated with Rac1 and SET in these protein complexes. Importantly, knock down of Rac1 prevented SET translocation in migrating cells, indicating Rac1 specifically recruits SET to inhibit PP2A at the leading edge of migrating cells (ten Klooster et al. 2007).

Investigation into the differences between a highly motile, metastatic Lewis lung carcinoma cell line and its non-metastatic variant, revealed that the metastatic cells had much lower PP2A activity (Young et al. 2000; Young et al. 2003). Treatment of the non-metastatic variant with OA increased phosphorylation of
paxillin, an adaptor molecule in focal adhesions, resulting in reduced cellular adhesion and increased cellular migration (Jackson et al. 2002). Analysis of paxillin immunoprecipitates from the metastatic cells revealed reduced PP2A associated with paxillin and also FAK compared to the non-metastatic cells (Young et al. 2003). A truncated form of the regulatory subunit PP2A-B’γ was discovered in the metastatic melanoma cells, and when this truncated subunit was transfected into the non-metastatic counterpart, increased cellular motility to the same levels as the metastatic cells was observed, due to inability of PP2A to de-phosphorylate paxillin (Ito et al. 2000).

Involvement of PP2A in these complexes suggests PP2A may not only have a role in tumourigenesis, but also in later stage migration and metastasis, and further experimentation is required to determine if PP2A has a role in metastatic breast cancer.

1.6.9. Cell cycle

PP2A is involved in both positive and negative regulation of the cell cycle. In normal cellular division, PP2A-B’γ translocates to the nucleus and de-phosphorylates the cyclin dependent kinase inhibitor p27, which stabilises p27 expression and regulates when cells enter S phase. Over-expression of PP2A-B’γ delayed cell cycle progression into S phase, whereas knockdown of PP2A-B’γ induced cellular proliferation (Lee et al. 2010). In contrast, de-phosphorylation of the dual specificity phosphatase Cdc25 by PP2A trimers containing PP2A-B’δ is required for the activation of Cdc25, which in turn activates Cyclin dependent kinase CDK1 (Margolis et al. 2006; Forester et al. 2007). As cyclin dependent kinases drive cell cycle progression, PP2A acts as a positive regulator of the cell cycle at the level of Cdc25. With regards to negative regulation, inhibition of PP2A by ST expression induces expression of Cyclin D1, required for progression through the G1 cell cycle checkpoint (Watanabe et al. 1996). More recent evidence suggests that PP2A is also crucial for allowing cells to exit mitosis, thus preventing continued cellular division (Manchado et al. 2010; Wurzenberger et al. 2011). CDK1 activation and
phosphorylation of target proteins drives cell cycle progression, while PP2A causes cells to exit mitosis by both deactivating CDK1 and also dephosphorylating its target proteins. Interestingly, PP2A-Bδ is the key regulatory subunit for inactivating CDK1 in Xenopus egg extracts (Mochida et al. 2009), while PP2A-Bα appears to be the key regulatory subunit in human cells (Schmitz et al. 2010). Overexpression of both PP2A-Bα and -Bδ can prevent mitotic exit in mouse fibroblasts (Manchado et al. 2010), thus demonstrating species specific regulation of PP2A by regulatory B subunits. The downstream targets of CDK1 known to be de-phosphorylated by PP2A include the pocket binding protein family members Rb, p107 and p130 (Kurimchak et al. 2012). In addition to mitotic exit, PP2A is also required for correct nuclear envelope reassembly, golgi apparatus reassembly, mitotic spindle formation and chromosome decondensation (Lowe et al. 2000; De Wulf et al. 2009; Schmitz et al. 2010; Torres et al. 2010; Wurzenberger et al. 2011). The mechanism of PP2A regulation of these processes remains under investigation, however PP2A-Bα also seems to be required for golgi apparatus reassembly (Schmitz et al. 2010). As PP2A is required for mitotic exit, protein kinase greatwall recruits two small binding proteins, endonuclease α and adenosine monophosphate-regulated protein 19 (ARPP19) to inhibit PP2A-Bα, thus allowing cell cycle progression (Gharbi-Ayachi et al. 2010; Mochida et al. 2010). As uncontrolled cell proliferation is a hallmark of cancer, dysregulation of PP2A in these mitotic events may play a role in tumourigenesis.

1.6.10. PP2A as hub of cellular signalling

As described throughout Section 1.6, PP2A regulates a number of signalling pathways involved in cellular proliferation, survival and migration, all of which have been implicated in the development and progression of cancer, including breast cancer. Indeed a number of lines of evidence suggest PP2A is a tumour suppressor and plays a key role in tumourigenesis.

As described in Section 1.2.5, multiple levels of cross talk exist between cellular signalling pathways involved in the progression of breast cancer. As PP2A is
involved in regulating all of these signalling pathways, alterations to PP2A function is likely to impact multiple signalling pathways and cellular responses (Figure 1.20). In addition, inhibition of PP2A in cancer could affect multiple signalling pathways. Indeed, it has recently been shown that the transforming effect of SV40 ST antigen in immortalised HEK-TER cells results from activation of the Akt, c-Myc and β-catenin pathways (Sablina et al. 2010). This indicates that PP2A may have an important role in cancer signalling, and that novel therapies targeting PP2A may simultaneously affect multiple signalling pathways.
Figure 1.20 PP2A regulates multiple signalling pathways involved in the progression of breast cancer.

This figure demonstrates that PP2A plays a central role in regulating numerous pathways involved in the progression of breast cancer. Note that many other protein interactions have not been included for simplicity, only key molecules in each pathway are depicted. Detailed explanation for PP2A regulation of ERK, Akt, p53, c-Myc, and cellular adhesion complexes is provided in Figures 1.14-1.20.
1.7. **Protein phosphatase 2A in cancer**

Numerous lines of evidence support the hypothesis that PP2A is a tumour suppressor. Initially, pharmacological inhibitors, DNA tumour viruses and also altered PP2A expression in tumour cell lines, all suggested that disruption to PP2A signalling might be important for tumourigenesis. More recently, investigations in human tumours have confirmed this, although the specific subunits and pathways they regulate are not fully elucidated.

1.7.1. **Pharmacological inhibition**

The initial evidence suggesting PP2A as a tumour suppressor was the discovery that the carcinogen, okadaic acid (OA), inhibited PP2A phosphatase activity (Bialojan et al. 1988). Topical application of OA causes tumours in mouse skin, and when added to drinking water induces glandular stomach cancer in rats (Fujiki et al. 2009). Although OA also inhibits the related phosphatase PP1, PP2A inhibition is about 200 times more potent than PP1 inhibition (Bialojan et al. 1988). Resolution of the crystal structure of PP2A bound to OA revealed a hydrophobic pocket in the catalytic subunit of PP2A, but not PP1, that covalently binds OA (Xing et al. 2006). This strong binding affinity explains the greater inhibition of PP2A by OA than PP1. A number of studies have used OA to induce cellular transformation (Meisinger et al. 1997; Ruvolo et al. 2002; Young et al. 2002; Chen et al. 2004). It has more recently been shown that OA also inhibits other PPP family members PP4 and PP6 to a similar extent as PP2A (Prickett et al. 2006). Thus most PP2A research now focuses more on targeted molecular inhibition of PP2A. Another PP2A inhibitor, Microcystin-LR acts as a liver tumour promoter when injected together with a carcinogen, diethylnitrosamine (DEN), into rats. That is, Microcystin-LR is not sufficient to induce tumour formation, but promotes tumour formation induced by other agents (Fujiki et al. 2009).

1.7.2. **DNA tumour viruses**

Tumour viruses also target PP2A to induce tumourigenicity (Pallas et al. 1990). SV40 infection has been linked to human mesothelioma, lymphoma, brain and
bone cancers, which correspond with tumours arising in mice infected with SV40 (Barbanti-Brodano et al. 2004). SV40 encodes two antigens, large T (LT) and small T (ST). LT disables tumour suppressors p53 and pRb. Co-expression of LT, hTERT (the catalytic subunit of telomerase) and activated Ras immortalises, but does not transform, human embryonic kidney (HEK) cells. Importantly, expression of ST in these immortalised cells (HEK-TER) is sufficient and necessary for transformation, as evidenced by anchorage independent growth and tumour formation in immune-deficient mice (Hahn et al. 2002). The specific inhibition of PP2A for the transforming ability of ST was demonstrated in two different ways. Firstly, expression of ST mutants that are no longer able to bind PP2A cannot transform the HEK-TER cells (Hahn et al. 2002). Secondly, treating these cells with OA increases the rate of cell proliferation and also induces anchorage independent growth, similar to the outcomes observed when transformed by ST (Chen et al. 2004). The HEK-TER cell model has been invaluable for determining which PP2A subunits and cellular signalling pathways are critical for PP2A mediated transformation (see Sections 1.7.5 and 1.7.6) (Chen et al. 2004; Chen et al. 2005b; Sablina et al. 2010). In addition, co-expression of SV40 antigens ST and LT together with hTERT and Ras also transforms human mammary epithelial cells (Elenbaas et al. 2001). However, further investigation into the specific regulatory B subunits or signalling pathways perturbed in these cells has not been performed. Interestingly, the power of ST as a transforming antigen was observed in mice with inducible expression of ST in the lactating mouse mammary gland, achieved using a milk protein promoter. About 10% of these mice developed breast tumours 10-17 months following pregnancy (Goetz et al. 2001). This tumourigenesis by ST expression alone is likely only to occur in mice as murine cells typically require fewer mutations for transformation than human cells (Rangarajan et al. 2004) and was probably aided by signalling pathways involved in remodelling the mammary gland during pregnancy, but nevertheless does demonstrate the transforming ability of PP2A inhibition.
ST displaces regulatory B subunits from the PP2A-Aα isoform, but not PP2A-Aβ (Zhou et al. 2003; Sablina et al. 2007), by binding to HEAT repeats 3-6 (Ruediger et al. 1994; Ruediger et al. 1999; Chen et al. 2004; Cho et al. 2007a). Small T expression reduces PP2A activity towards multiple substrates, including: myosin light chains, myelin basic protein, phosphorylase a, MEK, ERK and also peptide substrates, but does not inhibit PP2A de-phosphorylation of histone H1 (Yang et al. 1991; Sontag 2001; Chen et al. 2004; Chen et al. 2007). This suggests ST does not directly interfere with the catalytic site of PP2A-C, but whether ST inhibits PP2A activity towards multiple substrate due solely to the displacement of the regulatory B subunits is still unknown (Cho et al. 2007a). ST has been used extensively to elucidate the mechanism by which PP2A acts as a tumour suppressor in a number of cell types. Multiple pathways responsible for cellular growth, survival, apoptosis and adhesion are implicated in ST-mediated disruption of PP2A leading to cellular transformation (Sablina et al. 2008). These include: perturbation of the cytoskeleton (Sontag et al. 2006), activation of the PI3K/Akt pathway leading to anchorage independent growth (Yuan et al. 2002; Zhao et al. 2003; Rodriguez-Viciana et al. 2006) and activation of the Ras/ERK pathway (Sontag et al. 1993). The exact role that PP2A has in these pathways is under continued investigation, and is likely to be dependent on cell type.

Similarly, the polyoma middle T (PyMT) and small T (PyST) antigens also induce tumourigenicity by inhibiting PP2A (Cayla et al. 1993; Mumby 1995; Ichaso et al. 2001). PyMT transforms cells by recruiting a multi-protein complex consisting of PyMT, Src kinase, the adaptor protein Shc and also PP2A, which activates the ERK signalling pathway (Ichaso et al. 2001). However, a PyMT mutant unable to bind Src or Shc, but retaining the ability to bind PP2A was still able to activate the ERK pathway (Rodriguez-Viciana et al. 2006), suggesting PP2A is critical for maintaining the integrity of ERK pathway signalling. The PyST antigen has the same N-terminal sequence as PyMT, containing the PP2A binding region. However, unlike PyMT which is membrane associated, PyST is found in the nucleus and cytoplasm. Wild-type PyST can strongly stimulate
ERK, but not Akt, however PP2A binding deficient PyST failed to activate ERK (Rodriguez-Viciana et al. 2006). In addition, expression of PyMT in a transgenic mouse model, under the control of a mammary gland specific promoter, resulted in universal appearance of mammary tumours, with many mice also developing lung metastasis (Guy et al. 1992). The PyMT transgenic mouse model has now been established as a powerful model for human breast cancer progression, as a number of histological features and molecular alterations observed in human tumours are recapitulated in this model (Lin et al. 2003). Morphologically, these mice develop hyperplasia at 4-6 weeks of age, followed by intraepithelial neoplasia, then early and late carcinoma. At the molecular level, reduced expression of the estrogen and progesterone receptors as well as over-expression of ErbB2 and cyclin D1 in PyMT mammary tumours recapitulates human breast cancers associated with poorer prognosis (Lin et al. 2003). The targeting of PP2A by these DNA tumour viruses suggests that inhibition of PP2A activity is an efficient way to abrogate normal cellular signalling and transform cells. Hence, PP2A alterations in cancer may also contribute to cellular transformation.

1.7.3. PP2A inhibition by endogenous binding proteins

The cancerous inhibitor of PP2A (CIP2A) is over-expressed in head and neck squamous cell carcinoma, lung cancer, gastric cancer, serous ovarian cancer and also breast cancer (Juntila et al. 2007; Li et al. 2008; Come et al. 2009; Bockelman et al. 2011; Ma et al. 2011). Suppression of CIP2A protein expression inhibits cancer cell proliferation, anchorage independent growth and also tumour growth (Juntila et al. 2007; Ma et al. 2011). In addition, over-expression of CIP2A in HEK-TER cells substitutes for ST in transformation (Juntila et al. 2007).

SET, another inhibitor of PP2A, is activated by oncogenic tyrosine kinases in leukaemia, leading to progression of the disease (Neviani et al. 2005; Roberts et al. 2010). The BCR/ABL oncogene, a driver of disease in over 90% of chronic myeloid leukaemia patients, induces expression of SET to progress the disease
into the blast crisis phase (Neviani et al. 2005). Similarly, oncogenic mutations in the receptor tyrosine kinase, c-KIT, increase SET expression in acute myeloid leukaemia cells (Roberts et al. 2010).

Over-expression of α4 was found in 74/80 (88%) primary hepatocellular cancers, 21/25 (84%) primary lung cancers and 9/11 (82%) primary breast cancers; all compared to matched normal tissue from the same patients. In addition, over-expression of α4 transforms HEK-TER cells and also hepatocytes (Chen et al. 2011). Interestingly, the mechanism of increased α4 expression was found to be reduced expression of the microRNA miR-34b, which normally represses α4 expression. Reduced miR-34b was observed in 9/10 paired lung tumours.

1.7.4. PP2A activity in cancer patients

Very few studies have examined PP2A activity in human cancer samples. PP2A activity is measured by immunoprecipitation of one of the PP2A subunits, often PP2A-C, followed by in vitro incubation with a phosphorylated substrate. Substrates are commonly phosphorylated peptides, as whole protein substrates are more difficult and expensive to isolate. Thus, PP2A activity assays are not always representative of PP2A activity in vivo, especially considering the large variety of PP2A substrates in cells. In addition, large volumes of tumour tissues are required for sufficient PP2A protein isolation, and fresh samples, or at least fresh frozen samples, are required in order to maintain the integrity of PP2A multi-protein complexes. PP2A activity has been assessed in a number of leukaemia samples, as these tissues are much easier to process. A number of studies have found reduced PP2A activity in acute myeloid leukaemia (Yamamoto et al. 1999; Gallay et al. 2009; Cristobal et al. 2011; Yang et al. 2011). In addition, increased expression of the PP2A protein inhibitor SET, was found to cause reduced PP2A activity in chronic myeloid leukaemia (Neviani et al. 2005).
Increased phosphorylation of PP2A-C at Tyr\textsuperscript{307} has been observed in ErbB2 positive breast cancer cell lines and also breast tumours, however the ratio of P-Tyr\textsuperscript{307} to PP2A-C expression was not increased (Wong \textit{et al.} 2009). ErbB2 activation in cells leads to phosphorylation of Tyr\textsuperscript{307} through the ERK signalling pathway, but whether this leads to inactivation of PP2A activity in breast cancer has not been determined. Only one study to date has directly examined PP2A activity in breast cancer patient samples, and as these were not the main focus of the study, only a very small sample size was examined. In 10 breast cancer samples the overall PP2A activity was significantly lower than in the matched normal tissue (Kim \textit{et al.} 2009). Interestingly, the normal tissues demonstrated a wide range of PP2A activities, whereas the breast cancer samples were all clustered at a low value. As described below, alterations in PP2A holoenzyme composition, either via a reduction in subunit expression or PP2A-A subunit mutations, may be the most important mechanism of PP2A deregulation in breast cancer. Therefore, the relative activity of only a small subset of PP2A holoenzymes towards particular PP2A substrates in specific pathways, rather than global PP2A activity, might be more critical for breast tumourigenesis.

\subsection*{1.7.5. PP2A mutations in human cancers}

The first PP2A mutations to be reported in human cancer were mutations in the PP2A-A\textbeta subunit in lung and colon carcinomas (Wang \textit{et al.} 1998\textbf{b}). Other PP2A-A\textbeta mutations, and also mutations in PP2A-A\textalpha, were subsequently identified in multiple human cancers and are summarised in Table 1.3. The frequency of PP2A mutations has been much lower than initially hypothesised. At most 15\% of lung and colon tumours harbour a PP2A-A\textbeta mutation (Wang \textit{et al.} 1998\textbf{b}; Takagi \textit{et al.} 2000; Tamaki \textit{et al.} 2004). These mutations are not present in the adjacent normal tissue, suggesting that they may have a role in sporadic cancer. In a small number of B-cell chronic lymphocytic leukaemia samples, alternative splicing of PP2A-A\textbeta corresponded with reduced PP2A activity (Kalla \textit{et al.} 2007). In familial breast cancer, one particular PP2A-A\textbeta mutation (G90D) was present at a low frequency of 3\%, but this was still significantly higher than 0.3\%, identified in healthy controls. However this
Table 1.3 PP2A-A subunit mutations identified in human cancer.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Identified</th>
<th>Structural Domain</th>
<th>In vitro binding</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PP2A Aα</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E64G</td>
<td>Breast cancer</td>
<td>HEAT 2</td>
<td>Defective in B' but normal in B, B'', and C subunit binding</td>
</tr>
<tr>
<td>E64D</td>
<td>Lung cancer</td>
<td>HEAT 2</td>
<td>Defective in B' but normal in B, B'', and C subunit binding</td>
</tr>
<tr>
<td>Frame shift aa170</td>
<td>Breast cancer</td>
<td>HEAT 5-13</td>
<td>ND</td>
</tr>
<tr>
<td>Del 171-589</td>
<td>Breast cancer</td>
<td>HEAT 5-13</td>
<td>Binds no regulatory B subunits nor the C subunit</td>
</tr>
<tr>
<td>P179A</td>
<td>Melanoma</td>
<td>HEAT 5</td>
<td>Reduced B subunit binding, defective for B', but normal for B''. C subunit N.D.</td>
</tr>
<tr>
<td>R418W</td>
<td>Melanoma</td>
<td>HEAT 11</td>
<td>Binds no regulatory B subunits nor the C subunit</td>
</tr>
<tr>
<td>D492G</td>
<td>Equivalent to D504G in PP2A-Aβ</td>
<td>HEAT 13</td>
<td>ND. May destabilise protein fold</td>
</tr>
<tr>
<td><strong>PP2A Aβ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G8R</td>
<td>Lung Cancer</td>
<td>Before HEAT 1</td>
<td>Normal B’’ &amp; C binding</td>
</tr>
<tr>
<td>G15A</td>
<td>Colon cancer</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>A51D</td>
<td>Colon Cancer</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>G90D</td>
<td>Breast &amp; lung cancer</td>
<td>HEAT 2</td>
<td>Inhibited B56γ binding</td>
</tr>
<tr>
<td>Del exon 9</td>
<td>Breast cancer</td>
<td></td>
<td>Normal B’’ and C binding</td>
</tr>
<tr>
<td>P65S</td>
<td>Lung cancer</td>
<td>HEAT 2</td>
<td>Reduced B’’ binding</td>
</tr>
<tr>
<td>L101P / V488A</td>
<td>Lung cancer</td>
<td>HEAT 3 &amp; 12</td>
<td>Reduced B’’ &amp; C binding</td>
</tr>
<tr>
<td>Del 230-518</td>
<td>Lung cancer cell line</td>
<td>HEAT 6-12</td>
<td>ND</td>
</tr>
<tr>
<td>K343E</td>
<td>Lung cancer</td>
<td>HEAT 9</td>
<td>Normal B’’ &amp; C binding</td>
</tr>
<tr>
<td>Frame shift 422-601</td>
<td>Colon adenocarcinoma</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>V498E</td>
<td>Colon cancer</td>
<td>HEAT 13</td>
<td>ND</td>
</tr>
<tr>
<td>L499I</td>
<td>Colon cancer</td>
<td>HEAT 13</td>
<td>ND</td>
</tr>
<tr>
<td>V500G</td>
<td>Colon cancer</td>
<td>HEAT 13</td>
<td>ND</td>
</tr>
<tr>
<td>D504G</td>
<td>Lung cancer cell line</td>
<td>HEAT 13</td>
<td>Enhanced B’’ &amp; C binding</td>
</tr>
<tr>
<td>Frame shift 519-601</td>
<td>Lung cancer</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>V536A</td>
<td>Colon Cancer</td>
<td>HEAT 14,</td>
<td>Reduced C binding</td>
</tr>
<tr>
<td>W151R</td>
<td>Colon Cancer</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>V545A</td>
<td>Colon adenocarcinoma</td>
<td>HEAT 14</td>
<td>Reduced B’’ &amp; C binding</td>
</tr>
</tbody>
</table>

1The PP2A-A crystal structure has 15 HEAT repeats. Repeats 1-10 are involved in regulatory B subunit binding, while repeats 11-15 bind the catalytic PP2A-C subunit (Ruediger et al. 1992; Ruediger et al. 1994). Data obtained from references (Wang et al. 1998; Groves et al. 1999; Ruediger et al. 1999; Calin et al. 2000; Takagi et al. 2000; Ruediger et al. 2001b; Ruediger et al. 2001a; Tamaki et al. 2004; Esplin et al. 2006).
mutation was present in both the tumour and genomic DNA indicating either a potential risk factor or a polymorphism, therefore loss of the second allele may be required for enhanced tumourigenesis (Esplin et al. 2006). Interestingly, when genotyping further family members, this mutation also correlated with lung, colon and skin cancer (Esplin et al. 2006). Another study found a higher percentage of G90D mutations in breast cancer, with 7/30 (23%) sporadic breast tumours and 0/13 cell lines harbouring the mutation (Calin et al. 2000).

PP2A-Aβ appears to function as a traditional tumour suppressor, whereby loss of function of both Aβ alleles is required for cellular transformation (Wang et al. 1998b; Sablina et al. 2007). Ectopic expression of PP2A-Aβ mutants fail to transform HEK-TER cells still expressing wild-type PP2A-Aβ, suggesting these mutations cannot act as dominant oncogenes (Sablina et al. 2007). Indeed, in all 4 colorectal tumour samples identified to harbour a PP2A-Aβ mutation, loss of heterozygosity in the other allele was observed (Tamaki et al. 2004). Similarly, in 11 lung and colon tumour samples with mutated PP2A-Aβ, 4 lung cancer and 2 colon cancer samples had alterations in both alleles, while the remaining 5 samples still expressed one wild-type allele (Wang et al. 1998b). Functionally, 9 different PP2A-Aβ mutations failed to complex with and de-phosphorylate the GTPase RalA, a component of Ras and Raf-1 signalling complexes that drive cellular proliferation (Sablina et al. 2007). As RalA is not a substrate for wild-type PP2A-Aα, the mechanism by which these two isoforms induce cellular tumourigenicity are unique.

Mutations in PP2A-Aα have also been identified at much lower frequencies than initially hypothesised. Only 7/58 (12%) ovarian tumours (Jones et al. 2010), 1/24 (4%) primary lung tumours and cell lines and 1/14 (7%) melanoma cell lines harboured a mutation in PP2A-Aα (Calin et al. 2000). In addition, 2/30 (7%) primary breast tumours, but 0/13 breast cell lines had PP2A-Aα mutations (Calin et al. 2000).
The mechanism by which PP2A-Aα contributes to cellular transformation appears quite different to traditional tumour suppressors, including PP2A-Aβ. Complete ablation of PP2A-Aα protein is lethal to cells, inducing apoptosis (Chen et al. 2005b; Ruediger et al. 2011). However, reduction of PP2A-Aα expression to approximately 50% of normal levels induces transformation in HEK-TER cells. Ectopic expression of wild-type PP2A-Aα rescues this effect, while expression of PP2A-Aα mutants does not. However, ectopic expression of mutant PP2A-Aα in cells with sufficient wild-type PP2A-Aα produces no effect (Chen et al. 2005b). This suggests that PP2A-Aα functions as a tumour suppressor by haploinsufficiency, whereby reduction in wild-type PP2A-Aα expression, either by mutation of one allele (thus reducing the functional PP2A-Aα protein levels to half), or by an alternate mechanism, induces cellular transformation (Chen et al. 2005b).

The most common PP2A-A mutations are nucleotide substitutions of amino acids critical for regulatory subunit binding (Ruediger et al. 2001b; Ruediger et al. 2001a; Xu et al. 2006; Cho et al. 2007b) (Table 1.3). This suggests that disruption to the PP2A holoenzyme may have an important role in cellular transformation. Mutations in HEAT repeats 3-6 prevent regulatory B subunits from binding, whereas mutations in HEAT repeats 11-15 prevent both the catalytic PP2A-C and also regulatory B subunit binding (Ruediger et al. 2001b). Recently, much more information about the E64 mutation site has been discovered by introduction of PP2A-Aα E64D and E64G mutations into FVB mice (Ruediger et al. 2011). All mice with homozygous expression of either mutation grew normally, demonstrating these mutations do not affect cell viability, in contrast to homozygous deletion of PP2A-Aα which failed to produce any pups. Consistent with previous reports that mutations in PP2A-Aα prevent regulatory B subunit binding (Ruediger et al. 2001b) and that orphaned regulatory B subunits are degraded in cells (Strack et al. 2004), these mice demonstrated reduced expression of PP2A-B’α and -B’δ in the lung and PP2A-B’δ in brain. Other PP2A-B’ subunits were not examined, but are presumed to be degraded as well, as all PP2A-B’ subunit families share a
common PP2A-Aα binding domain. PP2A-Bα subunit expression and association with PP2A-Aα was not affected by these mutants, demonstrating these E64 mutations specifically inhibit PP2A-B’ subunit binding (Ruediger et al. 2011). Importantly, FVB mice are predisposed to developing lung cancer, and 10 months after injection with benzo[a]pyrene to enhance tumour formation, 48% of control mice had developed lung tumours. Expression of the E64D mutation increased the incidence of lung cancer to 72% of injected mice, suggesting that this PP2A-Aα mutation increases the risk of developing cancer (Ruediger et al. 2011).

Thus while mutation of PP2A-A in breast cancers occurs at quite a low frequency, the functional outcome of these mutations is altered holoenzyme composition through reduced regulatory B subunit binding. It may therefore be appropriate to consider both PP2A-Aα mutations and also reduced expression of PP2A subunits together as contributing to breast tumourigenesis.

1.7.6. Reduced expression of PP2A subunits

Even in the absence of mutations, PP2A expression is reduced in some human cancers (Colella et al. 2001; Suzuki et al. 2006; Mannava et al. 2011) and cancer derived cell lines (Suzuki et al. 2003b; Zhou et al. 2003). The PP2A-Aβ subunit is the most extensively studied subunit to date, although only in cancer cell lines. One study examined a panel of lung, colon, glioblastoma and breast cancer cell lines, but only made visual comparisons to normal lung and breast epithelial cells. Approximately 50% of these cancerous cell lines showed reduced protein expression of PP2A-Aβ, with 3/4 breast cancer cell lines clearly having reduced expression compared to normal human mammary epithelial cells (Zhou et al. 2003).

PP2A-Aα subunit expression was strongly reduced in about 40% of glioblastomas and oligodendrogliomas compared with normal brain tissue (Colella et al. 2001). Interestingly, PP2A-Bα and -C subunit expression remained constant across all samples. It has previously been shown that reduced
expression of the structural subunit results in proteasomal degradation of other
PP2A subunits (Strack et al. 2004). These brain tumours were also analysed for
mutations, however only silent mutations in PP2A-Aα were found in these
samples. In addition, a loss of heterozygosity did not explain the reduced protein
expression (Colella et al. 2001). One group has examined PP2A-Aα expression
in breast cancer, finding reduced expression in one breast cancer cell line,
MCF7, compared with quiescent normal human breast cells (Suzuki et al.
2003b), and importantly, also in breast tumours (Suzuki et al. 2006). In 28
matched pairs of breast tumour tissue and adjacent normal tissue, 27 (96%) of
the normal tissues expressed PP2A-Aα by immunohistochemistry, whereas only
12 (43%) of the tumour tissues demonstrated positive staining.

The functional effects of PP2A-Aα suppression have been investigated in the
HEK-TER model and also in PP2A-Aα knockout mice. Reduced expression of
PP2A-Aα by shRNA below one third of normal levels in HEK-TER cells
resulted in cell death by apoptosis, demonstrating that PP2A-Aα is critical for
cellular survival (Chen et al. 2005b). Supporting this, PP2A-Aα knockout mice
are embryonic lethal, and inducible suppression of PP2A-Aα to below one third
post-development caused severe disease and death due to liver apoptosis
(Ruediger et al. 2011). However, suppression of PP2A-Aα to approximately
50% of normal levels transforms HEK-TER cells, demonstrated by enhanced
proliferation, anchorage independent growth and tumour formation in mice
(Chen et al. 2005b). In the inducible PP2A-Aα knockout mouse model, reduced
levels of PP2A-Aα to approximately 50% of normal levels increased the
incidence of lung cancer by 50-60% when crossed with a mouse strain that
exhibit a predisposition to developing lung cancer (Ruediger et al. 2011).

As many of the PP2A-A subunit mutations prevent regulatory B subunit
binding, it is plausible that reduced expression of individual PP2A-B subunits
may also have a role in cellular transformation. Recently this has been shown in
melanoma, where 7 cell lines had reduced PP2A-B’α expression compared to
normal melanocytes, and immunohistochemical analysis of melanomas revealed
metastatic melanomas had much lower PP2A-B’α expression compared to primary melanomas (Mannava et al. 2011). The PP2A-B’γ subunit may also be important in cancer, as this subunit was unable to be detected in 10 lung cancer cell lines, while PP2A-Aα, -Bα and -Cα were expressed normally. In addition, retroviral mediated gene transfer of PP2A-B’γ3 into 3 of these cell lines suppressed cell proliferation and reduced anchorage independent growth (Chen et al. 2004).

Regulatory B subunit expression has not been investigated in other human cancers, but functional studies in the HEK-TER cell model have provided information regarding key regulatory B subunits involved in transformation of these cells, that warrant further investigation in cancers, including breast cancer. HEK-TER cells with suppressed PP2A-Aα retained no detectable PP2A-B’γ expression, adding weight to the importance of this particular subunit for the transformation of human cells (Chen et al. 2005b). In addition, suppression of PP2A-B’γ by shRNA transforms HEK-TER cells in a similar manner to suppression of PP2A-Aα or expression of the SV40 small T antigen (Chen et al. 2004; Chen et al. 2005b). Interestingly however, whole genome expression profiling with microarrays identified differences in genes altered by ST expression compared to suppression of PP2A-B’γ in HEK-TER cells (Moreno et al. 2004). This was also evident functionally, as cells with suppressed PP2A-B’γ still required serum for cell cycle progression and anchorage independent growth, whereas ST expressing cells were transformed even in the absence of serum (Moreno et al. 2004). These results suggest ST may transform cells by displacing additional key regulatory B subunits, or may even effect other as yet unidentified signalling molecules. With regards to the function of other regulatory B subunits in transformation, shRNA suppression of PP2A-B’α, and -B’’α (targeting PR72 and PR130) induced anchorage independent growth in HEK-TER cells. In contrast, knockdown of PP2A-Bα, -Bδ, -B’β, -B’δ, -B’e or -B’’β (PR48) had no transforming effect in HEK-TER cells (Chen et al. 2004; Sablina et al. 2010).
Forced alterations in the PP2A-C catalytic subunit have traditionally been difficult due to an autoregulatory mechanism in cells that maintains expression at reasonably constant levels (Baharians et al. 1998). However, PP2A-C shRNA expression by retroviral methods has recently been successful in suppressing the catalytic subunit by varying amounts (Sablina et al. 2010). Knockdown by 88% resulted in poor cellular proliferation, demonstrating PP2A-C is critical for normal cellular function. In contrast, suppression of PP2A-C by 44%, induced anchorage independent growth and tumour formation in mice to a similar extent as ST expression. Further, knockdown by only 15% induced intermediate effects, approximately half that observed by ST (Sablina et al. 2010).

In conclusion, PP2A-A mutations have been identified in breast cancer, albeit at a low frequency, and reduced expression of PP2A-A may also contribute to breast cancer progression. However, information about the expression levels of the regulatory B subunits in breast cancer is lacking. While a number of elegant studies demonstrating the functional effects of PP2A-A mutations and suppression of individual PP2A subunits have been performed in the HEK-TER model, information regarding the functional effects of PP2A alterations in a breast cancer model are required.
1.8. Models of breast cancer

1.8.1. Cell lines

Cell lines derived from human breast cancers have been used extensively to investigate breast cancer initiation and progression. Many of the original breast cancer cell lines were generated from pleural effusions, as they yielded high numbers of viable cells, and had less contaminating fibroblasts compared with attempts to generate cell lines from solid breast cancers (Cailleau et al. 1978). Comprehensive analysis of 47 of the earliest breast cancer cell lines revealed 6 were actually HeLa cervical carcinoma cells and one was not even of human origin (Engel et al. 1978). However, as cell culture and gene expression techniques have advanced, numerous studies have found similarities between cell lines and human tumours, with valuable information relating to hormone receptor function, gene expression and cell signalling pathway dysfunction provided by these model systems (Lacroix et al. 2004). Disadvantages of cell lines is the lack of heterogeneity observed in human breast cancers and also lack of local microenvironment, provided by the basement membrane and myoepithelial cells in the human breast (Bissell et al. 2002; Lacroix et al. 2004). Despite these disadvantages, comparison of breast cancer cell lines with normal human mammary epithelial cells is still a valuable tool for assessing cellular signalling proteins, such as the numerous subunits of PP2A, to determine if further investigation with patient samples and mouse models is warranted.

1.8.2. MCF10A three-dimensional culture system

Breast cancer exists in a three dimensional tissue architecture and is driven by cues from the surrounding microenvironment, including the basement membrane. Malignancies progress due to disruptions in homeostasis and tissue structure as the result of altered signalling pathways within this context. Indeed, many breast cancer cell lines, including MCF7, MDA-MB-231 and T47D, will demonstrate increased tumourigenicity in mice when co-injected with basement membrane proteins. This demonstrates the importance of microenvironmental cues on tumour metastasis (Elenbaas et al. 2001; Lacroix et al. 2004). The tissue specificity of the susceptibility gene BRCA1 indicates there is some tissue
specific signalling that contributes to breast cancer and hence the tissue context is vital for evaluation of the role mutated genes play in the mammary gland (Bissell et al. 2002). Most studies to date investigating signalling proteins in breast cancer have used two-dimensional (2D) monolayer culture systems, but lack of glandular architecture and microenvironmental cues means these cultures do not represent the in vivo environment (Bissell et al. 2001; Bissell et al. 2002; Debnath et al. 2005; Nelson et al. 2005; Hebner et al. 2008).

Human mammary epithelial cells (HMECs) harvested during reduction mastectomy surgeries for non-cancerous lesions can be grown in vitro when supplied with specific hormones and growth factors (Blatchford et al. 1999). The major draw-back to these cultures is that they reach senescence after 15-20 passages. This is a disadvantage for extended studies requiring longer selection processes, such as exogenous stable expression of oncogenes. Two spontaneously immortalised human mammary epithelial cell lines, MCF10A and HMT-3522-S1, demonstrate many of the same features of primary HMEC cultures including lack of tumourigenicity in nude mice, growth factor/hormone dependent proliferation, lack of anchorage independent growth in soft agar, and dome formation in confluent cultures (Soule et al. 1990; Weaver et al. 1995).

When primary human mammary epithelial cells or the immortalised cell lines are plated on a reconstituted basement membrane (BM) containing appropriate extracellular matrix (ECM) molecules, these cells will form three-dimensional (3D) spheroids, termed ‘acini’ (Figure 1.21). After an initial period of cell division, the cells will arrest proliferation and begin a process of structural differentiation. The loose group of cells secrete endogenous basement membrane proteins in response to contact with exogenous ECM proteins and express cell-ECM receptors on the outer (basal) surface. The golgi apparatus moves to the luminal (apical) side of the nucleus, resulting in a polarised cell layer (Debnath et al. 2005). A hollow lumen is then created via both apoptosis (Debnath et al. 2002; Mills et al. 2004) and autophagy (Mills et al. 2004) of cells not attached to the BM. The ultrastructural similarities of MCF10A 3D acini and normal breast lobular structures makes this a powerful model for
Figure 1.21 Formation of mammary acini in 3D culture.
Schematic (upper panel) and confocal images (bottom panel) of 3D acini formation. This figure is reproduced from Debnath et al. 2005 and Debnath et al. 2002.

1) Proliferating cells secrete endogenous basement membrane proteins eg. laminin 5.
2) Outer cells are polarised as evidenced by golgi apparatus (GM130) translocating to apical side of the nucleus.
3) Pro-survival signalling pathways (P-Akt) are induced in the outer cell layer.
4) Inner cells die by apoptosis as evidenced by active caspase 3 antibody.
5) A hollow lumen is now formed, and outer cells remain polarised.
6) Tranfection of MCF10A cells with oncogenes such as ErbB2 results in an altered acini phenotype that resembles histological features of breast tumours.
investigating alterations in the cancerous genotype within a 3D tissue context (Underwood et al. 2006).

Importantly, introduction of oncogenes into these cultures results in a phenotype similar to the histological changes observed when these oncogenes are present in breast cancer (Debnath et al. 2005). For example, when active ErbB2 is expressed in MCF10A cells, the 3D structures develop misshapen edges and filled lumen because ErbB2 confers both excessive proliferation and anti-apoptotic mechanisms to the cells (Debnath et al. 2002; Muthuswamy 2006). These in vitro features represent many facets of in vivo tumourigenesis. Early breast cancer lesions such as atypical ductal hyperplasia maintain a hollow lumen in the context of excessive cellular proliferation; whereas later stages of breast cancer, such as ductal carcinoma in situ, demonstrate varying levels of luminal filling. Thus, this method of cell culture is powerful to determine key signalling proteins involved in the development, as well as progression, of human breast cancer.

Tumourigenic cell lines such as MCF7 do not form these acini on reconstituted basement membranes, but rather form disorganised masses (Kenny et al. 2003). 3D cultures are able to readily distinguish between normal MCF10A or HMT-3522-S1 cells and tumourigenic cell lines, whereas only very minor differences are observed in 2D cultures (Petersen et al. 1992; Weaver et al. 1997; Kenny et al. 2007). Furthermore, recent gene expression profiling demonstrates not only are different genes expressed when cells are cultured in 2D compared to 3D, but they also respond differently to chemotherapies depending on culture type (Kenny et al. 2007). In fact, functional inhibitors of β1 integrin and EGFR only suppress protein expression in 3D, and not 2D cultures (Weaver et al. 1997; Bissell et al. 2002). It was later found that the β1 integrin and EGFR signalling pathways are able to cross-talk through the ERK signalling pathway, but only in 3D cultures. Cross-talk was not observed in 2D cultures that lack appropriate environmental signalling (Wang et al. 1998a). Furthermore, control of cellular polarity and proliferation by the Raf-1 and Akt
pathways could only be demonstrated in 3D cultures (Liu et al. 2004a). Indeed PI3K inhibitors could reverse the transformed phenotype of HMT-3522-T42 cells in 3D culture (Liu et al. 2004a).

Furthermore, 3D cultures represent an excellent compromise between animal models and traditional 2D cultures (Debnath et al. 2005). Mouse models of breast cancer offer more extensive contextual information, such as connective tissue, angiogenesis and whole organism signalling inputs. However, cell culture systems are much more appropriate for extensive experimental manipulation of signalling pathways and also the detailed biochemical and microscopic analyses required for investigating altered cellular signalling proteins. Expression of oncogenes or inhibition of tumour suppressor genes in 3D culture to recreate the transformation of normal cells into cancerous cells increases our understanding of the tumourigenic process and will enable us to model the reversion of these processes as a potential cancer therapy (Nelson et al. 2005). Thus, 3D cultures provide the appropriate structural and functional context for investigating the mechanisms and signalling pathways involved in organising cells into a tightly regulated architecture.

1.8.3. Animal models
While animal models provide more contextual information about the mammary gland micro-environment, which is critical for breast development and also initiation of cancer, mouse cells are much more readily transformed by PP2A inhibition than human cells (Rangarajan et al. 2004). This is evidenced by inducible SV40 ST expression alone causing mammary tumour formation in mice (Goetz et al. 2001), whereas at least four mutations: oncogenic Ras, hTERT, SV40 LT and ST, are required to transform human mammary epithelial cells (Elenbaas et al. 2001). Despite these limitations, the recently established PP2A-Aα mutation knock-in mouse model and PP2A-Aα wild-type knockout mice, have provided valuable information about PP2A in the whole organism as described in Sections 1.7.5 and 1.7.6 respectively.
1.8.4. Breast tumour samples

Breast tumour samples are an invaluable resource for assessing whether alterations in gene and protein expression that affects cellular signalling pathways in breast cancer cell lines are relevant in breast cancer patients. Commercial availability of breast tumour tissue arrays has made patient tumour tissue more accessible to laboratories focused on cellular signalling and tumour banks are becoming an invaluable resource for assessing large numbers of patient samples, including the rarer forms of breast cancer, as well as for correlating results with patient data stored at the banks. Disadvantages associated with tumour tissue relate mainly to integrity of the tissue, with formaldehyde fixing the main method of tumour preservation. While this method is acceptable for immunohistochemical studies, other techniques such as immunofluorescence to identify co-localisation of protein expression and also enzyme analysis, such as PP2A activity assays, require fresh samples or at least fresh frozen samples. In addition, late stage breast tumour samples may contain hundreds of genetic alterations, especially following chemotherapy (Kenemans et al. 2004) and thus are less informative about the key genes and proteins that initiate tumour development from normal human breast tissue.
1.9. Project objectives

The overarching hypothesis for this thesis was:
Protein phosphatase 2A has a role in breast cancer development and progression, with altered PP2A subunit expression or mutation leading to disruption of cellular signalling pathways that regulate normal human breast cells.

The aims of the project were to:

1) Characterise PP2A subunit expression in breast cancer cell lines and breast tumours compared with normal breast cells.
2) Generate a pannel of MCF10A cell lines with various proteomic alterations in order to investigate the function of PP2A in breast cells. These include: SV40 ST expression, knockdown of PP2A subunit expression with specific shRNA constructs and expression of PP2A-Aα mutants identified in cancers.
3) Determine the functional role of PP2A inhibition/mutation in cellular proliferation, anchorage independent growth and 3D acini cultures of human breast epithelial cells.
MATERIALS AND METHODS

2.1. Chemicals and Reagents

All standard laboratory chemicals and reagents were obtained from Sigma-Aldrich (Sydney, NSW) or Merk Pty Ltd. (Kilsyth, VIC) and were of molecular biology or research grade. All other reagents were obtained as stated throughout these methods.

2.2. Molecular Biology

2.2.1. Retroviral vectors

Retroviral plasmids integrate target DNA into the host cell genome, creating a stable transduction, whereby target DNA is propagated into the next generation of cells. As documented in Chapter 3, a number cell lines were generated by transduction with retroviral plasmids to either knock down the expression of PP2A subunits or to introduce mutant forms of the PP2A-Aα gene. The retroviral vectors used in this thesis are presented in Table 2.1 and vector maps are presented in Appendix 1. The SV40 ST PP2A binding deficient Mutant 3 (Mut3) (Sontag et al. 1993) was subcloned by Mrs Helen Carpenter into pBABE GFP from a pcDNA3 vector provided by Dr Jeff Holst (University of Sydney, NSW, Aus). The Mut3 sequence was amplified with PCR primers containing EcoRI and BamHI restriction sites and then ligated into pBABE GFP digested using the same restriction sites. Knockdown of regulatory B subunits and also the PP2A-Aα subunit was achieved using shRNA constructs as detailed in Table 2.2. A scrambled shRNA sequence in the PLKO.1 vector was obtained from Addgene inc (www.Addgene.org) as a control for shRNA infections and was subcloned by Mrs Helen Carpenter into pBABE GFP using AgeI and EcoRI restriction sites.

2.2.2. PCR reaction for subcloning PP2A-Aα mutants into pBABE puro

Five PP2A-Aα mutants: E64D, E64G, P179A, R418W and D492G were subcloned from pcDNA5/TO vectors into pBABE puro. More detail of these experiments are provided in Chapter 4. The pcDNA5/TO vectors containing PP2A-Aα mutations followed by an EE tag were a kind gift from Assoc. Prof. Stefan Strack (University of Iowa, Iowa, USA (Strack et al. 2004)). PCR
Table 2.1 Retroviral vectors used in this thesis.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Inserted Protein</th>
<th>Vector Name</th>
<th>Supplier</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBABE GFP</td>
<td>SV40 Small T antigen</td>
<td>Addgene plasmid 10673</td>
<td>Addgene Inc. Deposited by William Hahn</td>
<td>(Boehm et al. 2005)</td>
</tr>
<tr>
<td>pBABE GFP</td>
<td>Mutant 3</td>
<td>pBABE GFP Mut 3</td>
<td>Subcloned by Helen Carpenter</td>
<td>(Sontag et al. 1993)</td>
</tr>
<tr>
<td>pBABE GFP</td>
<td>Vector Control</td>
<td>Addgene plasmid 10668</td>
<td>Addgene Inc. Deposited by William Hahn</td>
<td>(Boehm et al. 2005)</td>
</tr>
<tr>
<td>pBABE Puro</td>
<td>Vector Control</td>
<td>Addgene plasmid 1764</td>
<td>Addgene Inc. Deposited by Bob Weinberg</td>
<td>(Boehm et al. 2005)</td>
</tr>
<tr>
<td>pBABE Puro</td>
<td>PP2A-Aα E64D</td>
<td>pBABE Puro PP2A-Aα E64D</td>
<td>Subcloned by Lauren Watt</td>
<td>(Strack et al. 2004)</td>
</tr>
<tr>
<td>pBABE Puro</td>
<td>PP2A-Aα E64G</td>
<td>pBABE Puro PP2A-Aα E64G</td>
<td>Subcloned by Lauren Watt</td>
<td>(Strack et al. 2004)</td>
</tr>
</tbody>
</table>
Table 2.2 shRNA vectors used in this thesis.

<table>
<thead>
<tr>
<th>Vector</th>
<th>PP2A Subunit</th>
<th>Vector Name</th>
<th>shRNA target sequence 5’→3’</th>
<th>Supplier</th>
<th>Reference</th>
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<td>pMKO.1 GFP</td>
<td>PP2A-Bα</td>
<td>Addgene plasmid 10679</td>
<td>AATGGATCTGACAGTGTG</td>
<td>Addgene Inc.</td>
<td>(Chen et al. 2004)</td>
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<td>PP2A-B’α</td>
<td>Addgene plasmid 10680</td>
<td>AACAGAAATAGTGCTTACAACA</td>
<td>Addgene Inc.</td>
<td>(Chen et al. 2004)</td>
</tr>
<tr>
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<td></td>
<td>Deposited by William Hahn</td>
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</tr>
<tr>
<td>pMKO.1 GFP</td>
<td>PP2A-B’γ</td>
<td>Addgene plasmid 10681</td>
<td>AAGATGAACCAACGTAGGAAG</td>
<td>Addgene Inc.</td>
<td>(Chen et al. 2004)</td>
</tr>
<tr>
<td></td>
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<td>Deposited by William Hahn</td>
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<td>pMKO.1 GFP</td>
<td>PP2A-Aα</td>
<td>pMKO.1 GFP Aα shRNA</td>
<td>GGTCAAGAGTCTGTGAA</td>
<td>Aα shRNA inserted into pMKO.1 GFP by Lauren Watt</td>
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<td>pMKO.1 GFP</td>
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<td>Addgene plasmid 1864</td>
<td>AGCGAGGGCGACTAACCTTAGG</td>
<td>Addgene Inc.</td>
<td>(Sarbassov et al. 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>scramble shRNA</td>
<td></td>
<td>Deposited by David Sabatini Subcloned from PLKO.1 to pMKO.1 by Helen Carpenter</td>
<td></td>
</tr>
</tbody>
</table>
primers with HindIII restriction sites were used to amplify the mutant PP2A-Aα sequences prior to subcloning into the retroviral vector pBABE puro (HindIII restriction site is underlined):

Forward: 5’- CGTTTAACCTTAAGCTTTAGGTTACCG-3’
Reverse: 5’- CTATGGAATGAAAGCTTTACGAGG-3’

To amplify the mutant PP2A-Aα sequence 1μg of template DNA together with 1x Pfu ultra reaction buffer (supplied), 1 mM dNTPs, 0.2 mM each forward and reverse primer and 2.5 U of PfuUltra® II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) were combined in nuclease free water to a final volume of 50 μl. PCR reactions were performed in a Mastercycler Pro (Eppendorf South Pacific, North Ryde, NSW, Aus) under the following conditions: a single denaturing step at 95°C for 2 min; followed by 30 cycles of 95°C for 1 min; 57°C for 1 min; 72°C for 3 min; followed by a final extension step of 72°C for 5 min. PCR reactions were purified to remove reaction buffers using a Wizard® SV Gel Clean-Up System (Promega, Madison, WI, USA) as described in Section 2.2.6, except the entire 50 μl reaction was combined with an equal volume of membrane binding buffer and loaded directly onto the SV Minicolumn assembly, rather than from an agarose gel slice. The purified product was eluted with 43 μl of nuclease free water to allow for subsequent restriction digest.

2.2.3. PP2A-Aα shRNA oligo annealing
PP2A-Aα forward and reverse oligos (Sigma Genosys, St Louis, MO, USA) were dissolved in annealing buffer (10 mM Tris pH 7.5-8.0, 50 mM NaCl, 1mM EDTA) to a final concentration of 0.1 mM. Oligos were annealed by adding 10 μl of each oligo to a PCR tube, which was heated to 95°C for 15 min and then allowed to cool to 25°C over a 60 min period, controlled by ramp speed on PCR machine. Annealed oligos were then cooled and stored at 4°C. As the oligos were ordered with overhang ends equivalent to an already digested product, the annealed oligos were ligated directly into pMKO.1 GFP as described in Section 2.2.9. The pMKO.1 GFP vector was digested as described in Section 2.2.4, with AgeI and EcoR1.
2.2.4. Restriction digests
The PCR products from Section 2.2.2 and also the pBABE puro vector were digested with HindIII. pMKO.1 GFP was digested with AgeI and EcoRI. To the 43 μl of purified PCR product in Section 2.2.2, 5μl of Buffer R (supplied) and 2 μl (20U) Hind III (Fermentas, Burlington, ON, Canada) were added for a final reaction volume of 50 μl. For pBABE puro and pMKO.1 GFP vectors 3.5 μg of vector DNA was combined with 5 μl Buffer R and 2 μl (20U) of each required restriction enzymes (Fermentas) with nuclease free water to a final volume of 50 μl. Each reaction was incubated at 37°C for at least 3 h, however the vector reaction was allowed to digest for 6 hours to ensure complete digestion. Digestion reactions were halted by adding 6x sample buffer (Fermentas), and then analysed by electrophoresis on a 1.5% agarose gel (Section 2.2.5).

2.2.5. DNA electrophoresis
DNA electrophoresis was performed on digested PCR reactions in order to remove digested end fragments and to purify digestion reaction products. In addition, PCR products and also the linearised vector were confirmed to be of correct size by DNA electrophoresis. The 1.5% agarose (Sigma) gels were made in 1x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) with 1 μg/ml ethidium bromide. Samples were electrophoresed in a horizontal DNA electrophoresis apparatus (Biorad, Hercules, CA, USA) at 90 V for approximately 30-40 min. DNA gels were imaged using an LAS 4000 (FujiFILM corporation, now available through GE Healthcare, Rydalmere, NSW, Aus). The size of DNA bands was compared to 100 bp and 1 kB markers (Fermentas, Burlington, ON, Canada) for PP2A-Aα PCR products and pBABE puro vector respectively.

2.2.6. DNA purification from agarose gel
Following separation by DNA electrophoresis, mutant PP2A-Aα PCR products and the pBABE puro vector were excised from the gel using a scalpel blade during visualisation on a Mini Transluminator (Biorad, Hercules, CA, USA). DNA was then purified from the agarose using a Wizard® SV Gel Clean-Up System (Promega, Madison, WI, USA). Each gel slice was weighed
in a 1.5 ml microtube and 1 μl membrane binding solution added for each 1 μg of gel. The gel was then dissolved in the membrane binding solution by heating to 65°C on a heat block with occasional vortexing until the gel slice was dissolved. The mixture was then transferred to an SV minicolumn assembly, equilibrated for 1 min at room temperature to facilitate DNA binding, and then centrifuged for 1 min at 16,000xg. The column was washed once with 700 μl of membrane wash solution and centrifuged for 1 min, then washed again with 500 μl of wash solution and centrifuged for 5 min. After each centrifugation the flow through was discarded. The column was then dried by centrifugation for 1 min with no other liquids present. DNA was eluted from the column by addition of 30 μl nuclease free water, equilibration for 1 min at room temperature, centrifugation for 2 min and the elute retained.

2.2.7. Nucleic acid quantification
DNA purified as in Section 2.2.6 and 2.2.10 or RNA as in Section 2.6.1 was quantitated by measuring the optical density (O.D.) using a Biophotometer at an absorbance of 260 nm (O.D$_{260}$) (Eppendorf South Pacific, North Ryde, NSW, Aus). Samples were diluted 1:30 (1 μl in 145 μl) and a concentration obtained in μg/ml from the biophotometer when after the appropriate dilution factor was set on the machine. The biophotometer also gives the absorbance ratio O.D$_{260}$/O.D$_{280}$ to indicate purity. Values ranging from 1.6-1.8 were considered acceptable.

2.2.8. Shrimp Alkaline Phosphatase treatment
As only one restriction site was used to insert the PP2A-Aα PCR products into the pBABE puro vector, the digested and purified vector was treated with Shrimp Alkaline Phosphatase (SAP, Promega, Madison, WI, USA) which removes the 5’ phosphate group. This prevents re-ligation of the vector when ligation of insert and vector is performed. The entire vector purification product obtained in Section 2.2.5 was combined with 1x SAP buffer (provided) and 2U SAP. The reaction was initiated by incubation at 37°C for 15min and then inactivated by heating to 65°C for 15 min. As complete inactivation occurs at this temperature, further purification was not required. Note: SAP is no longer available from Promega, but has been replaced with
thermosensitive alkaline phosphatase, TSAP. An identical protocol is used for TSAP reactions, except that heat inactivation requires 75°C.

2.2.9. Ligation of PCR reaction and vector
The mutant PP2A-Aα digested and purified PCR products were ligated into digested, purified, and SAP treated pBABE puro vector by a LigaFast™ Rapid DNA Ligation System (Promega, Madison, WI, USA). This system uses a unique 2x rapid ligation buffer (supplied) in a final volume of 10 μl. The final reaction for each mutant PP2A-Aα PCR product consisted of 1x reaction buffer (5 μl), 100 ng of vector (1.5 μl at 70 ng/μl), 30 U T4 DNA ligase (1 μl) and maximum volume (2.5 μl) of insert to ensure ligation efficiency, which was approximately 300 ng of the PCR reactions. Reactions were incubated for 5 min at room temperature. To amplify the mutant PP2A-Aα DNA, the ligation product was transformed into competent *Escherichia coli* cells. DNA from clones had to be sequenced to determine the orientation of the inserted PP2A-Aα mutants as only on restriction site was used and thus inserts could have been ligated in either direction.

2.2.10. Bacterial transformation, DNA expansion and purification
For transformation, 5 μl of ligation reaction was added to 50 μl of competent *Escherichia coli* cells (DH5α strain, Invitrogen, Carlsbad, CA, USA) and incubated on ice for 30 min. The cells were heat shocked in a water bath at 42°C for 45 sec to facilitate uptake of DNA and then placed on ice for a further 2 min. The cells were then allowed to recover by addition of 800 μl warm LB media (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0, autoclaved prior to use) and incubation at 37°C with gentle shaking for 1 hour. Following recovery, cells were centrifuged at 830x g, resuspended in 200 μl warm LB and 100 μl was added to 2x LB-AGAR plates with ampicillin (1.5% agar in LB media with 100 μg/ml Ampicillin) before overnight incubation at 37°C so that those bacteria containing the pBABE puro vector would form colonies (Note: pBABE puro contains both an ampicillin resistance gene for expansion in bacteria, and also a puromycin resistance gene for selection in mammalian cells). Plates with colonies were stored at 4°C.
In order to determine which colonies contained the PP2A-Aa mutant inserts, small scale DNA preparations were made, followed by digestion and DNA electrophoresis of fragments to rule out empty vector re-ligations, and then DNA sequencing to ensure correct insert orientation. Single colonies were picked from LB-AGAR plates, inoculated into 2 ml of LB media containing 100 μg/ml ampicillin and incubated overnight at 37°C with shaking. Cells were pelleted by centrifugation at 16,000x g for 1 min and the DNA was then purified using the Quicklyse Miniprep Kit (Qiagen, Doncaster, VIC, AUS). Small aliquots of culture were retained at 4°C for DNA expansion when positive cultures were determined. To each pellet, 400 μl of ice cold lysis solution was added and vortexed for 30 sec or until all cells were resuspended, followed by incubation at room temperature for 3 min. The lysed solution was then transferred to a spin column and centrifuged for 1 min. The column was washed once by addition of 400 μl of wash solution and centrifugation for 1 min, and then dried by centrifugation for 1 min after the flowthrough had been discarded. DNA was eluted with 40 μl of nuclease free water and centrifugation for 1 min. DNA was stored at 4°C. For each purified DNA product, 5 μl of sample was combined with 1 μl of loading buffer and electrophoresed as described in Section 2.2.5. The DNA concentration of any samples with correctly sized inserts was measured as described in Section 2.2.7 and sequenced as described in Section 2.2.11.

Once correctly oriented inserts were verified by sequencing, bacterial cultures were expanded further and purified to yield sufficient DNA for mammalian cell retroviral transduction. To ensure fresh starting material, bacterial cultures retained during mini DNA purification were restreaked on LB-AGAR plates with 100 μg/ml ampicillin and incubated at 37°C overnight. A 2ml starter culture of LB with 100 μg/ml ampicillin was inoculated with a fresh colony from the plates and incubated at 37°C with shaking for 8 hours. These 2 ml starter cultures were then added to 250 ml of LB with 100μg/ml ampicillin and grown overnight at 37°C. The following day, bacteria were harvested at 6,000x g for 15 min, and purified using a Maxi DNA purification kit (Qiagen). The pellets were thoroughly resuspended in 10 ml of buffer ‘P1’, followed by 10 ml of buffer ‘P2’ and incubated for 5 min at room temperature. Next, 10 ml
of chilled buffer ‘P3’ was added and incubated on ice for 20 min to form a precipitate which was removed from the DNA containing supernatant initially by centrifugation at 20,000x g for 35 min at 4°C and then filtration through a 0.45 μm filter disc. The purification column was equilibrated by passing ‘QBT’ buffer through the column by gravity. The supernatant retrieved from filtration by the filter disc was then loaded onto the column and allowed to drain by gravity. The column was then washed twice with buffer ‘QC’ and allowed to flow through. The DNA was then eluted off with 15 ml of buffer ‘QF’, and precipitated by addition of 10.5 ml isopropanol with mixing, followed by centrifugation for 30 min at 20,000x g. The resulting DNA pellet was then washed with 70% ethanol, centrifuged, air dried for 5-10 min, and finally resuspended in 150 μl of 1x TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The DNA concentration was then determined as in Section 2.2.7 and sequenced again as in Section 2.2.11 to ensure no mutations were introduced during the DNA expansion protocol.

2.2.11. DNA sequencing and analysis

Automated sequencing of Plasmid DNA (800 ng), or purified PCR product (30-50 ng) was performed at the Australian Genome Research Facility (AGRF, University of QLD, St Lucia, QLD, AUS). Details of primers for all sequencing reactions are provided in Table 2.3. Sequences were viewed, collated and figures created using Genious software (Drummond AJ 2010).
Table 2.3 Sequencing primers used in this thesis.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequencing Target</th>
<th>Oligonucleotide Sequence 5’→3’</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBABE 5’</td>
<td>5’→3’</td>
<td>pBABE vectors</td>
<td>CTTTATCCAGCCCTCAC</td>
<td>Determine direction of inserted PP2A-Aα mutants</td>
</tr>
<tr>
<td>PPP2R1A 425</td>
<td>5’→3’</td>
<td>PP2A-Aα</td>
<td>CCTCCCGCACCTCGGCTGC</td>
<td>Complete sequencing of PP2A-Aα to ensure mutants inserted into pBABE puro are correct and also when sequencing PP2A-Aα in breast cancer cell lines</td>
</tr>
<tr>
<td>PPP2R1A 725</td>
<td>5’→3’</td>
<td>PP2A-Aα</td>
<td>CCCTGGTGATGCCACACTCTG</td>
<td></td>
</tr>
<tr>
<td>PPP2R1A 1152</td>
<td>5’→3’</td>
<td>PP2A-Aα</td>
<td>TGAACATCATCTCTAACCCTG</td>
<td></td>
</tr>
<tr>
<td>PPP2R1A R 638</td>
<td>3’→5’</td>
<td>PP2A-Aα</td>
<td>CCTCTGACGACGGACT</td>
<td></td>
</tr>
<tr>
<td>PPP2R1A R 937</td>
<td>3’→5’</td>
<td>PP2A-Aα</td>
<td>CTCTCAGCTGACTGCGGAG</td>
<td></td>
</tr>
<tr>
<td>PPP2R1A UTR F</td>
<td>5’→3’</td>
<td>PP2A-Aα 5’ UTR</td>
<td>GTCTGACAGGAAAAGGACG</td>
<td>Sequences from the 5’ untranslated region for complete coding sequence in breast cancer cell lines</td>
</tr>
<tr>
<td>PPP2R1B UTR F</td>
<td>5’→3’</td>
<td>PP2A-Aβ 5’ UTR</td>
<td>GTGACCAGCAGGAGGAG</td>
<td></td>
</tr>
<tr>
<td>PPP2R1B 451</td>
<td>5’→3’</td>
<td>PP2A-Aβ</td>
<td>GATTGGTTCACCTCCTCGCAC</td>
<td></td>
</tr>
<tr>
<td>PPP2R1B 751</td>
<td>5’→3’</td>
<td>PP2A-Aβ</td>
<td>TTCGCTATATGGTGCGCTGAC</td>
<td></td>
</tr>
<tr>
<td>PPP2R1B 1152</td>
<td>5’→3’</td>
<td>PP2A-Aβ</td>
<td>GTTAAAGGATGAGTTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PPP2R1B R 673</td>
<td>3’→5’</td>
<td>PP2A-Aβ</td>
<td>GCTTCAGATGAACAGATTCA</td>
<td>Sequencing PP2A-Aβ in breast cancer cell lines.</td>
</tr>
<tr>
<td>PPP2R1B R 973</td>
<td>3’→5’</td>
<td>PP2A-Aβ</td>
<td>TTGCCCATTTAGAGATGAGGAGACC</td>
<td></td>
</tr>
<tr>
<td>PPP2R1B TV2</td>
<td>3’→3’</td>
<td>PP2A-Aβ TV2</td>
<td>AGGAGACCAAGTAGCAATG</td>
<td></td>
</tr>
</tbody>
</table>
2.3. Cell culture

2.3.1. Cell culture reagents and medias

Dulbecco’s modified eagle’s medium (DMEM), Roswell Park Memorial Institute media (RPMI), human mammary epithelial cell (HMEC) media and horse serum were supplied by Gibco® (Invitrogen, Carlsbad, CA, USA). Ham’s F12 media, Epidermal growth factor (EGF), Cholera toxin, insulin from bovine pancreas and hydrocortisone were obtained from Sigma-Aldrich (St Louis, MO, USA). HEPES and L-Glutamine were Hyclone® brand; supplied by Thermo Fisher Scientific, Roskilde, Denmark. Foetal calf serum (FCS) was supplied by Bovagen (Melbourne, Vic, Aus). Cultrex pathclear growth factor reduced (GFR) Basement membrane extract (BME) was obtained from R&D systems (Minneapolis, MN, USA). All reagents and medias used on cells were either purchased sterile or filtered through a 20 μM filter disc to sterilise. For many cellular experiments cells were washed in Phosphate Buffered Saline (PBS, 130 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄). Sachets containing premixed powdered PBS were purchased from Gibco (Invitrogen, Carlsbad, CA, USA), diluted in 1 L milliQ filtered water and sterilised by autoclaving.

All cell culture consumables including cell culture treated flasks, 10 cm dishes, tubes and serological pipettes were obtained from Greiner bio-one (Frickenhausen, Germany) unless stated otherwise. Cell culture treated plates of varying number of wells were purchased from Thermo Fisher Scientific (NUNC brand; Roskilde, Denmark).

2.3.2. Cell lines

Primary culture Human Mammary Epithelial Cells (HMEC) were purchased from Gibco® (Invitrogen, Carlsbad, CA, USA) and maintained in Gibco® HMEC media with supplements supplied. As instructed by manufacturers this media was never warmed to 37°C prior to use, but allowed to warm a little at room temperature to avoid cold shocking cells.
The MCF10A human breast epithelial cell line expressing the mouse ecotropic retroviral receptor (MCF10A ecoR) was kindly provided by Professor Roger Daly (Garvan institute of medical research, Sydney NSW). A number of medias were used with these cells depending on experimental conditions, and are summarised in Table 2.4.

All of the breast cancer cell lines were kindly provided by Professor Leonie Ashman (University of Newcastle, NSW), except for DU4475 which were provided by Dr Rick Thorne (University of Newcastle, NSW). MCF-7 breast cancer cells and Phoenix-Eco retroviral packaging cell lines were maintained in DMEM supplemented with 20 mM HEPES, 2 mM L-Glutamine and 10% foetal calf serum (FCS). Breast cancer cell lines MDA-MB-231, MDA-MB-468, HMT-3522-S2, HMT-3522-T42, T47D and DU4475 were maintained in RPMI supplemented with 20 mM HEPES, 2 mM L-Glutamine and 10% FCS. All cell cultures are adherent cells with the exception of DU4475, which is a suspension culture and were maintained at 37°C and 5% CO₂ in a humidified atmosphere.
Table 2.4 MCF10A medias.

MCF10A medias contained the following supplements in equal parts HamsF12/DMEM media:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Growth</th>
<th>Resuspension</th>
<th>Assay</th>
<th>Low serum</th>
<th>EGF stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>20mM</td>
<td>20mM</td>
<td>20mM</td>
<td>20mM</td>
<td>20mM</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>2mM</td>
<td>-</td>
<td>2mM</td>
<td>2mM</td>
<td>2mM</td>
</tr>
<tr>
<td>Horse serum</td>
<td>10% (v/v)</td>
<td>20% (v/v)</td>
<td>2% (v/v)</td>
<td>0.2% (v/v)</td>
<td>0.2% (v/v)</td>
</tr>
<tr>
<td>EGF</td>
<td>20ng/ml</td>
<td>-</td>
<td>5ng/ml*</td>
<td>-</td>
<td>50ng/ml</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>0.5µg/ml</td>
<td>-</td>
<td>0.5µg/ml</td>
<td>0.5µg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Cholera toxin</td>
<td>100mg/ml</td>
<td>-</td>
<td>100mg/ml</td>
<td>100mg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Insulin</td>
<td>10µg/ml</td>
<td>-</td>
<td>10µg/ml</td>
<td>10µg/ml</td>
<td>-</td>
</tr>
</tbody>
</table>

**Use**
- Propagation of MCF10A cells with full growth factors to allow rapid expansion
- Quenching trypsin during passaging to avoid using expensive growth media
- Establishment of 3D cultures, *5ng/ml EGF was added fresh each time cultures were replenished. Also used without EGF for growth assays as it contains low serum compared with growth media*
- For serum starving cells overnight prior to EGF stimulation
- High EGF concentration used for stimulation of cellular signalling. As cells were only treated for 1h, expensive reagents were not added.

DMEM = Dulbecco’s modified Eagle’s Medium, EGF = Epidermal Growth Factor
2.3.3. Revival of cell lines
Cells were revived from liquid nitrogen storage by thawing at room temperature with careful observation until just liquefied. Cells were then carefully mixed into 5-10 ml of warm media, or for MCF10A cells resuspension media, and centrifuged at 600x g for 3 min to remove Dimethyl sulfoxide (DMSO). The cellular pellet was then carefully resuspended in 13 ml of warm media and added to a T75 cell culture flask and left overnight to adhere to the flask. The following day the media was changed to remove any dead cells.

2.3.4. Passaging two-dimensional cultures
Adherent breast cancer cell lines were passaged every 2-3 days when cells reached 85-90% confluence. All reagents and medias were pre-warmed to 37°C. The following volumes are for a T75 cell culture flask. These volumes were doubled for cells in a T175 flask or halved for cells being maintained in a T25 flask. The cell monolayer was first washed with 4ml PBS and then incubated in 2 ml of 0.125% trypsin solution (Gibco brand; Invitrogen, Carlsbad, CA, USA, diluted from 0.5% to 0.125% in PBS) for 5 min at 37°C to detach cells from the flask. The trypsin was quenched with 4ml media containing 10% FCS and the cell mixture centrifuged at 800x g to remove the trypsin containing media. The cell pellet was resuspended as required for experiments, and 1:6-1:12 parts (to be confluent again in 2-3 days) added to a fresh flask with media. HMEC and MCF10A cells can be affected by higher concentrations of trypsin, and also grow in expensive medias, and thus were passaged as above with the following exceptions. Cells were washed twice with 4 ml PBS to remove all traces of media containing serum and incubated in 2 ml of either 0.025% trypsin for HMEC or 0.0625% trypsin for MCF10A. With this lower concentration of trypsin, incubation time at 37°C was increased to 15-20 min (checked regularly with gently agitation). The trypsin was quenched on HMEC cells with 6 ml 0.05% FCS in PBS, and with 4 ml resuspension media for MCF10A cells.

Unless stated otherwise in particular experiments, cells were counted before seeding for experiments by removing an aliquot of quenched trypsinised cells
prior to centrifugation to remove trypsin. These cells were loaded onto a haemocytometer and at least 3 squares counted. The total number of cells was determined by multiplying the average cell count by the total volume of cells/trypsin/quenching media (typically for a T75 = 2 ml trypsin + 4 ml quenching media = total 6ml) by 1x10^4. The cellular pellet centrifuged out of the trypsin/quenching media was then resuspended with media to the desired concentration of cells.

DU4475 cells grow as a suspension of cellular aggregates, and thus cannot be accurately counted. These cells were maintained by addition of media whenever cells appeared concentrated and media changed colour towards orange. Harvesting was performed by removing media containing cells, centrifugation at 800x g, washing 3 times in PBS and snap freezing pellets of unknown cell number in liquid nitrogen. As all experiments for these cells were performed following cellular lysis and adjustment for protein concentration, cell numbers were not required.

2.3.5. Cryopreservation of cell lines
Cells were cryopreserved from 85-90% confluent cultures in 40% FCS, or horse serum for MCF10A cells, and 10% DMSO in the same media used for growing each particular cell line. As Gibco® HMEC media does not contain serum, these cells were cryopreserved by adding 10% DMSO; however as they are a primary cell line, they did not thaw well. 1 ml of cells with DMSO were aliquoted into cryotubes (NUNC brand; Thermo Fisher Scientific, Roskilde, Denmark) and either wrapped in cotton wool or placed in a 5100 Cryo 1°C Freezing Container, "Mr. Frosty", containing 100% isopropyl-alcohol (Nalgene Brand; Thermo Fisher Scientific, Roskilde, Denmark) and stored at -80°C overnight to allow slow cooling of 1°C per minute. Cells were kept at -80°C for short term storage or moved to liquid nitrogen for long term storage.

2.3.6. Three-dimensional (3D) cell culture
Three-dimensional (3D) cultures were maintained essentially as previously described (Debnath et al. 2003), with some modifications. All cultures were
grown in 8 well chamber slides (NUNC brand; Thermo Fisher Scientific, Roskilde, Denmark) on Cultrex pathclear Growth Factor Reduced (GFR) Basement Membrane Extract (BME) (R&D systems, Minneapolis, MN, USA). BME was stored at -80°C long term, or -20°C for less than three months, and was thawed overnight on ice at 4°C. A 25 μl plug of BME was spread on the bottom of each chamber, with care not to form a meniscus up the sides of the well. Slides were incubated at 37°C for at least 15 min for the plug of BME to set. Cells to be cultured were removed from the flask with trypsin as described in Section 2.3.4. As a very low concentration of cells is required, at single cell suspension, cells were resuspended in 2ml of assay media after the trypsin containing quenching media was removed. The 2 ml suspension was pipetted approximately 25 times with a 1 ml pipette tip in order to break up any cellular clumps. A further 8ml of assay media was added, for a final volume of 10ml, and an aliquot of these were counted with a haemocytometer as described in Section 2.3.4. The cells were diluted to 1x10³ cells/ml and then diluted further 1:1 in 2x BME containing media (4% BME, 10ng/ml EGF in assay media). 400 μl of this mixture was added to each chamber slide, giving a final concentration of 200 cells/well in 1x BME media (2% BME, 5ng/ml EGF in assay media). The media was replenished every second day by removing half the volume of media and replacing with 200 μl of 1x BME media for up to 20 days. 3D cultures were analysed by light microscopy (Section 2.5.1) and also immunofluorescence (Section 2.8.1). The morphology of 3D acini were determined using a light microscope, and reported as percentage of acini of a particular morphology compared to the total number of acini for a particular field of view. The diameter of each acini was measured using Axiovision software (Zeiss).
2.4. Generation of MCF10A cell lines with altered PP2A

2.4.1. Retroviral supernatant from Phoenix-Eco packaging cells

When retroviral packaging cells such as Phoenix-Eco are transiently transfected with retroviral plasmids, they package the plasmid DNA into retroviral particles and release it into their growth media. Addition of this media to cells containing receptors for retrovirus takes up the retroviral particles containing the plasmid DNA and integrate it into their genome, thus creating a stable cell line that should propagate a DNA sequence of interest within the vector to subsequent generations of cells. Retroviral vectors containing sequence for either ectopic expression of entire proteins (Table 2.1) or short hairpin RNA (shRNA, Table 2.2) were transiently expressed in Phoenix-Eco cells by Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Phoenix-Eco cells were seeded at 3x10^6 cells/dish in 10 cm culture dishes and allowed to adhere overnight. The following day, for each plasmid sample, 80μl of Lipofectamine™ 2000 was mixed with 1 ml of Opti-MEM® (Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at room temperature. A further 1ml of Opti-MEM® was combined with 8 μg of DNA. The Lipofectamine™ 2000 mixture was then mixed with the DNA mixture and incubated at room temperature for 20 min before being added carefully to the Phoenix-Eco cells (Phoenix-Eco cells readily detach from the dish, so addition of media at the side of the dish is recommended). The media/Lipofectamine™ 2000 mixture was removed and replaced with 8 ml of fresh media 5 hours after infection. At 24 and 48 hours post infection, vectors containing green fluorescent protein markers were observed by fluorescent microscopy to assess infection efficiency, before media containing retroviral particles was removed with a 16 gauge needle and then passed through a 0.45μm filter disc. The resulting retroviral supernatant was snap frozen in liquid nitrogen and stored at -80°C until used to infect MCF10A cells.

2.4.2. Retroviral infection of MCF10A

MCF10A cells were seeded at 5x10^5 cells/dish 24 hours prior to infection so that they would be approximately 70% confluent at time of infection. Retroviral supernatants (section 2.4.1) collected at 24 and 48 hours post
infection were thawed and mixed prior to infection. For single infections, 2 ml of retroviral supernatant was combined with 6 ml of MCF10A growth media and 8 μg/ml polybrene (Chemicon, Temecula, CA, USA). For duel infections, 2 ml of each retroviral supernatant was combined with 4 ml of MCF10A growth media with 8 μg/ml polybrene. Cells were incubated in this retroviral containing media for 5 hours at 37°C and then replaced with fresh growth media. The following day, GFP expressing cells were selected by fluorescence activated cell sorting (FACS), performed by the Biomolecular Research Facility (University of Newcastle) on a FACS Aria. Cells were resuspended for sorting at 1x10^6 cells/ml in Hanks Buffered Salt Solution (Sigma) supplemented with 10% horse serum and 1 mM EDTA to both protect cells and prevent cell clumping. Following sorting, cells were maintained in growth media supplemented with 100 U/ml penicillin/streptomycin for one week to prevent contamination. Some cell lines were sorted twice to gain a higher percentage of GFP positive cells. The sorted GFP expressing cells were allowed to grow until they reached 85-95% confluence, when half of the cells were cryopreserved. In addition, a quarter of the cells were analysed by flow cytometry (see Section 2.4.3), while the remaining quarter were allowed to grow to confluence again. Cells expressing vectors with a puromycin resistance element were selected for by addition of 1 μg/ml puromycin (Sigma) until untransduced control cells treated equally were all dead, approximately 8-12 days. For MCF10A PP2A-Aα mutants which were dual infected with pMKO.1 GFP PP2A-Aα shRNA and pBABE-puro PP2A-Aα mutant DNA, cells were first selected for GFP expression by FACS, allowed to recover for 2-3 days, and then treated with 1 μg/ml puromycin.

2.4.3. Flow cytometry
Flow cytometry was used to measure GFP expression in MCF10A cells transfected with retroviral vectors containing a GFP marker. During passaging of cells, approximately 1x10^6 suspended cells were washed three times with cold PBA buffer (0.1% BSA, 0.1% sodium azide in PBS), and then resuspended in 500 μl of FACS fixative (2% glucose, 1% formaldehyde, 0.02% sodium azide in PBS). Cells were then incubated in the dark for at least 30 min at 4°C, and then kept at 4°C until analysis. GFP fluorescence was
detected using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the data analysed with CellQuest software (BD Biosciences).
2.5. **Analysis of altered cellular functions**

2.5.1. **Light microscopy**

Cells were imaged at a various stages of growth, including regular 2D culture, 3D cultures and scratch assays (Sections 2.3.4, 2.3.6 and 2.5.3 respectively). Light microscopy was performed on an Axiovert 200 inverted light microscope (Carl Zeiss Pty Ltd, North Ryde, NSW, AUS) and images analysed with Axiovision software (Zeiss).

2.5.2. **Cell proliferation assay**

To assess the proliferation rate of MCF10A cells transduced with PP2A-A mutations and/or shRNA constructs, the ability of cells to metabolise resazurin dye was measured. MCF10A cells were seeded in triplicate wells at 5x10^3 cells/well in 200μl of media into 4x 96 well plates. Cells were seeded in assay media without EGF, or in normal growth media. At 19 hours post seeding, 20 μl of resazurin mix (1.5 mM Reazurin, 0.02 mM Methylene Blue, 0.25 mM Potassium hexacyanoferrate (III), 0.25 mM Potassium hexacyanoferrate (II) trihydrate (all from Sigma) in PBS) was added to each well in one of the plates and incubated at 37°C in the dark for 5 hours. At 24 hours post seeding the fluorescence was measured at 544nm excitation/ 590nm emission on a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany). This procedure was repeated on the other plates on sequential days to obtain readings at 48, 72, a nd 96 hour s post seeding. The average fluorescence for each cell line was plotted against time using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA).

2.5.3. **Wound healing assays**

To measure cellular migration, the rate of closure of scratches made in confluent monolayers of cells was measured. Cells were seeded at 5.5x10^5 cells/well in 12 well plates and allowed to adhere overnight. Once cells were 100% confluent, scratches were made through the monolayer using a 1ml pipette tip. Wounds were photographed at 0, 12, 24, 36 a nd 48 hour s post scratching as described in Section 2.5.1. To quantitate wound width, 7 measurements were made down the length of the scratch, each 200 μm apart,
using Axiovision software (Carl Zeiss Pty Ltd, North Ryde, NSW, AUS). The average of the 7 measurements was plotted against time using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA).

2.5.4. Anchorage independent growth assays
To measure the ability of cells to proliferate without attachment to any protein matrix, cells were grown on a bed of agarose. A plug of 0.7% agarose (Sigma) in growth media was set on the base of 6 well culture plates. 5x10^3 cells/well were combined into 0.5% agarose in growth media, layered on top of the agarose plug and allowed to set. A further 2-3 ml of growth media was added to top of the cells, now set into the agarose, to prevent the agarose from drying out. These plates were incubated at 37°C for 20 days with no media changes. The plates were imaged on an LAS 4000 (FujiFILM corporation, now available through GE Healthcare, Rydalmere, NSW, Aus), and the number of colonies counted using Multi Gauge software (FujiFILM corporation).

2.5.5. Epidermal growth factor (EGF) stimulation
In order to investigate cellular signalling within breast cells, and whether alteration to PP2A affected this signalling, cells were serum starved overnight prior to stimulation with EGF. These cells were analysed by both western blotting (Section 2.7) and also for PP2A activity (Section 2.9). Cells were seeded at 5x10^5 cells/dish in a 10 cm dish 42-48 hours prior to cellular harvesting. Cells were then serum starved for 18 hours prior to treatment. Cells were serum starved in low serum media (see Table 2.4), and the following morning cells treated for 5 min, 15 min and 1 hour with EGF stimulation media (Table 2.4). A plate of cells not serum starved (maintained in normal growth media), and non-EGF treated (serum starved, but no EGF treatment) were also added as controls. Following treatment, cells were washed 3x with cold TBS (1mM Tris pH 8, 150mM NaCl) and treated as described for western blotting or PP2A activity assays.
2.6. Gene sequencing and mRNA expression analysis

2.6.1. RNA extraction and cDNA production

Total cellular RNA was extracted from cells using an RNeasy mini kit (Qiagen, Doncaster, VIC, AUS). Cells previously snap frozen in liquid nitrogen and stored at -80°C were thawed on ice and gently flicked to loosen cells. Cells were then lysed with 600 μl of RLT buffer (all buffers supplied), pipetted briefly to resuspend, loaded onto a QIAshredder spin column, and centrifuged for 2 min at full speed. 600 μl of 70% ethanol was added to the flow through and mixed well by pipetting. This mixture was then passed through an RNeasy spin column, in 2 batches by centrifugation for 20 sec at 15,000x g, and the flow through discarded. To eliminate genomic DNA contamination, on-column DNase digestion was then performed using the RNase-Free DNase Set (Qiagen). 350 μl of RW1 buffer was added to the RNeasy spin column, centrifuged for 20 sec at 15,000x g, and the flow through discarded. Next, 10 μl of DNase I stock solution was mixed with 70 μl of RDD buffer, added to the column and incubated at room temperature for 15 min. Following digestion, the column was washed with 350 μl of RW1 buffer, followed by 700 μl of RW1 buffer, and then 500 μl of RPE buffer, each centrifuged for 20 sec with the flow through discarded after each wash. A final wash with 500 μl of RPE buffer was centrifuged for 2 min before discarding the flow through. To dry the column, it was centrifuged without buffer for 1 min at full speed. Finally, the RNA was eluted with 30 μl of RNase-free water, applied directly to the centre of the column, and then centrifuged for 1 min at full speed. The RNA was then quantitated as described in Section 2.2.7.

To make cDNA, 10 μg of RNA with 2 μl of 0.5 μg/ml oligo dT primer (Sigma Genosys, St Louis, MO, USA) were made up to 18 μl with nuclease free water and incubated for 10 min at 65°C to allow primer annealing. The RNA was divided into 2 tubes, each with 1x reaction buffer (supplied), 10 U RNase inhibitor (Promega, Madison, WI, USA), 0.5 M dNTPs, and either 1 U of M-MLV reverse transcriptase (Promega) or 1 μl of nuclease free water as a negative control. For reverse transcription, the mixture was incubated at 42°C
for 1.5 hours, and then the enzyme was inactivated by incubation at 70°C for 10 min. The resulting cDNA was diluted in 180μl of nuclease free water and stored at -20°C.

2.6.2. Sequence analysis of PP2A-A
To determine if any of the cell lines used in this project harboured mutations within PP2A-A, isoform specific primers (Sigma Genosys, St Louis, MO, USA) were used to amplify PP2A-A isoforms (PP2A-Aα, PP2A-Aβ transcript variant 1, and PP2A-Aβ transcript variant 2), which were then sent for sequencing as described in Section 2.2.11. Only half of the entire 1800 bp product was amplified in each reaction using the isoform specific primers (Table 2.5), with an overlapping region in the middle. Each PCR reaction contained: 5 μl of cDNA (Section 2.6.1), 100 ng of each forward and reverse primer, 1 mM dNTP and 2.5 U of PfuUltra® II Fusion HS DNA Polymerase (Agilent Technologies, Santa Clara, CA, USA) with 1x reaction buffer (supplied) and nuclease free water in a final volume of 50 μl. PCR reactions were performed in a Mastercycler Pro (Eppendorf South Pacific, North Ryde, NSW, Aus) under the following conditions: a single denaturing step at 95°C for 2 min; followed by 30 cycles of 95°C for 1 min; 54°C for 1 min; 72°C for 3 min; followed by a final extension step of 72°C for 5 min.

2.6.3. Quantitative real-time PCR (qRT-PCR)
Isoform specific oligonucleotide primers (Sigma Genosys, St Louis, MO, USA) were used to assess the mRNA expression for individual PP2A subunits (Table 2.6). Ribosomal protein S18 (RPS18) was used as a reference gene. Each reaction was run in triplicate, containing 1 μl of cDNA, with 4pM primer in Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real Time PCR reactions were performed in a 7500 Real Time PCR System (Applied Biosystems) with the following cycling conditions: 50°C for 2min then 95°C for 10min; followed by 40 cycles of 95°C for 15 sec then 60°C for 1 min. The fold change in PP2A gene expression was compared to a human neuroblastoma cell line, SH-EP, using comparative quantitative algorithms (Applied Biosystems). The SH-EP cell line was used as a control
Table 2.5 PCR primers used to amplify PP2A-Aα and PP2A-Aβ.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Direction</th>
<th>Oligonucleotide Sequence 5’→3’</th>
<th>PCR Region</th>
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<tr>
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<td>PPP2R1A 725</td>
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</tr>
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<td>Rev</td>
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<td></td>
</tr>
<tr>
<td>PPP2R1B UTR F</td>
<td>Fwd</td>
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<td>Start of PP2A-Aβ including 5’ UTR</td>
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</tr>
<tr>
<td>PPP2R1B 751</td>
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<td>End of PP2A-Aβ including 3’ UTR.</td>
</tr>
<tr>
<td>PPP2R1B UTR R TV1</td>
<td>Rev</td>
<td>GCCCTTAATAGATCTCTGTAC</td>
<td>TV1/2 indicate transcript</td>
</tr>
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<td>variant specific primers</td>
</tr>
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<td>Sequencing Target</td>
<td>Gene Accession</td>
<td>Direction</td>
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<td>CATGAGCATATCTTCCGCC</td>
</tr>
<tr>
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<td>PPP2CB</td>
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<td>TCCCATACTTTGCAGACAT</td>
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<tr>
<td>PPP2R1A</td>
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<td>Rev</td>
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<td></td>
<td>Rev</td>
<td>GGCTCCTGTAGGTGTAT</td>
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<tr>
<td>PPP2R5B</td>
<td>NM 006244</td>
<td>Fwd</td>
<td>ACTCTGACAGACTGACGTG</td>
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<td></td>
<td>Rev</td>
<td>GAAACATCACCTCCTCTGG</td>
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<td>PPP2R5D</td>
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<tr>
<td></td>
<td></td>
<td>Rev</td>
<td>GACATCAGCTTCTGCTCCA</td>
</tr>
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</table>
as all primers had previously been optimised for mRNA expression in these cells and had robust expression of all subunits. The cycle threshold (Ct) of RSP18 was subtracted from the gene of interest to give a normalised average cycle threshold for each cell line (Avg ΔCT). The averaged cell cycle thresholds for the SH-EP cells were then subtracted from each cell line to give a relative threshold (ΔΔCT) with which to compare fold changes among the breast cancer cell lines. Relative gene expression was calculated as a fold difference by raising the number 2 to the negative power of the relative cycle threshold for each cell line (2^{−ΔΔCT}), as one cycle difference indicates twice as much starting template. Numerically:

\[
\text{Ct}_{\text{Gene of interest}} - \text{Ct}_{\text{RPS18}} = \text{Avg } \Delta CT
\]

\[
\text{Avg } \Delta CT_{\text{Breast cancer cell line}} - \text{Avg } \Delta CT_{\text{SH-EP}} = \Delta \Delta CT
\]

\[
\text{Fold difference (Relative to SH-EP)} = 2^{−ΔΔCT}
\]

Values were then plotted using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA).
2.7. Western blotting

2.7.1. Cell lysis and protein concentration

Adherent monolayers of cells grown in culture dishes were washed twice with cold PBS to remove media and then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris 1%, NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 10 μl/ml protease inhibitor cocktail (Sigma, Contains: 104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A)) for 30 min at 4°C with gentle rocking. Lysed cells were scraped from the dish, probe sonicated using a Soniprep 150 M SE Ultrasonic Disintegrator (MSE, Lower Sydenham, London, UK) for 10 sec at 5 µm amplitude, then centrifuged at 18,000x g for 10 min at 4°C to remove any remaining cellular debris. Cell lysates were either frozen at -80°C until all samples to be analysed were collected, or were used directly for western blotting. DU4475 cells grow in suspension and thus were centrifuged at 800x g, and washed 2x with cold PBS, before being either snap frozen or used fresh. Pellets were incubated in RIPA buffer for 30 min at 4°C with gentle rocking before sonication as above.

The protein concentration of cellular lysates was determined by BCA assay (Pierce, Rockford, Il, USA). Briefly, protein samples were diluted 1:2 in lysis buffer and compared against a standard curve generated from a series of known bovine serum albumin (BSA) standards, also diluted in lysis buffer. The protein concentration of samples and standards was determined by colourmetric assay, read at 562nm absorbance on a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany). Samples were diluted as required according to the protein concentration in lysis buffer, and then boiled for 5min in 5x SDS loading buffer (60 mM Tris pH 6.8, 25% glycerol, 2% SDS, 375 mM dithiothreitol, 0.1% bromophenol blue).

2.7.2. Western blotting

Proteins were separated on 12% resolving polyacrylamide gels (12% acrylamide/bis, 375 mM Tris pH 8.8, 0.1% SDS) with a 4% stacking gel (4% acrylamide/bis, 375 mM Tris pH 6.8, 0.1% SDS), at 80 V through the stacking gel, and then 110 V for approximately 3 hours using an Adjustable Slab Gel
Kit (CBS scientific, CA, USA) in electrophoresis running buffer (25mM Tris, 192 mM glycine, 0.1% SDS). The only exception to this was that protein lysates to be probed for SET were run on 18% resolving gels. Proteins were then transferred to nitrocellulose membrane either overnight at 0.2-0.3 A, or for 3 hours at 0.6-0.7 A with cooling at 4°C (GE Healthcare, Rydalmere, NSW, Aus) in boric acid transfer buffer (50mM boric acid, 2mM EDTA, pH 8.9.)

The membranes were stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) to ensure successful transfer and equal loading before blocking in 10% skim milk powder diluted in tris buffered saline-Tween 20 (TBS-T) buffer (1 mM Tris pH 8, 150 mM NaCl, 0.05% Tween-20) or 5% bovine serum albumin (BSA) in TBS-T for 1 hour. Membranes were then washed three times for 10 min in TBS-T. Primary antibodies were diluted in TBS-T, in some cases supplemented with 0.05% skim milk or 0.05% BSA (Table 2.7), and incubated for 1 hour or overnight at room temperature with vigorous shaking. Membranes were washed 3x 10 min in TBS-T before incubation in secondary antibody conjugated to horseradish peroxidise (HRP) (Table 2.8), diluted in TBS-T, for 1 hour at room temperature. Following another 3x 10 min TBS-T washes, protein bands were visualised by incubating the membranes for 5 min at room temperature in a developing solution (0.4 mM p-coumaric acid (Fluka brand, Sigma), 2.5 mM luminal (Fluka), 0.01% H2O2 in 100 mM Tris pH 8.8) and then imaging on an LAS 4000 (FujiFILM corporation, now available through GE Healthcare, Rydalmere, NSW, Aus). The density of bands were measured with Multi Gauge Software (FujiFILM corporation). Each individual band was outlined, including actin protein bands that were assayed under the same conditions as the band of interest. To account for differences in background staining, a region identically sized to the control band for each western blot was placed in an adjacent region with no protein bands, and the densitometry value for this region was subtracted from the densitometry value for each band on that membrane. Following background subtraction, the densitometry value for each protein band was normalised to actin expression to account for any alterations in protein
Table 2.7 Primary antibodies used for western blotting.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Antibody Name</th>
<th>Species</th>
<th>Supplier</th>
<th>Block</th>
<th>Concentration</th>
<th>Incubation time</th>
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</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Anti-Actin antibody</td>
<td>Rabbit</td>
<td>Sigma</td>
<td>10% SMP</td>
<td>1:1000</td>
<td>1 Hour</td>
</tr>
<tr>
<td>PP2A-A (α &amp; β isoforms)</td>
<td>PP2A Alpha Subunit (6F9) Monoclonal Antibody</td>
<td>Rat</td>
<td>Covance</td>
<td>10% SMP</td>
<td>1:1000</td>
<td>1 Hour</td>
</tr>
<tr>
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<td>PPP2R1B antibody</td>
<td>Sheep</td>
<td>Abcam</td>
<td>10% SMP</td>
<td>1:1000</td>
<td>1 Hour</td>
</tr>
<tr>
<td>PP2A-Bα</td>
<td>PP2A Bα antibody</td>
<td>Rabbit</td>
<td>Produced at IMVS FSQKGAVDDDVAEC (Chen et al. 2004)</td>
<td>10% SMP</td>
<td>1:500</td>
<td>1 Hour</td>
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<tr>
<td>PP2A-Bβ</td>
<td>PP2A Bβ antibody</td>
<td>Rabbit</td>
<td>Produced at IMVS GGGCPTGGNDFQW</td>
<td>10% SMP</td>
<td>1:500</td>
<td>1 Hour</td>
</tr>
<tr>
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<td>PPP2R5A antibody</td>
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<td>Novus Biologicals</td>
<td>10% SMP</td>
<td>1:500</td>
<td>1 Hour</td>
</tr>
<tr>
<td>PP2A-B’β</td>
<td>PPP2R5B antibody</td>
<td>Goat</td>
<td>Novus Biologicals</td>
<td>10% SMP</td>
<td>1:500</td>
<td>1 Hour</td>
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<td>1 Hour</td>
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<td>Species</td>
<td>Supplier</td>
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<td>Upstate</td>
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<td>I2PP2A (H-120)</td>
<td>Rabbit</td>
<td>Santa Cruz Biotechnology</td>
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<td>Chemicon</td>
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<td>Abcam</td>
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<tr>
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<th>Supplier details:</th>
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<td>Abcam: Sapphire Bioscience Pty Ltd, Waterloo, NSW, Aust</td>
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<td>Bethyl Laboratories: Montgomery, Tx, USA</td>
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<td>Chemicon: Millipore, Temecula, CA, USA</td>
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<td>Covance: Emeryville CA, USA</td>
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<tr>
<td>CST: Cell Signalling Technology, Danvers, MA, USA</td>
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<tr>
<td>Epitomics: Burlingame, CA, USA</td>
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</table>

Abbreviations: BSA; Bovine Serum Albumin, SMP; Skim Milk Powder

IMVS: Institute of Medical and Veterinary Science, Adelaide, SA.
Peptides for these antibodies were made at Auspep, Parkville, VIC
Novus Biologicals: Sapphire Bioscience Pty Ltd, Waterloo, NSW, Aust
Santa Cruz Biotechnology: Santa Cruz, CA, USA
Upstate: Upstate Brand, Millipore, Temecula, CA, USA
Table 2.8 Secondary antibodies used for western blotting.

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<td>#A9037</td>
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loading. If applicable, each band was then compared to a control cell line, as indicated in each figure. Membranes probed for p-Akt and p-ERK were stripped and re-probed for total Akt and ERK. Following visualisation, membranes were incubated 3x 20 min in secondary antibody stripping buffer (0.2 M glycine, 100 mM 2-Mercaptoethanol pH 2.6) heated to 54°C prior to each incubation. The membrane was rinsed briefly in TBS-T between each incubation. The membranes were then blocked again for 30 min in 5% BSA/TBS-T and then treated as described above. For these membranes, the level of background staining was equal across all membranes, so no background subtraction was performed. Rather than normalising to actin, densitometry for the phosphorylated proteins was expressed as a percentage of total protein.
2.8. Immunofluorescence

2.8.1. Two-dimensional (2D) immunofluorescence

Two-dimensional (2D) immunofluorescence was used to identify the location of PP2A subunits within breast cancer and normal breast cells. Sterile glass cover slips, 13mm in diameter, were placed in the bottom of 24 well plates. Cells were then seeded onto these coverslips at either 5x10^4 cells/well or 1x10^5 cells/well for cells to be sub-confluent or fully confluent at 36 hours post-seeding respectively. All immunofluorescent staining was performed within these 24 well plates. DU4475 is a suspension cell line, so these cells were washed 3x with PBS and then centrifuged onto slides using a Cytospin centrifuge (Shandon Scientific Ltd, England). These cells cannot be accurately counted (Section 2.3.4), but 100 μl of cells at approximately 2.5x10^5 cells/ml was spun onto the slide at 800 rpm for 5 min. Slides were coated with 50 μl of 100% FCS using the cytospin centrifuge prior to addition of cells, to aid attachment of the cells to the glass. Following cytospin centrifugation, DU4475 cells were treated identically to the other cell lines. Cells were initially rinsed three times in PBS to remove any media proteins before fixation for 10 min in 3.7% formaldehyde in PBS. Cells were washed 3x 5 min in PBS before permeabilisation in 0.1% Triton X-100 detergent diluted in PBS for 3 min. Cells were washed 3x 5 min in PBS before blocking in 10% FCS/PBS for 20 min at room temperature. Cells were then incubated in primary antibodies at 1:250 concentration in 5% FCS/PBS for 1h (Table 2.9. Note concentrations provided are for 3D IF in Section 2.8.2). Cells were then washed 3x 5 min in PBS before incubation in fluorescently labelled secondary antibodies diluted 1:1,000 in 5% FCS/PBS for 45 min in the dark (Table 2.10). All subsequent steps were performed in the dark to prevent bleaching of fluorescently labelled antibodies. Cells were washed 3x 5 min in PBS before a 5 min incubation in 4',6-Diamidino-2-phenylindole (DAPI) nuclear stain (1mg/ml in PBS. Invitrogen, Carlsbad, CA, USA). Following a 5 min wash in PBS, individual cover slips were removed from the wells with tweezers, inverted and placed onto a glass slide with a drop of Vectamount mounting media (Vector Laboratories Inc, Burlingame, CA, USA), then allowed to dry for 24 hours. For DU4475, a drop of vectamount was added to the cells and a
Table 2.9 Primary antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Antibody Name</th>
<th>Species</th>
<th>Supplier</th>
<th>Concentration (3D IF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP2A-A (α &amp; β isoforms)</td>
<td>PP2A Alpha Subunit (6F9) Monoclonal Antibody</td>
<td>Rat</td>
<td>Covance</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#MRT-204R</td>
<td></td>
</tr>
<tr>
<td>PP2A-Bα</td>
<td>PP2A Bα antibody</td>
<td>Rabbit</td>
<td>Produced at IMVS</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>FSQVKGAVDDDVAEC</td>
<td></td>
</tr>
<tr>
<td>PP2A-Bγ</td>
<td>PPP2R5C antibody</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#ab94633</td>
<td></td>
</tr>
<tr>
<td>PP2A-C</td>
<td>PP2A-C antibody</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#ab94633</td>
<td></td>
</tr>
<tr>
<td>PP2A-C</td>
<td>Anti-PP2A, C subunit, clone 1D6</td>
<td>Mouse</td>
<td>Upstate</td>
<td>1:50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#05-421</td>
<td></td>
</tr>
<tr>
<td>Proliferating Cells</td>
<td>Rabbit anti-Ki-67</td>
<td>Rabbit</td>
<td>Invitrogen</td>
<td>1:200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># 18-0191Z</td>
<td></td>
</tr>
<tr>
<td>Proliferating Cells</td>
<td>Mouse anti-Ki-67</td>
<td>Mouse</td>
<td>Invitrogen</td>
<td>1:200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># 18-0192Z</td>
<td></td>
</tr>
<tr>
<td>Apoptotic cells</td>
<td>Cleaved Caspase-3 (Asp175) Antibody</td>
<td>Rabbit</td>
<td>CST</td>
<td>1:200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#9661</td>
<td></td>
</tr>
<tr>
<td>Luminal Cells</td>
<td>GM130</td>
<td>Mouse</td>
<td>BD Biosciences</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#610823</td>
<td></td>
</tr>
<tr>
<td>Basal Cell membrane</td>
<td>RAT ANTI HUMAN CD49f</td>
<td>Rat</td>
<td>Serotech</td>
<td>1:200</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#MCA699</td>
<td></td>
</tr>
<tr>
<td>Basal Cell membrane</td>
<td>MOUSE ANTI HUMAN CD104</td>
<td>Mouse</td>
<td>Serotech</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>#MCA1456</td>
<td></td>
</tr>
<tr>
<td>EE tag (PP2A-A mutants)</td>
<td>Rabbit anti-glu-glu affinity purified polyclonal antibody</td>
<td>Rabbit</td>
<td>Chemicon</td>
<td>1:100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td># A83788</td>
<td></td>
</tr>
</tbody>
</table>

Supplier details:
Abcam, Sapphire Bioscience Pty Ltd, Waterloo, NSW, Aust
BD Biosciences; Franklin Lakes, NJ, USA
Chemicon Brand, Millipore, Temecula, CA, USA
Covance; Emeryville Ca USA
CST; Cell Signalling Technology, Danvers, MA, USA
IMVS; Institute of Medical and Veterinary Science, Adelaide, SA.
Peptides for IMVS antibodies were made at Auspep, Parkville, VIC
Invitrogen; Carlsbad, CA, USA
Table 2.10 Secondary antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 555 goat anti-rat IgG (H+L)</td>
<td>Molecular Probes, Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Alexa Fluor® 594 donkey anti-mouse IgG (H+L)</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488 donkey anti-mouse IgG (H+L)</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 594 goat anti-rabbit IgG (H+L)</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti-rabbit IgG (H+L)</td>
<td></td>
</tr>
</tbody>
</table>
coverslip placed on top. After 24 hours, coverslips were sealed with nail varnish and imaged by confocal microscopy as described in Section 2.8.3.

2.8.2. Three-dimensional (3D) immunofluorescence

At days 8, 14 and 20 post initial 3D culture set-up (Section 2.3.6), the cells were stained within the 8 well chamber slides. Media was removed and cells were immediately fixed in 2% formaldehyde in PBS for 10 min. Cells were then permeabilised in 0.5% Triton X-100 in PBS for 3 min before 3x 5 min washes in 100 mM glycine in PBS. Cells were blocked in 10% foetal calf serum (FCS) in 3D IF buffer (7.7 mM NaN3, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20 in PBS) for 1 hour at room temperature. Directly after blocking, cells were incubated with primary antibodies in 10% FCS/3D IF buffer at 4°C overnight. A list of primary antibodies and dilutions used is provided in Table 2.9. The following day, excess primary antibody was removed with 3x 20 min 3D IF buffer washes. Cells were then incubated in fluorescently labelled secondary antibodies diluted 1:1,000 in 10% FCS/3D IF buffer (Table 2.10) for 45 min at room temp in the dark. All subsequent steps were performed in the dark to prevent secondary antibodies from bleaching. Excess secondary antibody was removed with 3x 20min 3D IF buffer washes. If cells were to be co-stained with another antibody, the second round of primary antibody was added here, incubated at 4°C overnight, and then treated exactly the same for washes and then addition of the second round of secondary antibody. Following final washing to remove secondary antibody, cells were incubated for 5 min with DAPI nuclear stain (1mg/ml in PBS. Invitrogen, Carlsbad, CA, USA). DAPI was rinsed from cells with a 5min PBS wash, before chambers and gaskets were removed from the slides and the slides allowed to dry for 20 mins at room temp. A drop of prolong gold antifade mounting solution (Invitrogen, Carlsbad, CA, USA) was added to the cells, and a coverslip placed over the top. Due to the thickness of these specimens, they were allowed to dry for at least 48 hours before sealing with nail varnish. Cells were imaged by confocal microscopy as described in Section 2.8.3.
2.8.3. Confocal microscopy

All immunofluorescence imaging was performed by confocal microscopy on an Olympus Fluoview 1000 confocal microscope and analysed with Olympus Fluoview software (Olympus Europa GmbH, Hamburg, Germany). DAPI (blue) was imaged using an excitation wavelength of 405 nm, Alexa Fluor 488 fluorescently tagged secondary antibodies and also GFP (green) were imaged by excitation at 473 nm, while Alexa Fluor 555 fluorescently tagged secondary antibodies (red) were imaged at 559 nm. For 3D MCF10A cultures, acini were imaged by focussing through the centre of each acini. The proliferation rate of 3D acini was estimated by staining for Ki67, a marker of dividing cells, and the number of Ki67 positive cells per acini counted using ImageJ software ‘Analyse particles’ function (Freeware available at: http://rsbweb.nih.gov/ij/).
2.9. **PP2A activity assay**

2.9.1. **Immunoprecipitation**

In order to measure PP2A activity, PP2A was precipitated from total cellular protein lysates with antibodies and magnetic beads in a Precipitor™ (Abnova, Taipei, Taiwan). Cells were seeded at $5 \times 10^5$ cells/dish in a 10 cm plate 48 hours prior to harvesting. As a control to assure activity is due to PP2A, cells were treated with 1 uM okadaic acid (OA, Sigma) in normal growth media for 45 min prior to harvesting. Cells were harvested by addition of 500 μl of PP2A activity lysis buffer (20mM Imidazole, 2mM EDTA, 2mM EGTA) and immediately scraped into a 1.5 ml tube. Cells were then homogenised on ice with 40-50 strokes at 760 rpm on a Heidolph RZR2051 homogeniser (John Morris scientific Pty Ltd, Willoughby, NSW, Aus). The homogenate was then centrifuged at 18,000x $g$ for 10 min at 4°C to pellet cellular debris. The resulting supernatant was snap frozen in liquid nitrogen and stored at -80°C until all samples were collected. The protein concentration of cellular lysates was measured by BCA assay (Pierce, Rockford, Il, USA. Section 2.7.1). The Precipitor™ system uses magnetic beads in a 96 well deep sided plate, where the beads are mixed into the wells with plastic stirring rods, and transferred to subsequent wells using magnetic arms. In order to precipitate PP2A by adhesion onto the beads, the machine was programmed to perform the following tasks, and is run in a 4°C refrigerator. Firstly, beads from 20 μl of bead/PBS slurry were washed 3x 1 min in PP2A assay buffer (0.1mM CaCl2, 50mM Tris pH 7). The beads were then incubated with antibody used to precipitate out PP2A for 30 min. For these experiments either 2μg of PP2A-C antibody (Provided by Professor Alistair Sim against peptide, PHVTRRTPDYFL (Sim et al. 1998)) or 2 μg of rabbit anti-glu-glu affinity purified polyclonal antibody (Chemicon Brand, Millipore, Temecula, CA, USA) was used to precipitate exogenous PP2A-A mutants. The beads were then removed from the well with antibody and added to a well containing 250 μg of total protein lysate, where they were mixed for 2 hours. The beads were then washed 3x 5 min in TBS before being deposited in 20 μl of PP2A assay buffer. The beads with antibody, and now PP2A protein complexes, were then analysed in a colourmetic assay for PP2A activity.
2.9.2. **Colourmetric assay**

The 20 μl of magnetic beads retrieved from the PP2A immunoprecipitation were combined with 60 μl of a PP2A-specific phosphopeptide, (750μM; K-R-pT-I-R-R) and incubated for 10 min at 30°C with gentle agitation every 2 min. To detect the amount of phosphate released into the buffer, 25 μl of the IP/peptide mixture was added to half volume 96 well plates, in duplicate, with 100 μl of Malachite Green Phosphate Detection Solution (Upstate Brand, Millipore, Temecula, CA, USA) and incubated for 10 min at room temperature to allow colour change. The absorbance was measured compared to a control standard phosphate curve (PO₄ concentration range 0-2000 pmol), at 620 nm absorbance on a FLUOstar OPTIMA plate reader (BMG Labtechnologies, Offenburg, Germany). Concentration of released phosphate was determined using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA), by linear regression.
2.10. **Immunohistochemistry**

Human breast tissue arrays were purchased from Super Biochips (Seoul, South Korea). Each array consisted of 60 total core sections including 40 breast cancer cores of various breast cancer types; 10 matched lymph node cores, and 10 matched normal breast tissue samples. Tissue specimens were dewaxed in xylene for 2x 5 min, and then rehydrated for 5 min in 100% and then 80% ethanol. Slides were washed twice in PBS before endogenous peroxidise activity was quenched for 5 min in 1% H₂O₂. Slides were then washed 2x in PBS before antigen retrieval by microwaving slides in Vector Antigen Unmasking Solution (Vector Laboratories Inc, Burlingame, CA, USA) for 2 min 20 sec on 100% power and then 10 min on 50% power. Slides were allowed to cool for 30 min in unmasking solution before washing 2x 5 min in PBS. Tissues were then permeabilised for 10 min in 0.1% Triton X-100, washed 2x 5 min in PBS and then blocked for 1 hour in 10% goat serum in IHC buffer (1% BSA and 0.1% Tween-20 in PBS) at room temperature in a humidified chamber. Blocking solution was removed, and tissues incubated with primary antibody diluted at 1:10,000 in 10% goat serum/IHC buffer, overnight in a humidified chamber at 4°C. Antibodies used were PP2A-A Alpha Subunit (6F9) Monoclonal Antibody (Covance; Emeryville CA, USA), Anti-PP2A-C subunit clone 1D6 (Upstate Brand, Millipore, Temecula, CA, USA) and PP2A B56gamma3 clone TQ11-1G6 antibody (PP2A-B’γ, Novus Biologicals, Sapphire Bioscience Pty Ltd, Waterloo, NSW, Aust). Isotype immunoglobulin controls were also incubated at the same time as the primary antibodies. For the PP2A-A antibody, Rat IgG (Sigma) was used as a negative control, while for both the PP2A-C and PP2A-B’γ antibodies, ID4.5 mouse immunoglobulin G [IgG] 2a MAb against Salmonella typhi supplied by Professor Leonie Ashman (University of Newcastle, NSW), was used as a negative control. The following day, excess primary antibody was removed with 2x 5 min PBS washes, before incubation in biotinylated secondary antibodies diluted 1:222 in 10% goat serum/IHC buffer for 1 hour at room temperature in a humidified chamber. The biotinylated antibodies were from VECTASTAIN® ABC kit (Vector Laboratories Inc). Secondary antibodies were removed with 2x 5 min PBS washes, prior to incubation for 1 hour in
ABC solution from the VECTASTAIN® ABC kit. The ABC solution was removed with 2x 5 min PBS washes and then tissues were incubated in Vector DAB solution (Vector Laboratories Inc) for 1 min. DAB solution was removed with 2x 5 min PBS washes before nuclei were counterstained for 30 sec with Gills double strength hematoxylin solution (ProSciTech, Thuringowa, QLD, Aus), rinsed with running distilled water, ‘Blued’ for 30 sec with Scotts Tap Water Substitute (0.04M MgSO4 heptahydrate), and rinsed again with distilled water. Tissues were dehydrated by incubation in 80% and then 100% ethanol each for 5 min. Slides were cleared with 2x 5 min xylene incubations. Once slides were dry, one drop of Vectamount mounting reagent (Vector Laboratories Inc) was applied to the tissues, and then immediately a coverslip was added and sealed with nail varnish. Staining was verified by pathologist Doctor Barbara Young of the Hunter Area Pathology Service.

Slides were scanned using an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. The algorithm measures weak (1+), medium (2+) and strong (3+) staining intensities over the entire image. A score value was calculated using the positive staining percentages;

\[ \text{Score} = 1 \times (\% \text{ weak}) + 2 \times (\% \text{ medium}) + 3 \times (\% \text{ strong}). \]
2.11. **Statistical analysis**

All statistical analysis was performed by using a two-tailed students t-test or Mann Whitney U test as indicated for each figure in GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA). Averages are presented +/- standard error of the mean (SEM).
3. CHARACTERISATION OF PP2A IN BREAST CANCER

3.1. Introduction

A number of lines of evidence suggests that PP2A is a tumour suppressor. Inhibition of PP2A function by pharmacological agents, DNA tumour viruses, and also altered PP2A subunit composition have been demonstrated to transform human cells. PP2A exists in cells as a heterotrimeric protein. The structural PP2A-A subunit binds both the PP2A-C catalytic subunit and also a regulatory PP2A-B subunit. All PP2A subunits have multiple isforms.

Reduced expression of the PP2A-Aα and -Aβ subunit isoforms have been observed in one breast cancer cell line and also a number of brain, cervical, and colon cancer cell lines (Suzuki et al. 2003; Zhou et al. 2003). PP2A-A subunit expression was also found to be reduced in 40% of glioblastoma and oligodendroglioma brain tumours (Colella et al. 2001). Importantly, 16/28 breast tumours examined by immunohistochemistry were scored negative for PP2A-A staining, whereas only 1/28 matched normal samples demonstrated negative PP2A-A expression (Suzuki et al. 2005). This study is an excellent indicator that PP2A-A expression may be reduced in breast cancer. However, as these samples were only scored as positive or negative and were not compared to any other breast cancer characteristics; further studies are required to elucidate whether PP2A-A is reduced to varying degrees in some breast cancer specimens and further if reduced PP2A-A correlates with other features of breast cancer. Functionally, reduced expression of the PP2A-A subunit decreases the number of holoenzymes that can be formed with regulatory B subunits in cells, and subsequently results in proteasomal degradation of the unbound regulatory B subunits (Strack et al. 2004; Chen et al. 2005; Ruediger et al.). In addition, as DNA tumour viruses bind to the same site on PP2A-A as the regulatory B subunits, they transform cells through displacement regulatory B subunits, rather than direct inhibition of the catalytic activity of the enzyme (Zhou et al. 2003; Cho et al. 2007a; Sablina et al. 2007). Indeed, there is increasing evidence that disruption of PP2A holoenzyme composition is an important mechanism by which the tumour suppressive function of PP2A is impaired. As regulatory B subunits target the holoenzyme to specific substrates (McCright et al. 1996;
altered expression of PP2A subunits may affect PP2A activity towards particular substrates without altering the total cellular PP2A activity.

Further, other mechanisms of altering PP2A function may also be important in cancer. Up-regulation of the nuclear oncprotein SET drives disease progression in chronic myeloid leukaemia (Neviani et al. 2005; Perrotti et al. 2006), while the α4 protein has been demonstrated to positively regulate cell spreading, migration and apoptosis (Kong et al. 2004; Kong et al. 2007). The catalytic PP2A-C subunit can also undergo post-translational modification, resulting in altered PP2A function. Tyrosine phosphorylation at Tyr$^{307}$ potently inhibits PP2A activity (Chen et al. 1992), and increased P-Tyr$^{308}$ is observed in both chronic and acute myeloid leukaemia (Neviani et al. 2005; Roberts et al. 2010). In contrast, methylation at Leu$^{309}$ appears to aid regulatory B subunit binding, particularly the PP2A-B subunit family (Cho et al. 2007b). Thus, PP2A function may be altered in multiple ways, resulting in disruption to key cellular signalling pathways involved in breast cancer progression.

Mutations in the structural PP2A-A subunit have been identified in multiple cancers including breast cancer (Ruediger et al. 2001b; Ruediger et al. 2001a). The rate of PP2A-A mutation is quite low (Calin et al. 2000), but the functional effect of these mutations is interesting. The majority of mutations are point mutations in regions critical for subunit binding. Thus, a number of these mutations result in defective binding of regulatory B subunits (Ruediger et al. 2001b; Ruediger et al. 2001a; Xu et al. 2006; Cho et al. 2007b) as well as impaired phosphatase activity (Chen et al. 2005). Therefore, while the mutation rate of PP2A-A is quite low, as these mutations disrupt PP2A holoenzyme composition, it may be beneficial to consider altered PP2A function, either by mutation, reduced subunit expression, or other mechanism of PP2A regulation, when assessing the role of PP2A as a tumour suppressor in breast cancer.

The expression of regulatory PP2A subunits in breast cancer has not yet been determined. In addition, other mechanisms of PP2A regulation including
post-translational modification of PP2A-C and expression of endogenous PP2A inhibitory proteins remains to be examined in breast cancer. This chapter aimed to firstly determine PP2A subunit expression, PP2A-C post-translational modification, expression of PP2A binding proteins and also PP2A activity in a panel of breast cancer cell lines. Finally, PP2A subunit expression was assessed in human breast tumours by tissue arrays and compared with clinical characteristics of the cancers to further determine the role of PP2A in breast cancer.
3.2. Breast cancer cell lines

This thesis employed a number of cell lines in different cell culture techniques to characterise PP2A expression in normal breast and breast cancer. Table 3.1 contains a summary of these cell lines including the status of some important receptors and signalling proteins.

3.2.1. Human mammary epithelial cells

Primary human mammary epithelial cells (HMECs) were used as controls for PP2A subunit protein and mRNA expression levels as well as PP2A activity. The HMECs used in this thesis were obtained from Gibco® (Invitrogen, Carlsbad, CA, USA) and they are cultured directly from normal, human reduction mammoplasty tissue. While HMECs are the ideal control cells for breast cancer studies, these cells reach senescence after approximately 15-20 passages in culture, and thus they are not suitable for long term experiments such as stable expression of PP2A mutations.

3.2.2. MCF10A

MCF10A cells were generated by passage of human mammary epithelial cells from fibrotic disease, which is not a cancerous disease, in low calcium media. These cells are immortal and continually proliferate in culture, but do not form tumours in immune-deficient mice (Soule et al. 1990). High resolution gene mapping of the MCF10A cells, compared to the mortal HMECs they were derived from, showed that the immortal MCF10A cells had lost one copy of the BRCA2, RB and BCL2 genes. Both copies of genes CDKN2A and CDKN2B were also deleted, which encode cyclin dependent kinase inhibitor 2A (p16) and multiple tumor suppressor 2 (MTS-2) respectively. These proteins regulate the Rb and p53 pathways. Gene gains were observed in the ERBB2 (Her2/neu) gene that encodes the ErbB2 receptor, PIK3CA which encodes the catalytic subunit of PI3K, and also MYC that encodes c-Myc (Worsham et al. 2006).
Table 3.1 Breast cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Source</th>
<th>Tumour Type</th>
<th>Tumourigenic</th>
<th>ER</th>
<th>PR</th>
<th>HER2(^1)</th>
<th>EGFR(^1)</th>
<th>p53(^2)</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>HMEC</td>
<td>Primary Breast</td>
<td>-</td>
<td>No</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>(Neve et al. 2006; Subik et al. 2010)</td>
</tr>
<tr>
<td>MCF10A</td>
<td>Primary Breast</td>
<td>Fibrotic disease</td>
<td>No</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MCF7</td>
<td>Pleural effusion</td>
<td>Invasive ductal</td>
<td>Weakly</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>WT</td>
<td>(Soule et al. 1973; Engel et al. 1978; Lacroix et al. 2004; Neve et al. 2006; Subik et al. 2010)</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Pleural effusion</td>
<td>Adenocarcinoma</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mut</td>
<td>(Cailleau et al. 1978; Engel et al. 1978; Neve et al. 2006; Subik et al. 2010)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Pleural effusion</td>
<td>Adenocarcinoma</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Mut</td>
<td>(Cailleau et al. 1978; deFazio et al. 2000; Ripple et al. 2005; Neve et al. 2006)</td>
</tr>
<tr>
<td>HMT-3522-S2</td>
<td>Primary Breast</td>
<td>Fibrotic disease</td>
<td>Very Weakly</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>(Briand et al. 2001; Rizki et al. 2008)</td>
</tr>
<tr>
<td>HMT-3522-T42</td>
<td>HMT-3522-S2 cells</td>
<td>Passaged tumourigenicity</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>(Briand et al. 2001; Rizki et al. 2008)</td>
</tr>
<tr>
<td>T47D</td>
<td>Pleural effusion</td>
<td>Invasive ductal</td>
<td>Weakly</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Mut</td>
<td>(Engel et al. 1978; Lacroix et al. 2004; Neve et al. 2006)</td>
</tr>
<tr>
<td>DU4475</td>
<td>Skin metastasis</td>
<td>Undifferentiated</td>
<td>Yes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>WT</td>
<td>(deFazio et al. 2000; Lacroix et al. 2004)</td>
</tr>
</tbody>
</table>

\(^1\)Receptor status shown is determined from protein levels. [ ] indicates inferred from mRNA as protein levels are not known.
\(^2\) P53 mutation status was obtained using the COSMIC database available at [http://www.sanger.ac.uk/genetics/CGP/CellLines/](http://www.sanger.ac.uk/genetics/CGP/CellLines/) 25/01/2012.
Due to their non-tumourigenic status, MCF10A cells were also considered as controls for PP2A subunit protein and mRNA expression, and PP2A activity experiments. One of the most important features of MCF10A cells is their ability to form spherical acini in a three-dimensional culture model. These acini are functionally similar to human breast ducts (Underwood et al. 2006) and also to three-dimensional cultures of primary HMECs (Soule et al. 1990). Therefore MCF10A three-dimensional cultures were employed to assess PP2A expression in a model of the human breast duct.

3.2.3. MCF7
The MCF7 cell line, one of the most commonly utilised breast cancer cell lines, was originally isolated in 1973 from a plural effusion (Soule et al. 1973). MCF7 cells are considered an established model of both estrogen and progesterone receptor positive breast cancer as they originally required estrogen supplementation for growth (Jones et al. 2000). However, over time and with continual passaging in different laboratories, many MCF7 stocks now have varied responses to estrogen (Jones et al. 2000). The MCF7 cells used in this thesis were not supplemented with estrogen.

3.2.4. MDA-MB-231 and MDA-MB-468
The MDA-MB cell lines were all established from metastatic tumours isolated from patients at the M. D. Anderson Hospital and Tumor Institute (Cailleau et al. 1978). They have varied features and tumourigenic potential. Both MDA-MB-231 and MDA-MB-468 were isolated from pleural effusions and form tumours in mice (Cailleau et al. 1978).

3.2.5. HMT-3522-S2 and HMT-3522-T42
The HMT-3522 cell line was originally isolated from fibrotic disease with no suspicion of malignancy (Briand et al. 1987). This cell line was later termed HMT-3522-S1 and is not tumourigenic. Prolonged propagation of the HMT-3522-S1 cell line without epidermal growth factor resulted in cellular transformation, giving rise to the HMT-3522-S2 cell line, which is very weakly tumourigenic. Continued passaging in EGF-free media resulted in increased
expression of the EGF receptor. Tumours formed by HMT-3522-S2 cells were retrieved from mice, re-passaged in vitro, and re-transplanted into mice. A second round of in vitro/in vivo passaging resulted in the HMT-3522-T42 cell line which is highly tumourigenic (Briand et al. 1996; Briand et al. 2001). This series of breast cancer cell lines is useful as expression of proteins can be assessed at increasing stages of metastasis.

3.2.6. T47D
Like MCF7, the T47D cell line was isolated from a pleural effusion, and expressed both the estrogen and progesterone receptors (Keydar et al. 1979). Also, as for MCF7, in our laboratory these cells proliferate without estrogen supplementation.

3.2.7. DU4475
The DU4475 cell line, isolated in 1979, has a fascinating suspension morphology, where cells grow in clumps with ‘serpentine outgrowths’ (Langlois et al. 1979). These cells do not express estrogen or progesterone receptors, but still retain some epithelial characteristics, including tight junctions between the cells. As this cell line was isolated from a skin lesion, and forms aggressive tumours in mice, it is representative of highly metastatic breast disease.
3.3. Results

3.3.1. PP2A subunit protein expression is varied in a panel of breast cancer cell lines

A panel of breast cancer cell lines was employed in order to determine if the relative expression of PP2A subunits is altered in breast cancer. Whole cell lysates were separated on acrylamide gels, transferred to nitrocellulose membranes and probed with anti-PP2A subunit antibodies. For all subunits, PP2A expression was normalised to actin as a loading control and compared with HMEC to determine if any subunits have significantly altered expression in cancerous cell lines compared to normal mammary epithelial cells.

Expression of the catalytic subunit, PP2A-C, as measured with an antibody that recognises both the α and β isoforms of the subunit, is relatively constant across the panel of cell lines (Figure 3.1A). PP2A-C expression is slightly increased in MCF10A cells and decreased in the HMT-3522-T42 cells. In contrast, expression of the structural PP2A-A subunit is decreased in a number of the breast cancer cell lines (Figure 3.1B). The MCF7 and HMT-3522-S2 cell lines demonstrate decreased expression with an antibody targeting both the PP2A-Aα and -Aβ isoforms of the PP2A-A subunit, but no significant change in -Aβ alone, suggesting it is the -Aα isoform that is reduced in these cells. Interestingly, T47D cells show increased PP2A-Aβ expression, and the DU4475 cells have very high PP2A-Aβ levels. In contrast, the antibody recognising both PP2A-Aα and -Aβ shows less than 50% expression in the DU4475 cells compared to HMECs, suggesting that PP2A-Aα is greatly reduced in these cells. The HMT-3522-T42 cells also demonstrate significant reductions in PP2A-A expression, as determined by both antibodies, with expression levels only approximately 50% of HMECs.

In the PP2A-B family, PP2A-Bα expression is significantly reduced in all of the cancerous cell lines, compared to HMECs, while the immortalised MCF10A mammary epithelial cells have similar expression to HMECs (Figure 3.2A). The MDA-MB-468 and HMT-3522 cell lines demonstrated the greatest reductions, down to nearly 50% of HMEC levels. Expression of the PP2A-Bδ subunit has
Figure 3.1 Altered expression of PP2A subunit proteins in a panel of breast cancer cell lines.

Whole cell lysates were analysed for PP2A subunit expression by western blotting with specific antibodies as indicated in each graph. Protein bands were quantitated by normalising to actin as a loading control and then compared to HMEC expression. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01 compared to HMEC using a students t-test. A representative blot for each subunit is shown below the graph.

A) PP2A-C expression normalised to actin.

B) For PP2A-A two different antibodies were used as indicated by the blots.
Figure 3.2 Altered expression of PP2A subunit proteins in a panel of breast cancer cell lines.
Whole cell lysates were analysed for PP2A subunit expression by western blotting with specific antibodies as indicated in each graph. Protein bands were quantitated by normalising to actin as a loading control and then compared to HMEC expression. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01, *** p<0.001 compared to HMEC using a students t-test. A representative blot for each subunit is shown below the graph.
reduced expression in all of the cell lines compared to HMECs, except for the MCF7 cells which have significantly increased protein expression (Figure 3.2B). The PP2A-Bβ and -Bγ subunits were not examined, due to known neuronal specific enrichment (Zolnierowicz et al. 1994).

In the PP2A-B′ family, DU4475 cells have nearly double the PP2A-B′α protein expression compared to HMECs. The other cell lines also demonstrated some increases in PP2A-B′α expression, however this was not significant over 3 independent experiments (Figure 3.2C). Two breast cancer cell lines, MCF7 and HMT-3522-S2, exhibited increased PP2A-B′β protein expression, whereas the MDA-MB-468 and DU4475 cell lines had reduced expression (Figure 3.2D).

The PP2A-B′ subunit family isoforms also had quite varied expression across the panel of breast cancer cell lines (Figure 3.3). The known 55kDa band of PP2A-B′α was reduced in the HMT-3522-T42 cell line (Figure 3.3A). A lower molecular weight protein (approximately 45kDa) also reacts strongly with the PP2A-B′α antibody, but interestingly was only observed in the breast cancer cell lines MCF7, MDA-MB-468, T47D and DU4475, but not the HMEC or MCF10A cells. Whether this band is the second transcript variant of PP2A-B′α with the shorter 5′ UTR, a cleavage product of PP2A-B′α, or a non-specific protein is not known.

The expression of PP2A-B′β was slightly increased in 2 breast cancer cell lines, MCF7 and MDA-MB-231, with a larger increase observed in the DU4475 cells (Figure 3.3B).

The PP2A-B′γ subunit demonstrated the most significant and comprehensive reduction in expression of all of the subunits examined (Figure 3.3C). The immortalised MCF10A mammary epithelial cells have about 50% PP2A-B′γ expression compared to HMECs, whereas in the breast cancer cell lines expression is reduced to only about 25% of HMEC expression.
Figure 3.3 Altered expression of PP2A subunit proteins in a panel of breast cancer cell lines.
Whole cell lysates were analysed for PP2A subunit expression by western blotting with specific antibodies as indicated in each graph. Protein bands were quantitated by normalising to actin as a loading control and then compared to HMEC expression. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01, ***p<0.001 compared to HMEC using a students t-test. A representative blot for each subunit is shown below the graph. Figures with blue and green bars are two different bands from the same blots displayed on the same graph.
PP2A-B’δ has three known transcript variants. The 70kDa variant 1 shows increased expression in four of the breast cancer cell lines: MDA-MB-231, MDA-MB-468, T47D and DU4475; whereas the HMT-3522-S2 cell line has slightly reduced expression (Figure 3.3D). PP2A-B’δ variants 2 and 3 have predicted molecular weights of 66 and 58kDa respectively. Two strongly reactive bands were observed on the PP2A-B’δ western blots (Figure 3.3E) along with the 70kDa band, however these have lower molecular weights of approximately 48 and 45 kDa. Whether these bands are further isoforms, degraded variants, or due to non-specific antibody binding remains to be determined. Every cell line demonstrated reduced expression of these bands, compared with HMEC. The non-tumourigenic MCF10A, has about 50% the expression of HMECs, and the very weakly tumourigenic HMT-3522-S2 is further reduced to about 25%. All of the other breast cancer cell lines, including the HMT-3522-T42 which are a more tumourigenic line derived from the HMT-3522-S2, have less than 10% expression compared to HMECs.

Interestingly, the 55kDa PP2A-B’ε isoform is only expressed at very low levels in HMEC cells, but more strongly expressed in the MCF10A cells and breast cancer cell lines (Figure 3.3F, blue bars). A strongly reactive 37kDa protein band shows reduced expression in the MCF10A, MDA-MB-231, MDA-MB-468 and HMT-3522-T42 cell lines compared with HMECs, whereas MCF7 cells demonstrate increased expression (Figure 3.3F, green bars). Again, whether this is a transcript variant or non-specific reactivity remains to be determined. A summary of all alterations in protein expression observed in the panel of breast cancer cell lines is presented in Table 3.2.

3.3.2. PP2A subunit mRNA expression does not correlate with protein expression

To determine if the altered expression of PP2A subunit proteins in breast cancer cell lines is due to altered gene expression, mRNA from the panel of breast cancer cell lines was isolated and reverse transcribed into cDNA. Quantitative real time PCR analysis was performed to determine the relative levels of PP2A subunit mRNA expression compared to normal HMEC cells. All analysis was
Table 3.2 Summary of PP2A protein expression in breast cell lines compared with HMEC.

<table>
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<tr>
<th>PP2A subunit</th>
<th>MCF10A</th>
<th>MCF7</th>
<th>MDA- MB-231</th>
<th>MDA-MB-468</th>
<th>HMT-3522-S2</th>
<th>HMT-3522-T42</th>
<th>T47D</th>
<th>DU4475</th>
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<th>Total ↑</th>
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<td>Aβ</td>
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<tr>
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<td>↓↓</td>
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<td>-</td>
<td>↑↑</td>
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<td>2/8</td>
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</table>

↓ p<0.05, ↓↓ p<0.01, ↓↓↓ p<0.001 (and also for ↑)
normalised to a neuronal SH-EP cell line as the primers used had previously been optimised for mRNA expression in these cells and they display robust expression of all subunits.

Isoform specific primers were designed to amplify the α and β isoforms of both PP2A-C and PP2A-A independently. The mRNA expression for PP2A-C is quite varied (Figure 3.4A), with the PP2A-Cα isoform increased in HMT-3522-S2 and DU4475 cell lines, but decreased in MDA-MB-468 and T47D cells. The PP2A-Cβ isoform is reduced in MCF7, MDA-MB-468 and T47D cell lines. No alterations in mRNA expression were observed in the MCF10A and HMT-3522-T42 cell lines which demonstrated slight variations in protein expression (Figure 3.1A). No correlation was observed between mRNA expression for either isoform and total PP2A-C protein expression (Appendix 2a A-B).

The PP2A-A subunit demonstrated marked reductions in mRNA expression (Figure 3.4B), with the PP2A-Aα isoform reduced approximately two fold in the MDA-MB-468 and HMT-3522-S2 cell lines compared with HMECs. All of the breast cancer cell lines have reduced PP2A-Aβ mRNA expression compared with HMECs, with the non-tumourigenic MCF10A showing similar expression to HMECs. Interestingly, only the HMT-3522-T42 cells showed reduced PP2A-Aβ protein expression (Figure 3.1B). Thus, for PP2A-A no correlation between mRNA and protein expression was observed (Appendix 2a C-D).

In the PP2A-B family, PP2A-Bα mRNA expression is reduced to approximately half HMEC levels in 3 of the breast cancer cell lines: MDA-MB-468, HMT-3522-T42 and T47D (Figure 3.5A). PP2A-Bδ mRNA expression is relatively constant, although the MDA-MB-468 cells have about half the mRNA expression of HMECs, whereas HMT-3522-S2 cells demonstrate increased mRNA expression. All of the breast cancer cell lines have reduced PP2A-Bα and PP2A-Bδ protein expression compared with HMECs, except for MCF7 cells which have increased PP2A-Bδ expression PP2A-(Figure 3.2A and B). Neither subunit demonstrated correlation between mRNA and protein expression (Appendix 2b A-B).
Figure 3.4 Altered expression of PP2A subunit mRNA in a panel of breast cancer cell lines by real-time PCR.

RNA was extracted from cells and mRNA reverse transcribed using oligo dT primers. Real time PCR reactions were performed using isoform specific primers in a SYBR Green detector assay. All cell lines were normalised to a neuronal SH-EP cell line as the primers used had previously been optimised for mRNA expression in these cells and they display robust expression of all subunits. Error bars are SEM for at least three different experiments. * p<0.05, ** p<0.01, ***p<0.001 compared to HMEC using a students t-test.
Figure 3.5 Altered expression of PP2A subunit mRNA in a panel of breast cancer cell lines by real-time PCR.

RNA was extracted from cells and mRNA reverse transcribed using oligo dT primers. Real time PCR reactions were performed using isoform specific primers in a SYBR Green detector assay. All cell lines were normalised to a neuronal SH-EP cell line as the primers used had previously been optimised for mRNA expression in these cells and they display robust expression of all subunits. Error bars are SEM for at least three different experiments. * p<0.05, ** p<0.01, *** p<0.001 compared to HMEC using a students t-test.
Interestingly, in the PP2A-B” family, PP2A-B”α mRNA expression does not match the pattern of protein expression levels (Figure 3.5C and 3.2C), whereas PP2A-B”β has a very similar pattern of mRNA and protein expression (Figures 3.5D and 3.2D). For PP2A-B”α, MCF10A, MCF7 and DU4475 demonstrated reduced mRNA expression (Figure 3.5C), but none of these cell lines have altered protein expression compared with HMECs (Figure 3.2C). PP2A-B”β on the other hand, displayed increased mRNA expression in the MCF7 and HMT-352-S2 cells, but decreased expression in MDA-MB-468 cells (Figure 3.5D). Similarly, MCF7 and HMT-3522-S2 cells have increased PP2A-B”β protein expression, whereas MDA-MB-468 cells have decreased protein expression (Figure 3.2D). However, linear regression analysis reveals no significant correlation between mRNA and protein expression for either of these PP2A subunits (Appendix 2b).

With regards to the PP2A-B’ subunit family isoforms, mRNA expression does not correlate with protein expression (Appendix 2c and 2d). PP2A-B’α is increased at the gene level in MCF10A, MDA-MB-468, HMT-3522-S2 and DU4475 cell lines (Figure 3.6A), but none of these cell lines have significantly increased PP2A-B’α protein expression of the expected 55kDa protein (Figure 3.3A). However, the lower 45kDa reactive band was increased in the MCF7, MDA-MB-486, T47D and DU4475 cells (Figure 3.3A). That the mRNA is significantly increased in some of these cell lines may indicate that this is a true PP2A-B’α protein that is either alternatively spliced, truncated or cleaved. PP2A-B’β mRNA expression is increased in HMT-3522-T42 cells and decreased in MDA-MB-468 cells (Figure 3.6B), but neither of these cell lines have altered PP2A-B’β protein expression (Figure 3.3B). PP2A-B’γ expression is increased at the gene level in HMT-3522-S2, HMT-3522-T42 and DU4475 cells (Figure 3.6C), but conversely all cell lines have reduced PP2A-B’γ protein expression compared to HMECs (Figure 3.3C). All of the cell lines except for MDA-MB-468 have significantly reduced PP2A-B’δ mRNA expression (Figure 3.6D), which does not match PP2A-B’δ transcript variant 1 protein expression (Figure 3.3D). Interestingly, all of the cell lines do have reduced expression of an unidentified pair of protein bands that react strongly with the
Figure 3.6 Altered expression of PP2A subunit mRNA in a panel of breast cancer cell lines by real-time PCR.

RNA was extracted from cells and mRNA reverse transcribed using oligo dT primers. Real time PCR reactions were performed using isoform specific primers in a SYBR Green detector assay. All cell lines were normalised to a neuronal SH-EP cell line as the primers used had previously been optimised for mRNA expression in these cells and they display robust expression of all subunits. Error bars are SEM for at least three different experiments. * p<0.05, ** p<0.01, ***p<0.001 compared to HMEC using a student's t-test.
PP2A-B’δ subunit antibody in HMEC cells (Figure 3.3F), but whether these are real isoforms of PP2A-B’δ remains to be determined. PP2A-B’ε mRNA expression was varied with the MDA-MB-468 cell lines having reduced expression; while MCF7, HMT-3522-S2 and HMT-3522-T42 cell lines have nearly 1.5 times the gene expression of HMEC cells (Figure 3.6E). This pattern of mRNA expression is not similar to the protein expression for either of the protein bands that react with the anti-PP2A-B’ε antibody (Figure 3.3F).

A summary of mRNA expression for all PP2A subunits examined is presented in Table 3.3. Comparison of gene expression with protein expression levels in Table 3.2 reveals that, for the most part, altered mRNA expression does not explain the changes in PP2A subunit protein expression observed in the panel of breast cancer cell lines.
Table 3.3 Summary of PP2A mRNA expression in breast cell lines compared with HMEC.

<table>
<thead>
<tr>
<th>PP2A subunit</th>
<th>MCF10A</th>
<th>MCF7</th>
<th>MDA-MB-231</th>
<th>MDA-MB-468</th>
<th>HMT-3522-S2</th>
<th>HMT-3522-T42</th>
<th>T47D</th>
<th>DU4475</th>
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↓ p<0.05, ↓↓ p<0.01, ↓↓↓p<0.001 (and also for ↑)
3.3.3. Mutations in PP2A-A do not account for reduced regulatory B subunit protein expression

Mutations in PP2A-A have been shown to prevent regulatory B subunit binding (Ruediger et al. 2001b; Ruediger et al. 2001a; Zhou et al. 2002), and result in their subsequent degradation (Ruediger et al.), presumably by proteosomal degradation (Strack et al. 2004). To determine if mutations in PP2A-A contributes to the reduction in regulatory B subunit expression observed in Section 3.2.1, mRNA was isolated from all of the breast cancer cell lines and reverse transcribed into cDNA. Isoform specific primers were used to amplify and sequence the full length PP2A-Aα gene and the two transcript variants of PP2A-Aβ in two overlapping sections. The only alteration identified in any of the sequences was a silent mutation in the PP2A-Aα subunit, a substitution of nucleotide 154 C→G, in the HMT-3522-T42 cell line (data not shown). No mutations were found in either transcript variants of PP2A-Aβ. Thus, mutations do not contribute to the observed regulatory B subunit down-regulation.

3.3.4. Post-translational methylation of PP2A-C is reduced in breast cancer cell lines, but phosphorylation of PP2A-C does not change

Post-translational methylation of PP2A-C at leucine 309 (M-PP2A-C) does not alter PP2A activity, but is important for regulatory B subunit binding. In particular, PP2A-B family subunits require M-PP2A-C for holoenzyme formation in vivo, whereas PP2A-B’ subunits will bind non-M-PP2A-C (Longin et al. 2007). The methylation status of PP2A-C in the panel of breast cell lines was determined using an antibody that specifically recognises M-PP2A-C. A number of the breast cell lines have reduced M-PP2A-C when compared with non-methylated PP2A-C expression. MCF10A, MCF7, HMT-3522-S2 and T47D all show significant reductions in the ratio of M-PP2A-C to non-methylated PP2A-C compared with HMECs, with MDA-MB-468 cells being just beyond significance (P= 0.0554) (Figure 3.7A). All of these cell lines have reduced expression of either PP2A-Bα or -Bδ (Figure 3.2A and 3.2B), suggesting that altered PP2A-C methylation may have an effect on PP2A-B subunit family isoform levels. However as PP2A-Bα is reduced in all of the breast cancer cell lines, but not MCF10A cells, and PP2A-B’δ is increased in
Figure 3.7 PP2A-C post-translational modifications in a panel of breast cancer cell lines. Whole cell lysates were analysed for PP2A-C subunit post-translational modification by western blotting with antibodies that specifically recognise modified forms of PP2A-C. Protein bands were quantitated by normalising to actin as a loading control, expressed as a percentage of non-methylated PP2A-C and then compared to HMEC expression. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01 compared to HMEC using a students t-test.

A) Methylated Leu$^{309}$ PP2A-C

B) Phosphorylated Tyr$^{307}$ PP2A-C

A representative blot for each PP2A-C modification is shown below the graph.
MCF7 cells, other mechanisms must also be in play to regulate PP2A-B subunit protein expression.

Tyrosine phosphorylation of PP2A-C at tyrosine 307 (P-PP2A-C) inactivates the catalytic activity of PP2A in vitro (Chen et al. 1992), and is a proposed mechanism to remove inhibitory regulation of growth factor signalling pathways by PP2A (Chen et al. 1994). In this panel of breast cancer cell lines, the level of P-PP2A-C was constant across all breast cancer cell lines compared to HMECs (Figure 3.7B).

3.3.5. Expression of PP2A binding proteins is altered in breast cancer
PP2A activity can be inhibited by the endogenous protein inhibitor SET, and increased expression of this protein in leukaemia drives disease progression (Li et al. 1996; Neviani et al. 2005; Roberts et al. 2010). Western blotting with an anti-SET antibody was used to determine if increased expression of this protein may contribute to the progression of breast cancer (Figure 3.8A). Increased SET expression was observed in MCF7, MDA-MB-231, MDA-MB-468, T47D and DU4475 breast cancer cell lines. Thus, increased SET expression may play a role in the progression of breast cancer. The α4 protein binds directly to monomeric PP2A-C, inhibiting PP2A activity towards numerous substrates (Inui et al. 1998). In the panel of breast cancer cell lines α4 protein expression was increased in MDA-MB-468 cells, but reduced in T47D and DU4475 cells, as determined by western blotting with an anti-α4 antibody (Figure 3.8B).

3.3.6. PP2A activity is increased in breast cancer cell lines
As PP2A is a proposed tumour suppressor (Janssens et al. 2005), and altered protein expression of PP2A subunits was identified in a number of breast cancer cell lines (Section 3.3.1), PP2A activity was also determined in these breast cancer cell lines. PP2A activity was measured by firstly immunoprecipitating the PP2A-C subunit from whole cell lysates, followed by in vitro incubation with a phosphopeptide that is specifically de-phosphorylated by PP2A. Released phosphate was measured by a colourimetric malachite green assay and the results are presented in Figure 3.9. In direct contrast to what would be expected
Figure 3.8 Altered expression of the PP2A binding proteins.
Whole cell lysates were analysed for PP2A binding protein expression by western blotting. Protein bands were quantitated by normalising to actin as a loading control and then comparison to HMEC expression. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01, ***p<0.001 compared to HMEC using a students t-test.

A) Endogenous protein inhibitor SET
B) PP2A-C binding protein α4

A representative blot for each protein is shown below the graph.
Figure 3.9 PP2A activity is increased in a number of breast cancer cell lines. PP2A-C was immunoprecipitated from whole cell lysates and incubated with a specific phosphopeptide substrate for 10mins. Release of phosphate from the peptide was determined by colourmetric assay. Addition of 10nM okadaic acid (OA) to the immunoprecipitate of an identical MCF10A sample demonstrates this assay likely measures PP2A activity. Error bars are SEM for three independent experiments. * p<0.05, ** p<0.01, ***p<0.001 compared to HMEC using a students t-test.
for a tumour suppressor, PP2A activity towards this particular phosphopeptide substrate is increased in all cell lines compared to HMEC. This increase was statistically significant for the MCF10A, MDA-MB-468, T47D and DU4475 cell lines. Confirmation of the specificity of this reaction for PP2A activity was aided by reduced activity upon addition of the PP2A inhibitor, okadaic acid (OA) (although it is noted that OA also inhibits PP4 and PP6).

3.3.7. Sub-cellular location of PP2A subunits in breast cancer

To further characterise the role of PP2A in breast cancer, the sub-cellular location of the PP2A-A and PP2A-C subunits was investigated by immunofluorescence. PP2A functions in many cellular processes and the location of the enzyme may provide a clue as to the role of PP2A in these cells. Cells were grown on glass cover slips prior to fixing, permeabilisation, and protein detection using specific anti-PP2A subunit antibodies followed by fluorescently labelled secondary antibodies. Proteins were analysed while cells were still sub-confluent and demonstrated cellular proliferation, and also once cells reached confluence and ceased proliferating due to contact inhibition. Cellular proliferation was confirmed using a Ki67 antibody labelling (Appendix 3).

Primary HMEC cultures are a heterogeneous mixture of luminal epithelial cells and myoepithelial cells. Luminal epithelial cells are the inner, milk producing cell layer, while myoepithelial cells have a contractile function and are located between the luminal epithelial cells and the basement membrane. Myoepithelial cells express α6β4 integrins to adhere to the basement membrane, but luminal epithelial cells do not express these particular integrins (Jones et al. 1997). PP2A-C is highly expressed in the cytoplasm of luminal epithelial cells, but not in myoepithelial cells, identified by α6 integrin expression (Figure 3.10). In contrast, PP2A-A is highly expressed at the periphery of the myoepithelial cells with low PP2A-C expression (Figure 3.11), but is also present at the periphery of the of the luminal epithelial cells as demonstrated by co-localisation with PP2A-C (yellow on merged image). PP2A-A also co-localises with β4 integrin
Figure 3.10 PP2A-C subunit expression in primary human mammary epithelial cells.
HMECs were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

HMECs contain two cell types: myoepithelial cells which stain for α6 integrin (red) as they attach to the basement membrane and also luminal epithelial cells that do not stain for α6 integrin. PP2A-C (green) demonstrates greater expression in luminal epithelial cells.
Figure 3.11 PP2A subunit expression in primary human mammary epithelial cells.
HMECs were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) has a similar filamentous, peripheral pattern of expression to α6 integrin (Figure 3.10), in myoepithelial cells that only weakly express PP2A-C, but is also co-localised (yellow on merge image) with PP2A-C (green) in luminal epithelial cells.
in HMEC cells, confirming that PP2A-A is expressed in the myoepithelial cells (Appendix 4). When cells were incubated with fluorescently labelled secondary antibodies alone in an identical procedure to immunofluorescence with anti-PP2A subunit antibodies, no background fluorescence was observed due to nonspecific adherence of these antibodies to cellular proteins (Figure 3.12). In contrast to the pattern of PP2A expression in HMEC cells, PP2A-A and PP2A-C are highly expressed in the nuclei of sub-confluent MCF10A cells, as well as the cytoplasm (Figure 3.13). These cells are highly proliferative, as demonstrated by Ki67 staining (Figure 3.14 and Appendix 3); whereas HMEC cells proliferate much more slowly and only a few Ki67 positive cells were observed even in sub-confluent cultures (Appendix 3). PP2A-A staining also showed a number of punctuate nuclear bodies in some cells. Interestingly, when MCF10A cells reach confluence, PP2A-A and PP2A-C expression is reduced and no longer nuclear (Figure 3.15). These images were taken on the same exposure to allow qualitative comparison. Confluent MCF10A cells cease proliferating due to contact inhibition, and very little Ki67 staining is observed in these cells (Figure 3.14 and Appendix 3).

T47D and MCF7 cells are characteristic of luminal epithelial breast cancer (Gordon et al. 2003). T47D cells grow in small clumps of cells, with PP2A-A and PP2A-C strongly expressed at the membrane of the peripheral cells in these clumps (Figure 3.16, arrows). Interestingly, PP2A-A and PP2A-C are expressed in the nuclei of these peripheral cells, but not in the nuclei of the central cells. Ki67 staining reveals the peripheral cells are highly proliferative, whereas the inner cells are not (Appendix 3), which corresponds with the observed nuclear expression of PP2A in proliferating MC10A cells (Figure 3.13). Cytoplasmic PP2A staining is also observed in the T47D cells, with good correlation between PP2A-A and PP2A-C expression (yellow on merged image Figure 3.16). PP2A-A is also present in nuclear bodies as observed for MCF10A cells. In contrast, PP2A-A expression in MCF7 cells was not detected in the nuclei, nor were any nuclear bodies identified (Figure 3.17). Interestingly, PP2A-C was still expressed in the nuclei of some MCF7 cells that were not surrounded by
Figure 3.12 Fluorescent secondary antibody controls.
HMEC cells were grown on glass coverslips; fixed, permeabilised and during primary antibody incubation these cells were incubated with buffer alone, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.
Figure 3.13 PP2A subunit expression in MCF10A cells.

MCF10A cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-C (green) are expressed in both the nucleus and the cytoplasm of sub-confluent, proliferating MCF10A cells. PP2A-A is also present in nuclear bodies (arrows).
Figure 3.14 Confluent MCF10A cells cease to proliferate.

Cells were grown on glass coverslips; fixed, permeabilised and proliferating cells detected with anti-Ki67 antibody, followed by secondary fluorescently labelled antibody. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

Sub-confluent cultures (top) demonstrate greatest cellular division at the edges of groups of cells, with the inner cells ceasing to proliferate. In confluent cultures (bottom), most cells have ceased to proliferate, as only a few cells demonstrate Ki67 staining.
Figure 3.15 PP2A subunit expression in confluent MCF10A cultures.
MCF10A cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

When MCF10A cells reach confluence and stop proliferating, PP2A-A (red) and PP2A-C (green) expression is no longer nuclear. Although PP2A-C still demonstrates some cytoplasmic expression, PP2A-A has substantially reduced expression.
Figure 3.16 PP2A subunit expression in T47D cultures.
T47D cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

T47D cells grow as cohesive groups of cells, and PP2A-A (red) and PP2A-C (green) are expressed in the nucleus, cytoplasm and also along the peripheral membrane (arrows) of the proliferating cells at the edges of cohesive groups. In addition, PP2A-A is also present in nuclear bodies.
Figure 3.17 PP2A subunit expression in MCF7 cultures.

MCF7 cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

MCF7 cells demonstrate predominantly cytoplasmic staining of PP2A-A (red) and PP2A-C (green). PP2A-A is not expressed with PP2A-C in the nuclei of proliferating cells, but does have increased expression at the cell membrane.
other cells. Despite lack of co-localisation in the nucleus, PP2A-A and PP2A-C co-localised in the cytoplasm of MCF7 cells (yellow on merged image, Figure 3.17), with slightly more PP2A-A expressed at the cell membrane. In addition, confluent cultures of both T47D and MCF7 cells showed reduced expression of both PP2A-A and PP2A-C with no nuclear expression, as was observed for MCF10A confluent cultures (Appendix 5).

The HMT-3522-S2 cell line also had a similar staining pattern to MCF10A, with PP2A-C clearly expressed in the nuclei of cells that were relatively free from contact with other cells and PP2A-A predominantly expressed in the nuclear bodies (Figure 3.18). Once cells became more confluent, PP2A expression was primarily cytosolic (right side of Figure 3.18 and also Appendix 5). In contrast to their parental HMT-3522-S2 cells, HMT-3522-T42 cells did not display nuclear PP2A expression even in sub-confluent cultures (Figure 3.19). These cells appeared much more fibroblastic, with some filamentous staining patterns demonstrated by PP2A-C and higher expression of PP2A-A at the peripheral edge of the cells. PP2A-A expression was co-localised with β4 integrin staining in these cells (Appendix 6), suggesting a role for PP2A in cellular adhesion. Interestingly, while PP2A-A expression is qualitatively reduced in confluent HMT-3522-S2 cells, PP2A-A and also PP2A-C expression is qualitatively increased in confluent HMT-3522-T42 cells (Appendix 5).

The MDA-MB cell lines did not reach confluence on glass coverslips, rather they continued to detach and float into the media. This feature is observed in MDA-MB-468 cells even in tissue culture flasks (data not shown). The MDA-MB-231 cells had a dramatically different morphology on glass cover slips; with a much smaller, rounded appearance and very little cytoplasm, compared to their normal morphology in culture flasks which is more fibroblastic-like with cellular spreading (Appendix 7). Thus the PP2A expression observed in these cells may not be representative of their true expression patterns under normal culture conditions. PP2A subunit expression was observed in the cytoplasm and membrane of MDA-MB-231 cells, with PP2A-A staining mostly present at the cell membrane, while PP2A-C was more evenly distributed (Figure 3.20).
**Figure 3.18 PP2A subunit expression in HMT-3522-S2 cultures.**

HMT-3522-S2 cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

In HMT-3522-S2 cells PP2A-A (red) and PP2A-C (green) are expressed in both the nucleus and the cytoplasm of sub-confluent, proliferating MCF10A cells. PP2A-A is also present in nuclear bodies. Cells on the right are more closely packed and do not express nuclear PP2A.
Figure 3.19 PP2A subunit expression in HMT-3522-T42 cultures.

HMT-3522-T42 cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

HMT-3522-T42 demonstrate a more fibroblastic morphology with PP2A-A (red) staining having both cytoplasmic and also peripheral edge staining (open arrow). PP2A-C (green) is not as highly expressed at the peripheral edges, but rather demonstrates predominantly cytoplasmic expression, with some filamentous staining (closed arrow).
Figure 3.20 PP2A subunit expression in MDA-MB-231 cultures.

MDA-MB-231 cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-C (green) are not expressed in the nuclei of sub-confluent MDA-MB-231 cells, but rather both subunits are predominantly expressed in the cytoplasm, with some membrane staining demonstrated by PP2A-A. The enlargement presented is not from this figure, but a 2.5x zoom using the same objective.
Similarly in MDA-MB-468 cells, PP2A expression was observed in the cytoplasm, but both subunits demonstrated uniform expression, with no apparent peripheral edge staining (Figure 3.21). No nuclear PP2A staining was observed in either MDA-MB cell lines.

Finally, as DU4475 is a suspension cell line, these cells were fixed to glass coverslips using a cytopsin centrifuge. PP2A expression was again present in the cytoplasm of these cells, but not the nuclei, with higher expression in some cells compared with others in the same culture (Figure 3.22). Some regions of expression were observed at the membrane of these cells, perhaps indicating a role in cell-cell contact, as these suspension cells do not make extracellular adhesions.

Thus, a number of breast cancer cell lines demonstrate PP2A expression in the nucleus during cellular proliferation. When cells become confluent and cease proliferating due to contact inhibition, PP2A expression is often reduced and no longer nuclear. These results show that compared to HMEC cells, where PP2A-A is expressed primarily at the periphery, and PP2A-C is expressed primarily in the cytoplasm, other breast cancer cell lines do demonstrate altered sub-cellular expression of PP2A subunits, and hence may play a role in breast cancer progression.
**Figure 3.21 PP2A subunit expression in MDA-MB-468 cultures.**

MDA-MB-468 cells were grown on glass coverslips; fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-C (green) are both highly expressed in the cytoplasm of MDA-MB-468 cells.
Figure 3.22 PP2A subunit expression in DU4475 cultures.

As DU4475 cells grow as floating aggregates, cells were adhered to glass coverslips by cytospin centrifugation. The cells were then fixed, permeabilised and proteins detected with anti-PP2A antibodies as indicated, followed by secondary fluorescently labelled antibodies, as for the other adherent cell lines. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-C (green) only demonstrate cytoplasmic expression with no nuclear expression.
3.3.8. PP2A expression in an MCF10A three-dimensional culture model of breast development is associated with integrin expression and apoptosis.

MCF10A cells cultured on a bed of extracellular matrix proteins form spherical, three-dimensional (3D) structures, termed ‘acini’, with a hollow lumen (Debnath et al. 2003). The MCF10A cells are plated at a very low density to form the acini from single cells. For the first 1-8 days these cells rapidly proliferate, forming a solid ball of cells (Figures 3.23 top panels and 3.24). From about day 8, the outer cells begin to polarise, as demonstrated by α6 integrin expression on the basal side of the outer layer of cells and the golgi apparatus localising to the luminal side of the outer layer of cells (Figure 3.24). The inner cells die by apoptosis, leaving a hollow lumen at day 20, surrounded by a layer of fully polarised MCF10A cells (Figure 3.20 lower panels and Figure 3.25). Specific staining of the structures indicated was determined by control experiments with secondary fluorescently labelled antibodies alone, which demonstrates that the fluorescently labelled antibodies do not bind to any cellular or matrix proteins in a non-specific manner (Figure 3.26). Importantly, these acini are similar to the structures formed by primary HMECs when cultured under the same conditions, only much larger (Figure 3.27) (Petersen et al. 1992). They are also similar to a cross-section of normal human breast ducts (Bissell et al. 2003). In addition, breast cancer cell lines demonstrate very different morphologies, indicative of increased cellular proliferation, inability to form cell-cell adhesions and also invasive potential (Figure 3.27) (Kenny et al. 2007).

PP2A is expressed in a peripheral ring around the acini and also in the cells at the centre of the lumen in developing acini at day 8 (Figure 3.28). PP2A-A and PP2A-C co-localise with integrins (Figures 3.29 and 3.30) at the basal membrane of the outer layer of MCF10A cells, indicating a possible adhesion role for PP2A in breast development. In addition, the luminal staining for PP2A-A co-localises with cleaved caspase 3 (Figure 3.31), a marker of cellular apoptosis, indicating PP2A may have a further role in clearance of the lumen by apoptosis. As development continues, and differentiation occurs, PP2A-A is still present in the peripheral ring and also in the clearing lumen (Figure 3.32), with
Figure 3.23 MCF10A three dimensional culture model.
Light microscope images of MCF10A acini, cultured from single cells on a bed of extracellular matrix proteins for up to 20 days as a model of mammary gland development. At days 5 and 8 cells are proliferating as a solid sphere of cells. At day 13 and 16 the forming hollow lumen is evident as a peripheral ring of cells becomes defined against a shadowy centre of apoptosing cells. Day 20 images demonstrate cleared lumens and the peripheral layer of polarised cells. All images were taken at 20x magnification except for day 20 as indicated.
Figure 3.24 Polarisation of MCF10A acini – Day 8.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were imaged with DAPI (blue). Scale bar = 50μm.

Day 8 acini express α6 integrin (red) at the periphery where cells are in contact with the basement membrane. GM130 (green) is a golgi marker, demonstrating that cells are still present in the centre of the developing acini, but are beginning to polarise in the outer layer of cells.
Figure 3.25 Polarisation of MCF10A acini – Day 20.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were imaged with DAPI (blue). Scale bar = 50μm.

By day 20 α6 integrin (red) continues to be expressed at the periphery, and now cells are fully polarised with GM130 expression (green) on the luminal side of the nuclei. A mostly hollow central lumen is indicated by lack of nuclei (blue) in the centre of the acini.
MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and during primary antibody incubation these cells were incubated with buffer alone, followed by secondary fluorescently labelled antibodies. Nuclei were imaged with DAPI (blue). Scale bar = 50μm.

Figure 3.26 3D Fluorescent secondary antibody controls.
Figure 3.27 Breast cancer cell lines in 3D culture.
Light microscope images of breast cancer cells cultured from single cells on extracellular matrix proteins for up to 20 days as a comparison to MCF10A 3D cultures.
A) MCF10A acini at day 20 have a polarised layer of cells surrounding a hollow lumen.
B) Human mammary epithelial cells grow into spherical acini in a similar manner to MCF10A, but are much smaller, due most likely to a lower proliferation rate.
C) MCF7 cells demonstrate discohesive structures with detached cells in the surrounding matrix.
D) MDA-MB-231 cells display an invasive phenotype with elongated projections out into the surrounding extracellular matrix. These structures were so large that they were discontinued at day 14.
Figure 3.28 PP2A expression in MCF10A acini – Day8.
MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-C (green) is expressed at the periphery of developing 3D acini and also within cells at the centre of the lumen at day 8.
**Figure 3.29** PP2A-C expression co-localises with α6 integrin in MCF10A acini – Day 8.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50 μm.

Co-staining for α6 integrin (red) and PP2A-C (green) demonstrates co-localisation at the periphery of acini (yellow on merge image), indicating PP2A may be involved in attachment of acini to the extracellular matrix.
Figure 3.30 PP2A-A expression co-localises with β4 integrin in MCF10A acini – Day 8.
MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50 µm.

PP2A-A (red) and β4 integrin (green) are expressed at the periphery of developing 3D acini.
Figure 3.31 PP2A-A expression co-localises with cleaved caspase 3 in MCF10A acini – Day 8.
MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

Co-staining for PP2A-A (red) and cleaved caspase 3 (green), a marker of apoptotic cells, demonstrates co-localisation (yellow on merge image), indicating PP2A may be involved in apoptotic clearing of the acini lumen.
Figure 3.22 PP2A expression in MCF10A acini – Day 14.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were imaged with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) expression is still present at the periphery and co-localises with PP2A-C (green) in the cells clearing the lumen. PP2A-C expression at the membrane changes to be more baso-lateral and only present in some cells (arrows).
co-localisation between PP2A-A and cleaved caspase 3 still evident (Figure 3.33). PP2A-C expression however, becomes less basal in the peripheral cells, with more cytoplasmic staining and a baso-lateral expression pattern in some cells (Figure 3.32). This baso-lateral PP2A-C expression in only some cells becomes more pronounced at day 20 (Figure 3.36), perhaps indicating this is a differentiated phenotype for PP2A-C which is slightly different to the continuous peripheral ring staining observed in the developing acini at day 8. PP2A-C is still expressed in the clearing lumen of acini at day 14 (Figure 3.32).

Expression of regulatory B subunits was also investigated at day 14, and PP2A-Bα was co-localised with PP2A-A at both the periphery of the acini, and also to a lesser extent in the clearing luminal cells (Figure 3.34). PP2A-B’γ was more co-localised with PP2A-A in the clearing luminal cells, but also demonstrated basal expression in the outer cell layer (Figure 3.35).

Interestingly, at day 20, PP2A-A expression is qualitatively reduced at the basal membrane of the outer cells, and as the lumen are now cleared, demonstrate no luminal staining (Figure 3.36). As mentioned above, PP2A-C has more baso-lateral expression in the outer cell layer, but this is not present in all cells, making a discontinuous peripheral ring. Co-staining for PP2A-C and α6 integrin shows that PP2A-C still co-localises with α6 integrin at the basal membrane (Figure 3.37), but not to the same extent as in developing acini.

Thus, PP2A may have a role in cell-matrix adhesion in developing acini, and also in clearing the lumen by apoptosis. In contrast, PP2A does not co-localise with a marker of cellular proliferation, Ki67, expressed in the outer cell layer of developing acini (Appendix 8).

3.3.9. PP2A-A expression is reduced in human breast tumour tissue

While cancer cell lines give an indication of features of the tumours they are derived from, and are a useful starting point for initial investigation, cell lines are not always a true representative of in situ tumours (Lacroix et al. 2004). Thus, human breast tissue arrays, consisting of formalin fixed, paraffin
Figure 3.33 PP2A-A expression co-localises with cleaved caspase 3 in MCF10A acini – Day 14.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were imaged with DAPI (blue). Scale bar = 50μm.

Co-staining for PP2A-A (red) and cleaved caspase 3 (green), a marker of apoptosing cells, demonstrates co-localisation (yellow on merge image), indicating PP2A may be involved in clearing of the lumen by apoptosis.
Figure 3.34 PP2A-Bα expression in MCF10A 3D acini – Day 14.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-Bα (green) expression is co-localised (yellow on merge) at both the periphery of developing acini and also to a lesser extent in the lumen.
Figure 3.35 PP2A-B’γ expression in MCF10A 3D acini – Day 14.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) and PP2A-B’γ (green) expression is co-localised (yellow on merge) predominantly in the lumen of acini, but also in the periphery.
Figure 3.36 PP2A expression in MCF10A acini – D20.

MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

PP2A-A (red) expression is greatly reduced in day 20 acini and PP2A-C (green) expression only demonstrates baso-lateral staining in the periphery of some cells.
Figure 3.37 PP2A-C in the periphery of MCF10A acini – Day 20.
MCF10A cells were cultured from single cells on extracellular matrix proteins for up to 20 days as a model of mammary gland development. Acini were fixed, permeabilised and proteins detected with specific antibodies as indicated, followed by secondary fluorescently labelled antibodies. Nuclei were labelled with DAPI (blue). Scale bar = 50μm.

Co-staining for α6 integrin (red) and PP2A-C (green), still demonstrate co-localisation (yellow on merge image), but PP2A-C is not present to the same extent as α6 integrin.
embedded breast tumour tissue cores, together with matched normal tissue, were analysed for PP2A subunit expression by immunohistochemistry with antibodies for PP2A-A and -C subunits (Figure 3.38, Appendix 9). To assess the specificity of these antibodies, the tissue arrays were also incubated with isotope control antibodies, which demonstrated low background staining levels (Appendix 10). Positive staining was confirmed by a pathologist and the slides were scanned with an Aperio Scancope.

In normal breast epithelium, PP2A-A and PP2A-C subunit expression is greater in the luminal epithelial cell layer, compared with the underlying myoepithelial layer (Figure 3.39). In addition, PP2A also demonstrates some basal membrane staining of the myoepithelial cells, adjacent to the basement membrane, similar to that observed in the MCF10A 3D acini (Figures 3.28, 3.35 and 3.36). PP2A subunits were expressed in both the cytoplasm and the nucleus of both cell types, and clear demarcations of the nuclei were difficult to distinguish when DAB staining alone was examined (Figure 3.39C,D and also Appendix 11).

The tissue arrays comprised of 40 breast tumour cores, 10 lymph node cores and also 9 normal breast cores, which were matched with tumour cores (Appendix 12). For two patients, matched normal, tumour and lymph node cores were available. For PP2A-C, one of the normal cores could not be analysed due to poor tissue quality. PP2A subunit expression was analysed for staining intensity using the Aperio colour deconvolution algorithm. As normal epithelial ducts comprise such a small area of a total core, these were traced prior to analysis, and only the traced sections analysed (Figure 3.40B,C, Appendix 13). In addition, while some tumour and lymph node cores were highly homogenous, and thus the whole core was analysed with no tracing, some tumours were clearly identifiable against the background stroma and so were also traced. The colour deconvolution algorithm is able to determine regions of weak (1+), medium (2+) and strong (3+) staining intensities over the entire image. A score value was calculated for each core or traced region using the positive staining percentages:

\[
\text{Score} = 1 \times (\% \text{ weak}) + 2 \times (\% \text{ medium}) + 3 \times (\% \text{ strong}).
\]
Figure 3.38 PP2A-A protein expression in breast cancer tissue arrays.

Human tissue arrays were assessed for PP2A-A subunit expression by immunohistochemistry using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm.

A whole slide stained with anti-PP2A-C antibody is presented in Appendix 9.

Negative control slides incubated with isotype matched antibodies demonstrating background staining levels are presented in Appendix 10.
Figure 3.39 PP2A subunit protein expression in normal breast tissue.
Immunohistochemical analysis of normal breast tissue in tissue arrays using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). All images are from core # 58.

A) PP2A-A is expressed in the cytoplasm and nucleus of epithelial cells lining the breast ducts, with strong staining in the luminal epithelial cells and less staining in the cytoplasm of the myoepithelial cells, but clear basal expression near the basement membrane (arrows).
B) DAB only for PP2A-A demonstrates staining in both the nucleus and cytoplasm.
C) Isotype antibody control for PP2A-A antibody shows negative staining.
D) PP2A-C is also expressed in epithelial cells lining the ducts, and demonstrates some basal membrane staining of the myoepithelial cells at the basement membrane (arrows).
E) DAB only for PP2A-C demonstrates staining in both the nucleus and cytoplasm.
F) Isotype antibody control for both the PP2A-C antibodies also has no non-specific staining.
Figure 3.40 PP2A protein expression in breast cancer tissue arrays.

Human tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A-A antibody (brown). Nuclei are stained with hematoxylin (blue). Slides were scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm.

A) Tissue array stained for PP2A-A. The top samples are breast tumour tissues (40), the blue box marks the lymph node tissues (10), and the red box contains the normal matched tissue for some tumours.

B) For normal epithelium cores (black box on A), and also tumour samples that were not homogenous, ducts or tumour tissue was traced prior to analysis.

C) Higher magnification section indicated in (B) to demonstrate tracing of breast ducts (green line).

D) Negative staining with isotype control for PP2A-A antibody in the same region as (C).
PP2A-C expression remained constant across the different tissue types (Figure 3.41A), whereas PP2A-A expression was significantly reduced in tumour and lymph node cores compared with normal epithelium (Figure 3.41B). These results are comparable to what was observed in the panel of breast cancer cell lines (Figure 3.1). Interestingly, three outliers in the tumour tissues with much higher PP2A-A expression (Figure 3.41B) were all identified as ductal carcinoma in situ (DCIS). In addition, one mucinous carcinoma also demonstrated high PP2A-A expression. In contrast, a number of the invasive ductal carcinomas had very low PP2A-A expression (Figure 3.42). These results indicate that PP2A expression may vary with tumour type, or even progression.

Direct comparison of the matched normal cores and lymph nodes revealed that PP2A-A expression is reduced in 8/9 matched samples (Figure 3.43A). A paired t-test of these matched samples revealed the reduction in PP2A-A expression is statistically significant. Example images demonstrating the reduced PP2A-A expression in matched normal and tumour tissue show that PP2A-A expression is still predominantly cytosolic in breast tumours, with no increase in nuclear expression (Figure 3.42C-D, Appendix 11a,b shows PP2A-A only without the hematoxylin staining). In addition, when PP2A-A expression was examined a set of matched normal, tumour and lymph node cores from the same patient, PP2A-A expression was reduced in both the tumour and the lymph node compared to normal tissue (Figure 3.44). One core demonstrated both tumour and adjacent normal tissue as confirmed by a pathologist (Figure 3.45). The tumour tissue on the lower left of the core has much lower PP2A-A expression than the normal breast ducts in the upper right. This result strongly suggests that PP2A-A expression is reduced in tumour tissue compared to normal tissues as any possible variation in tissue processing of different samples is eliminated. Together this data suggests that expression of PP2A-A is lower in breast tumours compared with normal breast ducts.

Nuclear PP2A-A and/or PP2A-C expression was observed in proliferating cells in a number of breast cancer cell lines (Figures 3.13, 3.16, 3.17, 3.18), however while it is likely that the breast tumour cells also have a higher rate of
Figure 3.41 Quantitation of PP2A protein expression in breast cancer.

Human breast tumour tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Error bars are SEM of all cores for each tissue type. *p<0.05, ***p<0.001 using a Mann Whitney U test compared to normal epithelium.

A) PP2A-C expression was constant across normal epithelium, tumours and lymph nodes.

B) PP2A-A expression is significantly reduced in breast tumour and lymph node cores compared with normal epithelium.
Figure 3.42 PP2A-A expression is reduced in some invasive ductal carcinomas compared to pre-invasive ductal carcinoma in situ or tumour and lymph node compared to normal tissue in a set of matched patient samples.

Human tissue arrays were analysed for PP2A-A subunit expression by immunohistochemistry using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Cores for DCIS (Ductal carcinoma in situ) are #15 (left) and #18 (right), Mucinous carcinoma is #39, IDC (Invasive ductal carcinoma) are #9 (left) and #29 (right).
Figure 3. PP2A-A expression is reduced in breast tumours compared to matched normal tissue.

Human tissue arrays were analysed for PP2A-A subunit expression by immunohistochemistry using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm.

A) Core score for all matched normal and breast tumour tissues on the array demonstrates reduced expression in 8/9 tumours compared with the normal epithelium.

B) Grouped analysis of the paired cores in (A) shows the reduced expression of PP2A-A is statistically significant. Error bars are standard error of the mean, **p<0.01 using a paired t-test.

C) Example normal breast epithelium, core #57, compared with matched tumour tissue, core #13.

D) Example normal breast epithelium, core #54, compared with matched tumour tissues, core #10.
Figure 3.44 PP2A-A expression is reduced in tumour and lymph node compared to normal tissue in a set of matched patient samples.

Human tissue arrays were analysed for PP2A-A subunit expression by immunohistochemistry using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Normal sample is core #52, Tumour is core #31 and Lymph node is core #41. All are from the same patient.
Figure 3.45 PP2A-A expression is reduced in tumour cells adjacent to normal ducts within an individual core.

One interesting example of breast tumour tissue core #33, has normal ducts in the top right corner (enlarged on top right), on the same core as the tumour on the bottom left corner (enlarged on top left). Below each enlargement is a colour representation of the staining intensity, with dark blue = negative, yellow = 1+, orange = 2+ and red = 3+. The tumour (lower left) has lower PP2A-A expression than the normal ducts (lower right).
proliferation than the normal epithelial cells, no increase in nuclear expression was observed in breast tumours.

PP2A-C expression in matched samples is variable, with 3/8 pairs demonstrating increased PP2A-C expression in the tumours, while 3/8 pairs have decreased PP2A-C expression compared to normal, and 2/8 demonstrate very similar expression levels (Figure 3.46A). Combined analysis of the paired cores averaged out these differences, revealing no difference in PP2A-C expression between breast tumours and normal epithelium (Figure 3.46B). Figure 3.46C shows one of the paired cores with reduced PP2A-C expression in the tumour, while Figure 3.46D shows an example of paired cores with increased PP2A-C expression in the tumour core. Interestingly, this pair of cores demonstrated nuclear expression of PP2A-C, in both the tumour tissue and also the normal epithelium (Appendix 11d).

Immunohistochemistry with a PP2A-B'y antibody was attempted, but very strong staining in plasma cells made optimisation difficult. Compared to these cells, infiltrated throughout the tissues, both normal and tumour breast tissue had very low staining levels (Appendix 14). Further work with alternate antibodies and/or antigen retrieval methods may be required.

To determine if PP2A expression is associated with specific tumour characteristics, the samples were analysed according to ER, PR, ErbB2 and p53 status (Appendix 12). Interestingly, tumours that do not express the estrogen receptor (ER-) have reduced PP2A-A expression (Figure 3.47A), but this was just beyond significance (p-value = 0.0564, Mann-Whitney U test). PP2A-C did not demonstrate significant alterations in expression between ER positive (ER+) and ER- tumours (Figure 3.47A). Neither PP2A-A nor PP2A-C expression showed any associations with progesterone receptor (Figure 3.47B), the tumour suppressor p53 (Figure 3.48A) or the ErbB2 receptor (Figure 3.48B) expression. PP2A expression was also not altered in ‘triple negative’ breast cancer, which do not express the ER, PR or ErbB2 receptor (Figure 3.49). In addition, PP2A
subunit expression did not vary across tumour stage (Figure 3.50A) or predict survival outcome (Figure 3.50B).
Figure 3.46 PP2A-C expression is variable in matched normal breast and breast tumours.

Human tissue arrays were analysed for PP2A-C subunit expression by immunohistochemistry using anti-PP2A antibodies (brown). Nuclei are stained with hematoxylin (blue). Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm.

A) Core score for all matched normal and breast tumour tissues on the array demonstrates varied expression between tumours and normal epithelium.

B) Grouped analysis of the paired cores in (A) shows no overall difference between tumour and normal epithelium.

C) Example normal breast epithelium, core #58, compared with matched tumour tissue, core #34.

D) Example normal breast epithelium, core #52, compared with matched tumour tissues, core #31.
Figure 3.47 PP2A expression compared with hormone receptor status.
Human breast tumour tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Error bars are SEM of all cores for each tissue type.

*p<0.05.

A) PP2A-A expression is reduced in estrogen receptor (ER) positive breast tumours compared with ER negative tumours. PP2A-C expression is not significantly different in ER positive or negative tumours.

B) Neither of the PP2A subunits demonstrate significantly altered expression in progesterone receptor (PR) positive or negative tumours.
**Figure 3.48 PP2A expression compared with signalling protein expression.**

Human breast tumour tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Error bars are SEM of all cores for each tissue type.

**A)** PP2A subunit expression is not altered depending on p53 status of breast tumours.

**B)** PP2A subunit expression is not altered by ErbB2 expression either.
Figure 3.49 PP2A expression is not altered in triple negative; ER, PR, ErbB2 negative breast cancer.

Human breast tumour tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Error bars are SEM of all cores for each tissue type.
Figure 3.50 PP2A expression does not correspond with progression of disease.

Human breast tumour tissue arrays were analysed for PP2A subunit expression by immunohistochemistry using anti-PP2A antibodies. Slides were then scanned with an Aperio Scanscope and PP2A subunit expression was quantitated using the Aperio Colour Deconvolution algorithm. Error bars are SEM of all cores for each tissue type.

A) PP2A subunit expression is constant across the stages of breast cancer in these patients.
B) PP2A expression did not predict survival outcome.
3.4. Discussion

In order to investigate the role of PP2A in breast cancer, PP2A subunit expression and regulation was examined in a panel of breast cancer cell lines. The structural PP2A-A subunit together with the regulatory PP2A-Bα, -Bδ and -B’γ subunits demonstrated marked reductions in protein expression in the breast cancer cell lines compared with normal breast epithelial cells. The reductions in regulatory B subunit expression could not be explained by altered gene expression, nor mutations in PP2A-A that would prevent holoenzyme formation. While the expression of the catalytic subunit remained quite stable, reductions in post-translational methylation, but not phosphorylation of PP2A-C was observed. Interestingly, expression of the endogenous PP2A inhibitor SET was increased in a number of breast cancer cell lines, while expression of the α4 protein was unaltered. Surprisingly, PP2A activity towards a specific phosphopeptide substrate was increased in a number of the cell lines. Examination of PP2A subunit sub-cellular location in breast cancer cells as well as a 3D MCF10A culture model revealed co-localisation of PP2A subunits with integrins at the cellular membrane and also with apoptotic cells in 3D acini. Importantly, investigation of PP2A subunit expression in breast tumours confirmed that PP2A-A is reduced in the majority of breast cancers compared to normal breast tissue.

PP2A-A expression was reduced in the breast cancer cell lines: MCF7, HMT-3522-S2, HMT-3522-T42 and also the highly invasive DU4475 cell line (Figure 3.1B). Consistent with these results, two previous studies have demonstrated reduced PP2A-A expression in the MCF7 cell line compared to primary human breast epithelium (Suzuki et al. 2003; Zhou et al. 2003). Reduced PP2A-A expression was also identified in breast tumour samples (Figure 3.41B), which has also been previously demonstrated (Suzuki et al. 2006), confirming the relevance of PP2A-A as a tumour suppressor in breast cancer. The strength of the previous study was that all 28 tumour samples were matched with normal epithelial tissue from the same patient. However, the weakness of this study was that PP2A-A expression was only reported as positive or negative; with 27/28 (96%) normal breast and 12/28 (43%) breast
tumours being scored as positive for PP2A-A expression (Suzuki et al. 2006). The results obtained using the Aperio Deconvolution algorithm in this thesis revealed a wide range of PP2A-A expression levels in breast tumours, with scores between 47 and 139 out of a possible 300, with a median value of 80 ± 30 (SD). In comparison, the values in normal epithelium ranged from 38 to 182 with median 142 ± 41, thus the use of the algorithm to generate an intensity score provides greater information about the nature of altered PP2A-A expression in breast tumours. In addition, patient data available with the tissue arrays used here revealed that PP2A-A expression is reduced in ER- tumours (Mean of ER- was 79, compared with ER+ mean of 103, p value = 0.0564 using Mann Whitney U test) which is a marker of poorer prognosis, compared with ER positive tumours (Figure 3.48A). While this did not reach statistical significance, it does suggest a trend that may be significant if more samples were analysed. A link between ER expression and PP2A has previously been demonstrated in breast cancer cell lines, where 3 ER negative cell lines had lower PP2A activity than 3 ER positive cell lines (Gopalakrishna et al. 1999). Two of the ER+ cell lines, MCF7 and T47D, and one of the ER- cell lines, MDA-MB-231, were included in the panel of breast cancer cell lines examined for PP2A activity in Figure 3.9; where the MDA-MB-231 cells did trend towards a lower PP2A activity than the ER+ cell lines. Most interestingly, treatment of ER positive cell lines with the phosphatase inhibitor okadaic acid (OA) resulted in reduced ER mRNA and protein expression, suggesting PP2A may regulate ER expression. The mechanism of ER regulation by PP2A was not entirely unravelled, but is likely to involve the 3'UTR as expression of exogenous ER lacking this region was unaffected by OA (Keen et al. 2005). If future studies confirm the reduced PP2A-A expression in ER- tumours, this would suggest that reduced expression is not only important for tumourigenesis, but may also contribute to the loss of ER expression in breast cancer.

The two main functional effects of reduced PP2A-A expression in cells are somewhat paradoxical; cellular death by apoptosis and cellular transformation. Reduction of PP2A-Aα expression below approximately one third of normal levels induces apoptosis (Chen et al. 2005; Ruediger et al. 2011). However,
reduced expression of PP2A-Aα to approximately half of normal levels induces cellular transformation in immortalised HEK-TER cells (Chen et al. 2005) and also increases the incidence of lung cancer in mice with a predisposition to developing the disease (Ruediger et al. 2011). Reduced expression of PP2A-Aβ also immortalises HEK-TER, most likely via deregulation of the small GTPase RalA (Sablina et al. 2007). The reduction in PP2A-A expression observed in breast cancer cell lines and breast tumours suggests that investigation into the functional effects of reduced PP2A-A expression in breast cancer cells is worth pursuing. PP2A-A mRNA expression was examined as a possible mechanism for reduced PP2A-A protein expression in the breast cancer cell lines, however no direct correlations between mRNA and protein expression were observed (Appendix 2a C-D). PP2A-Aβ mRNA expression was reduced in all of the cell lines, and also at the protein level in one cell line, but increased protein expression was observed in two cell lines. Furthermore, the antibody that detects both PP2A-Aα and -Aβ is reduced in four of the cells lines, only one of which demonstrated reduced PP2A-Aβ, suggesting that the PP2A-Aα isoform is reduced in three of the cell lines. However, of these, only the HMT-3522-S2 cells demonstrated reduced PP2A-Aα mRNA expression. Together this suggests that expression of PP2A-Aα and -Aβ are regulated post-transcriptionally.

PP2A-B’γ protein expression demonstrated extensive and comprehensive reduction across the panel of breast cancer cell lines (Figure 3.3C). Unfortunately, immunohistochemical analysis of the breast tumour tissue arrays with the PP2A-B’γ antibody was hampered by high expression levels in plasma cells (Appendix 14) and required further experimental optimisation. Functionally, suppression of PP2A-B’γ expression in HEK-TER cells causes cellular transformation (Chen et al. 2004), and this subunit regulates a number of signalling pathways aberrantly activated in breast cancer including Akt (Rocher et al. 2007; Kim et al. 2009), p53 (Koma et al. 2004; Moreno et al. 2004; Li et al. 2007; Shouse et al. 2008; Shouse et al.), ERK (Letourneux et al. 2006) and Wnt (Seeling et al. 1999). PP2A-B’γ also regulates the cell cycle, by translocating to the nucleus and increasing levels of the CDK inhibitor p27,
preventing cells from entering the cell cycle until the G1/S cell cycle checkpoint is completed (Lee et al.). Thus, the reduced expression of PP2A-B’γ in breast cancer cell lines is perhaps not surprising and warrants further analysis to determine its functional significance; as performed in Chapter 5 of this thesis.

The PP2A-Bα subunit also demonstrated reduced protein expression in all of the breast cancer cell lines. This subunit is involved in regulation of survival signalling through Akt (Kuo et al. 2008); cell proliferation in the ERK pathway (Ory et al. 2003; Adams et al. 2005); mitotic exit from the cell cycle (Schmitz et al. 2010), the growth inhibitory effects of TGF-β (Griswold-Prenner et al. 1998); the cell cycle through interactions with microtubules (Sontag et al. 1995); and also β-catenin in the Wnt pathway (Zhang et al. 2009). In all of these pathways PP2A-Bα prevents excessive cellular survival and proliferation, hence down-regulation of this subunit is a potential mechanism by which cancer cells enhance their proliferation and evade cell death. The functional role of PP2A-Bα knock down in breast tumourigenesis is investigated in Chapter 5.

Investigation into a potential mechanism for reduced PP2A regulatory B subunit expression revealed that the pattern of mRNA expression (Figures 3.5 and 3.6) does not fit with the pattern of reduced protein expression (Figures 3.2 and 3.3). In addition, no mutations in the PP2A structural subunit were identified that would prevent binding of the regulatory B subunits to PP2A-A. Mutations in PP2A-A or reduced PP2A-A expression can result in degradation of the regulatory B subunits by the proteasome (Strack et al. 2004), presumably as they are no longer protected by a structural subunit. While PP2A-A expression was reduced in 4/7 breast cancer cell lines (Figure 3.1B), PP2A-Bα and -B’γ expression was reduced in all of the breast cancer cell lines (Figures 3.2A and 3.3C), indicating an additional mechanism of regulation must be present in at least some of the breast cancer cell lines with normal PP2A-A protein expression.

PP2A-C protein expression levels were relatively constant across the panel of breast cancer cell lines (Figure 3.1A), and also in the breast tissue arrays (Figure
It has previously been shown that PP2A-C levels within cells are tightly controlled by an auto-regulatory mechanism (Baharians et al. 1998). Therefore, unaltered PP2A-C expression was an expected result, and altered PP2A-C expression is not a hypothesised mechanism of PP2A holoenzyme alteration. PP2A-C post-translational modifications regulate PP2A activity, and thus may contribute to altered PP2A function in breast cancer. Methylation of PP2A-C at Leu^{309} (M-PP2A-C) was reduced in 4/7 breast cell lines (Figure 3.7), including the non-tumourigenic MCF10A cell line. As M-PP2A-C is required for binding of the PP2A-B family in vivo this reduction in methylation is a possible mechanism for reduced PP2A-Bα and -Bδ expression observed in the cell lines (Figure 3.2A,B). While a reduction in PP2A-C methylation may not explain the reduction of PP2A-Bα and -Bδ in all of the breast cancer cell lines, this mechanism together with other mechanisms that reduce regulatory B subunit expression, such as suppression of PP2A-A, may jointly account for the reduction of these subunits across all of the breast cancer cell lines. Phosphorylation of Tyr^{307} did not change across the panel of breast cancer cell lines, and hence no role for this modification in breast cancer is predicted based on the results presented here.

PP2A activity can be inhibited by a number of binding proteins. The expression of SET and α4 proteins were examined in the panel of breast cancer cell lines to determine if they may have a role in breast cancer. Interestingly, SET expression was increased in all of the breast cancer cell lines compared with HMECs, but this was only significant for 5/7 breast cancer cell lines. SET was first discovered as an inhibitor of PP2A in myeloid leukaemia cells (Li et al. 1996), and most research to date on SET has been in leukaemia. The BCR/ABL oncogene, a driver of chronic myeloid leukaemia, inactivates PP2A via increased SET expression (Neviani et al. 2005). Importantly, pharmacological reactivation of PP2A in these cells suppresses growth, colony formation and leukaemogenesis, as well as enhancing apoptosis and differentiation (Neviani et al. 2005). Similar results were also observed in acute myeloid leukaemia cells expressing the oncogene c-KIT (Roberts et al.). Increased SET expression has now been observed in a range of leukaemias and indeed specific SET inhibitors
such as COG112 are being developed as anti-leukaemic agents (Switzer et al. 2011). In contrast, the role of SET in solid tumours is less well characterised to date with one study reporting SET promotes tumourigenesis in colorectal adenocarcinoma (Jiang et al. 2011), and another reporting high expression of SET in Wilms’ tumour, but not renal cell carcinoma, adult polycystic kidney disease or transitional cell carcinoma (Carlson et al. 1998). Increased SET expression in breast cancer cell lines indicates this PP2A regulatory protein may play a role in breast cancer progression, but further examination of expression levels in breast tumours is required. In contrast, ω4 expression is increased in one breast cancer cell line, but decreased in two others.

The most surprising result observed was that PP2A activity was increased in all of the cell lines compared with HMEC, although this was significant for only 4 of the cell lines, including the MCF10A cells (Figure 3.9). As PP2A is a proposed tumour suppressor, and PP2A-α expression was reduced in a number of the cell lines, these results were unexpected. A few possibilities for this perplexing data may be hypothesised. PP2A plays a role in regulating the cell cycle (Sontag et al. 1995; Okamoto et al. 2002; Lee et al.), and PP2A activity associated with microtubules demonstrates a rapid increase during S phase (Sontag et al. 1995). Thus, it is possible that the immortalised MCF10A and breast cancer cell lines have increased activity due to increased cell division compared to the HMEC cells which have a much lower proliferation rate (Appendix 3). One major limitation of this activity assay is that it only measures PP2A activity against one peptide substrate. PP2A has numerous intracellular substrates, with PP2A activity being regulated by a number of different mechanisms in multiple cellular signalling pathways at any particular time. Thus, it may be that for breast cancer progression, only a small portion of the total cellular PP2A activity needs to be disrupted. For example, only PP2A holoenzymes with PP2A-B’γ target p53 (Li et al. 2007; Shouse et al. 2008; Shouse et al.) and only PP2A-B’α holoenzymes target c-Myc (Arnold et al. 2006; Arnold et al. 2008). It may be that small pools of PP2A holoenzymes are disrupted in the progression of breast cancer, and indeed an overall increase in PP2A activity could be an effort to restore vital function to these small pools.
This hypothesis however is pure speculation, and would be difficult to demonstrate with the current resources available as to date very few specific and robust regulatory B subunit antibodies exist. Generation of new regulatory B subunit antibodies would allow immunoprecipitation of specific pools of PP2A holoenzymes for analysis. Further activity assays with a range of PP2A substrates, including signalling proteins known to be altered in breast cancer may provide more clues as to whether PP2A activity towards multiple substrates is increased in breast cancer. However, one investigation did examine PP2A activity in 10 breast tumours compared with matched normal tissue, and found that overall PP2A activity was significantly lower in tumours than in the matched normal tissue (Kim et al. 2009). This study used the same peptide substrate as was used to examine PP2A activity in the panel of breast cancer cell lines herein, and might suggest that for PP2A activity, breast cancer cell lines are not representative of breast tumours. Thus, further investigation is required to determine if PP2A activity levels are reduced in human breast cancer patient samples. However, these results should still be analysed with care as to whether total PP2A activity is the critical determinant of PP2A involvement in breast cancer, or if smaller pools of PP2A with specific regulatory B subunits are more important.

Examination of PP2A expression by immunofluorescence in 2D breast cultures of normal human mammary epithelial cells revealed PP2A expression was co-localised with integrins (Figures 3.10, Appendix 4). PP2A promotes the formation of focal adhesions with the extracellular matrix (Mulrooney et al. 2000; Suzuki et al. 2005), and it is possible that more invasive breast cancer cells, such as MDA-MB-231 and MDA-MB-468 cells (Figures 3.20 and 3.21) have reduced PP2A expression at the peripheral membrane as they become less adhesive to extracellular supports. In addition, PP2A also associated with integrins at the basement membrane in the 3D MCF10A cultures (Figures 3.29 and 3.30). Interestingly, a number of cell types including fibroblasts, mouse mesenchymal cells and mammary epithelial cells, have less focal adhesions when grown on a 3D gelatinous matrix rather than a rigid 2D matrix coated with extracellular matrix proteins (Wozniak et al. 2004). It is suggested that cells can
sense the rigidity of the extracellular environment through focal adhesions via contractility of the cytoskeleton (Wozniak et al. 2003). This may be one of the mechanisms that switches mammary epithelial cells from a proliferative state in 2D to a differentiated state in 3D cultures (Wozniak et al. 2004). The expression of PP2A subunits at the basement membrane decreased as the cells differentiated from day 8 through 14, with the lowest expression observed at day 20, especially for PP2A-A (Figures 3.28-3.37). While PP2A-C expression was still evident at day 20, it was no longer a continuous ring surrounding the acini (Figures 3.36). Thus it is possible that less PP2A is required at the basement membrane to maintain the reduced number of focal adhesions. This hypothesis would require further examination of other focal adhesion proteins, such as IQGAP1 and paxillin, by immunofluorescence in the 3D MCF10A cultures.

Nuclear expression of PP2A subunits was observed in the proliferating cells of a number of the breast cell lines (Figures 3.13, 3.16 and 3.18) but not in the HMEC cells (Figure 3.10), confluent breast cancer cell lines (Appendix 5) or the MCF10A 3D acini (Figure 3.28 and 3.32). PP2A has been shown to both positively and negatively regulate the cell cycle (Margolis et al. 2006; Forester et al. 2007; Lee et al. 2010), and thus the increased expression of PP2A in the nuclei of dividing breast cancer cell lines may indicate PP2A regulates cellular proliferation in breast cancer. The nuclear bodies stained by the PP2A-A antibody are interesting and are similar in appearance to PML nuclear bodies. These are macromolecular structures that control apoptosis, cell proliferation and senescence (Salomoni et al. 2002). Intriguingly, the PML protein has been shown to recruit both PP2A and phosphorylated Akt to these nuclear bodies where PP2A inactivates Akt. Reduced expression of PML is associated with cancer (Trotman et al. 2006). The PP2A nuclear bodies were observed in the non-tumourigenic MCF10A cells, and the weakly tumourigenic HMT-3522-S2 and T47D cells (Figures 3.13, 3.16 and 3.18), but were not present in MCF7 or more tumourigenic MDA-MB-231, MDA-MB-468, HMT-3522-T42 or DU4475 cell lines (Figures 3.17 and 3.19-3.22). This would support the theory that reduced expression of PML is associated with cancer. However, the HMEC cells also showed no PP2A-A staining of these bodies, thus further work is required.
to definitely determine the nature of these PP2A containing nuclear bodies, and their role, if any, in breast cancer. Another potential hypothesis is that the nuclear bodies may be nucleoli. Nucleolar localisation of PP2A-C was previously reported in human lung-derived MRC-5 cells (Brewis, 1993), although no co-staining with specific nucleoli markers was performed to confirm this. The PP2A-B subunit family however, has been identified in the nucleoli of budding yeast in connection with mitotic exit processes (Wang et al. 2006). As PP2A subunits have also been identified in these processes in human cells (Schmitz et al. 2010), this also presents an avenue of further investigation.

Finally, PP2A-A and PP2A-C is co-localised with cleaved caspase 3 expression, a marker of cellular apoptosis, in the clearing lumen of MCF10A 3D acini (Figures 3.31 and 3.33). PP2A is known to regulate cellular apoptosis, chiefly through activation of p53 and inactivation of Bcl2, in a number of cell lines (Gjoerup et al. 2001; Ruvolo et al. 2002; Chiang et al. 2003; Xin et al. 2006; Boudreau et al. 2007; Deng et al. 2009) including MCF7 cells (Bertoli et al. 2009), and thus altered PP2A function may be a mechanism by which breast cancer cells avoid apoptosis.

In conclusion, PP2A expression, activity and also various known mechanisms of PP2A regulation were assessed in a panel of breast cancer cell lines. The most striking result was the observed reductions in PP2A-A expression in both the breast cancer cell lines and also patient samples. This is compelling evidence that reduced PP2A-A expression may contribute to breast cancer. In addition, a number of regulatory B subunits also demonstrated altered expression, the most significant being reduced PP2A-Bα, -Bδ and PP2A-B’γ expression. PP2A-A mutations were not observed in any of the cell lines used. Thus, even though mutations in PP2A-A occur at a relatively low frequency, these results indicate that alternate methods of PP2A disruption, such as decreased PP2A-A, or regulatory B subunit expression, may occur in a significant proportion of breast cancers. It may therefore be worth considering PP2A function, as opposed to only PP2A mutations, when examining the role of PP2A in human cancers.
4. ESTABLISHMENT OF CELL LINES WITH KNOCKED DOWN OR MUTATED PP2A SUBUNITS

4.1. Introduction

As established in chapter 3, PP2A-A expression is reduced in breast cancer cell lines, and also in human breast tumours. In addition, a number of regulatory B subunits have reduced expression in a panel of breast cancer cell lines, particularly PP2A-Bα and -B’γ. Although no PP2A-A mutations were identified in the 9 cell lines examined, the PP2A-A mutations identified at a low frequency in breast cancer prevent regulatory B subunit binding and lead to their degradation (Calin et al. 2000; Ruediger et al. 2011). In order to investigate the functional effects of reduced PP2A subunit expression and PP2A-A mutations in breast cancer, specific alterations to PP2A subunit expression in a breast cancer model is required. The MCF10A human mammary epithelial cell line was chosen for these experiments as MCF10A cells are immortal, but not tumourigenic (Soule et al. 1990). Thus, unlike minimally replicating primary human mammary epithelial cells (HMECs), which senesce after approximately 15 population doublings, MCF10A cells allow longitudinal studies following stable PP2A manipulations. This chapter details the generation of cell lines with reduced expression of PP2A subunits or introduced PP2A-A mutations, with the functional effects of these alterations presented in Chapters 5 and 6 respectively.

The SV40 small T antigen (ST) is a valuable tool for investigating the role of PP2A subunits within cells. The binding site for ST on PP2A-A overlaps with the regulatory B subunit binding region, and has been shown to displace PP2A-B and -B’’ subunits from the holoenzyme in vitro (Chen et al. 2007), as well as the PP2A-Bα and -B’γ subunits from PP2A holoenzymes in vivo (Chen et al. 2004).

In chapter 3, significant reduction in the expression of the PP2A-Bα and -B’γ subunits was identified in all of breast cancer cell lines. Knockdown of these subunits in the immortalised HEK-TER model system leads to cellular transformation in a similar manner to ST expression (Chen et al. 2004; Chen et al. 2005; Sablina et al. 2007a). In contrast, reduced PP2A-B’α expression was only observed in one breast cancer cell line, and this subunit was not able to transform HEK-TER cells (Sablina et al. 2010). In order to investigate the effects of selectively targeting individual regulatory B
subunits in breast cancer, MCF10A cells were transfected with short-hairpin RNA (shRNA) constructs to knockdown the protein expression of the PP2A-Bα, -B’α and -B’γ subunits.

Mutations in the structural PP2A-A subunit have been identified in multiple cancers including breast cancer (Ruediger et al. 2001b; Ruediger et al. 2001a). Although mutations have been identified in both the PP2A-Aα and PP2A-Aβ subunits, PP2A-Aα mutations are predicted to induce haploinsufficiency, whereas PP2A-Aβ functions as a traditional tumour suppressor requiring inactivation of both alleles (Chen et al. 2005; Sablina et al. 2007b). Despite the mutation rate of PP2A-Aα being quite low in tumours (Calin et al. 2000), as mutations often prevent binding of other PP2A subunits, they nevertheless provide an excellent research tool for teasing out the functional effects of reduced regulatory B subunit binding (Ruediger et al. 2001a).

Five different PP2A-Aα mutations are expressed in MCF10A cells in this thesis: E64D, E64G, P179A, R418W and D492G. Investigations examining the PP2A-Aα mutations E64D, E64G and R418W in HEK-TER cells first prompted the haploinsufficiency theory of PP2A-Aα mediated transformation (Chen et al. 2005). Ectopic expression of these mutants surprisingly resulted in unchanged PP2A activity and lack of transformed phenotype. However, when the wild-type endogenous PP2A-Aα was down-regulated in HEK-TER cells by shRNA, to approximately 50% of original PP2A-Aα expression, a corresponding reduction in PP2A activity was observed together with a tumourigenic phenotype. These cells grew approximately 20% faster than control cells, were capable of anchorage independent cell growth in soft agar, and were able to form tumours in immune-deficient mice. Re-introduction of wild-type PP2A-Aα (by shRNA resistant gene constructs) rescued the tumourigenic phenotype, whereas the PP2A-Aα mutants did not. This suggests that the level of functional PP2A-Aα expression is critical for maintaining normal cellular growth. The authors proposed haploinsufficiency as an alternate theory of tumour suppression, where mutations in one PP2A-Aα allele leave only 50% of PP2A-Aα functional, which was the level shown in this study to induce tumourigenicity (Chen et al. 2005). To investigate if a similar mechanism can transform breast epithelial cells, MCF10A cells were co-transfected with PP2A-Aα shRNA to suppress wild-type PP2A-Aα, together with shRNA resistant PP2A-Aα mutants.
As MCF10A cells are notoriously difficult to transfect with DNA constructs, retroviral infection was chosen to transduce DNA into the MCF10A cell genome to generate stable cell lines. As these are a human cell line, and human retroviral manipulation poses a safety risk, MCF10A cells containing an ecotropic receptor for mouse retrovirus were utilised (Provided by Professor Roger Daly, Garvan institute of medical research, Sydney NSW). Selection for the ecotropic receptor was achieved by selection with gentamycin/G418, rendering these cells resistant to G418 (Brummer et al. 2006), and thus selection of cells positive for DNA constructs must use alternative selection strategies. In this case, either selection for co-expression of GFP, or selection for puromycin resistance was utilised.
4.2. Results

4.2.1. Generation of MCF10A cells expressing the SV40 small T antigen

A retroviral approach was used to insert the ST sequence into the MCF10A genome (Section 2.4). This produces a stable infection, where subsequent generations should not lose ST expression. As a negative control, a C-terminal truncation of ST unable to bind PP2A, named Mut3 (Sontag et al. 1993) was also transduced into MCF10A cells. The pBABE GFP vector (Appendix 1), used for both ST and Mut3, contains a GFP sequence downstream of the inserted gene of interest, but is expressed as a separate polypeptide. Thus, GFP expression indicates that the gene of interest is also expressed, so cells positive for GFP expression were isolated by fluorescent activated cell sorting (FACS) to select for cells expressing ST or Mut3. Both ST and Mut3 cells were sorted twice to generate populations that were over 99% GFP positive by flow cytometry (Figure 4.1A). Expression of ST and Mut3 was also confirmed by RT-PCR of mRNA isolated from these cells using vector primers directly adjacent to the inserted sequences (Figure 4.1B), which also shows that Mut3 is a truncation of ST. Western blotting using an anti-SV40 antibody revealed protein expression of both ST and the smaller Mut3 (Figure 4.1C), however expression levels were very low, requiring a 48 hour primary antibody incubation and double the standard secondary antibody concentration.

H-Ras-V12 expressing MCF10A cells were also generated as a positive control. The vector for H-Ras-V12 expression contained a puromycin resistance gene as a selection marker rather than GFP. Retroviral infected cells were maintained in puromycin until all non-infected control cells were dead, and western blotting with an anti-Ras antibody demonstrates the increased expression of Ras in these cells compared with untransduced controls (Figure 4.1D).

As ST binds to PP2A-Aα in the same region as regulatory B subunits (Ruediger et al. 1999; Cho et al. 2007), can displace regulatory B subunits in vitro and in vivo (Chen et al. 2004; Chen et al. 2007), and displaced regulatory B subunits can be degraded in cells (Strack et al. 2004); the expression of individual regulatory B subunits was determined in ST expressing MCF10A cells by western blotting (Figure 4.2).
Figure 4.1 Generation of SV40 small t antigen and RAS expressing MCF10A cell lines.

A) Flow cytometry profile of GFP expression in MCF10A cells transduced with ST or Mut3 after sorting by FACS. Negative control was UTD MCF10A cells.

B) RT-PCR of SV40 ST and Mut3 expressing MCF10A cells with vector primers.

C) Western blot of SV40 ST and Mut3 expressing cell lines with media from TIB-230 hybridoma cell line which produces anti-SV40 antibodies, and also anti-PP2A-A and -C subunit antibodies as indicated.

D) RAS expressing MCF10A cell line probed with RAS, anti-PP2A-A and -C subunits as indicated.

UTD = Untransduced MCF10A cells
Figure 4.2 PP2A subunit expression in MCF10-ST cells.
Western blots of whole cell lysates from MCF10A-Mut3 and -ST expressing cells for PP2A subunits as indicated. Actin expression was used as a control. Percentages indicate the expression of PP2A subunits in ST cells compared with Mut3 relative to actin.
PP2A-Bα, -Bδ, -B′α, -B′γ and -C subunits demonstrate reduced expression in MCF10A-ST cells compared with -Mut3, while PP2A- -B′′β remain constant.

4.2.2. Generation of MCF10A cells with reduced PP2A subunit expression

4.2.2.1. Regulatory B subunits
A retroviral approach was employed to introduce shRNA constructs for PP2A-Bα, -B′α and -B′γ into the MCF10A cell genome (Section 2.4). The pMKO.1 GFP vector for these constructs also contained a GFP selection gene. The cells expressing GFP were selected for by two rounds of FACS to generate populations that were 95% GFP positive (Figure 4.3A). Western blotting for individual regulatory B subunits and also PP2A-A and -C was performed to determine the extent of regulatory B subunit knockdown by shRNA (Figure 4.3B). PP2A-Bα expression was reduced to 79% of vector controls, with -B′α and -B′γ reduced to 47% and 46% respectively. These cells were analysed for a range of transformed characteristics including proliferation rate, anchorage independent growth and three-dimensional acini formation as described in Chapter 5.

4.2.2.2. PP2A-Aα
The shRNA sequence for PP2A-Aα used in this thesis has been previously published (Strack et al. 2004). This sequence was chosen to match the PP2A-Aα mutants used in this thesis which are resistant to this particular shRNA sequence (Section 4.2.3). Multiple attempts were made to subclone the PP2A-Aα shRNA sequence from a pSUPER shRNA retroviral vector into pMKO.1 GFP (Provided by Assoc. Prof. Stefan Strack, University of Iowa, Iowa, USA). The PP2A-Aα sequence was required in pMKO.1 GFP to use GFP as a selection marker as pSUPER does not contain a selection gene. For an unknown reason, the ligation and transformation of a correctly sized PCR product from pSUPER and the digested pMKO.1 GFP vector consistently yielded Escherichia coli (E. coli) clones that did not contain the PP2A-Aα shRNA insert. As an alternate method, considering the shRNA sequence is quite short, oligo sequences with overhanging ends that mimic digestion with Age1 and EcoR1 were designed (Figure 4.4 and Section 2.2.3). Annealing of the oligos produced a 65bp product (Figure 4.5A) which was then ligated into the pMKO.1 GFP vector (Figure 4.4A). PCR primers specific for pMKO.1 GFP were used to amplify a 250bp region.
Figure 4.3 Generation of MCF10A cell lines expressing PP2A regulatory subunit shRNA constructs.

A) GFP expression in shRNA expressing MCF10A cell lines by flow cytometry.

B) Western blots of whole cell lysates from regulatory B subunit shRNA expressing MCF10A cells for individual B subunits and Actin as indicated. Percentages beside each regulatory B subunit blot are percentage expression as determined by densitometry compared to vector control cells, and relative to actin expression.
**Figure 4.4 PP2A-Aα shRNA.**

A) Annealed oligos containing the PP2A-A target sequence with overhang for direct insertion into pMKO GFP. Red arrow indicates location of insertion into pMKO.1 GFP. The pMKO.1 GFP shRNA vector map is from Addgene website (www.addgene.org).

B) Short hairpin structure that targets PP2A-Aα.

Figures were created using Geneious software (Drummond et al. 2010).
Figure 4.5 Introduction of PP2A-α RNAi into the pMKO-GFP vector.
A) 12.5% polyacrylamide gel showing annealed α RNAi oligos.
B) 250bp PCR product purified for sequencing PP2A-α shRNA oligos inserted into pMKO GFP.
C) Sequencing result of pMKO PCR product containing PP2A-α shRNA oligos as compared to the original oligo sequences.
D) Efficient transfection of pMKO GFP with PP2A-α shRNA into retroviral packaging cell line, Phoenix-Eco, as demonstrated by GFP expression.
of vector containing the insert (Figure 4.5B), which was subsequently sequenced. The PP2A-Aα shRNA sequences remained intact, although the restriction sites used to insert them were destroyed (Figure 4.5C). The pMKO.1 GFP vector containing the newly inserted PP2A-Aα shRNA was transfected into Phoenix-Eco retroviral packaging cells, and efficient vector expression was confirmed by a high proportion of GFP expressing cells (Figure 4.5D). The supernatant of these cells contain retroviral particles that was subsequently used to infect MCF10A cells (Section 2.4). MCF10A cells infected with PP2A-Aα shRNA pMKO.1 GFP were also infected with retroviral supernatant containing empty vector pBABE puro as controls for PP2A-Aα mutants (Section 4.2.3). As an additional control, MCF10A cells were also infected with a scrambled shRNA sequence that does not match any known proteins (Sarbassov et al. 2005) (Table 2.2). The scrambled shRNA was also co-infected with the pBABE puro empty vector construct.

Following infection, cells expressing GFP were isolated by FACs, and then treated with puromycin for 8 days, the time required for non-infected MCF10A cells treated with puromycin to die. The doubly selected cells were then plated into 6 well plates at a very low density and allowed to grow into individual colonies. A number of these individual colonies were then picked and expanded. These clones were analysed for GFP expression by flow cytometry and also PP2A-A expression by western blotting (Figure 4.6). For the PP2A-Aα shRNA cells (Figure 4.6A) clone 5 had the highest GFP expression and also the lowest PP2A-A expression, with less than 20% expression compared to untransduced controls. These cells proliferated very slowly and eventually died. They were not assessed for any markers of apoptosis, but the cells appeared to have apoptotic vacuoles and membrane blebbing (Appendix 15). This is consistent with previous reports that reduction of PP2A-Aα below approximately 1/3 expression results in cellular apoptosis (Strack et al. 2004; Chen et al. 2005; Ruediger et al. 2011). Clones 1 and 4 were chosen for further experimentation with regulatory B subunit shRNA expressing MCF10A cells as described in Chapter 5. Clone 1 was chosen as it had approximately 30% PP2A-Aα expression but still proliferated well. No clones demonstrated the near 50% reduction shown previously to induce tumour formation in HEK-TER cells, however clone 4 was chosen as an intermediate level of PP2A-Aα expression with approximately 70% expression compared to vector controls.
Figure 4.6 PP2A-Aα shRNA clones.

A) Clonal populations of MCF10A cells infected PP2A-Aα shRNA were isolated from single cell colonies and examined for GFP and PP2A-A protein expression. The top graph (green bars) shows the mean GFP fluorescence for each clone determined by flow cytometry. Overall PP2A-Aα protein expression was determined by western blotting on whole cell lysates with anti-PP2A-A antibody, the densitometry for each clone is presented in the lower graph (blue bars). A representative blot is shown beneath the graph.

B) Selection of scrambled shRNA cell clones with figures as described for (A).

GFP = Green fluorescent protein. UTD = untransduced MCF10A cells. BC = Bulk culture before clonal selection.
For the scrambled shRNA expressing cells, after the initial round of FACS, over 96% of the sorted population were positive for GFP expression with a high mean fluorescence (Figure 4.6B). However, following puromycin selection and clonal expansion, all of the clones generated had very weak GFP expression. Clone 3 was resorted by FACS, but still only 35% of the population expressed GFP, and at quite low levels (Figure 4.6B). In addition, these cells demonstrated an altered cellular morphology, with increased spreading (Appendix 16). Due to these alterations in cellular morphology and the low percentage of GFP expressing cells, the MCF10A scrambled shRNA expressing cells were not used for further analysis.

4.2.2.3. PP2A subunit expression in MCF10A shRNA cell lines
Knockdown of PP2A-A in cells can lead to reduced expression of regulatory B subunits, due to lack of structural PP2A-A subunit to protect them from proteasomal degradation (Strack et al. 2004; Chen et al. 2005; Ruediger et al. 2011). For this reason MCF10A cells expressing both PP2A-A and also regulatory B subunit shRNA constructs were analysed for expression of other regulatory B subunits (Figure 4.7). PP2A-A knockdown results in slightly reduced expression of PP2A-Bα and -B’α, with greater reductions in -B’β and -B’’β. PP2A-Aα shRNA #4 also demonstrated reduced expression of PP2A-B’γ, whereas PP2A-Aα shRNA #1 did not. Knockdown of PP2A-Bα and -B’α resulted in reduced PP2A-B’β subunit expression, with little effect on other regulatory B subunits. PP2A-B’γ shRNA reduces the protein expression of a number of the regulatory B subunits including PP2A-Bα, -Bβ, -B’α and -B’β. The two bands for PP2A-B’γ are likely to be transcript variants 1 and 3, with variant 3 being the lower molecular weight. This was the isoform found to have reduced expression in breast cancer cell lines (Section 3.3.1). The shRNA construct targets all transcript variants of PP2A-B’γ.

4.2.3. Generation of MCF10A cells with PP2A-Aα mutations
It has previously been shown that ectopic expression of cancer associated PP2A-Aα mutants alone in HEK-TER cells produces little phenotype, presumably as the endogenous PP2A-Aα protein is still able to function normally despite the presence of the mutant protein. However, expression of PP2A-Aα mutants in combination with suppressed endogenous PP2A-Aα results in a transformed phenotype
Figure 4.7 Regulatory B subunit expression in MCF10A cells with knockdown of PP2A subunits.

Western blots of whole cell lysates from regulatory B subunit and PP2A-α shRNA expressing cells for PP2A subunits and actin as indicated. Percentages indicate the expression of PP2A subunits in shRNA expressing MCF10A cells compared with vector control cells relative to actin.
(Chen et al. 2005). In human cancers, mutation of one allele may reduce the functional PP2A-Aα expression by half, leading to cellular transformation in a similar manner to reduced expression of endogenous wildtype PP2A-Aα levels in the absence of mutation. To investigate whether PP2A-Aα mutations can transform immortalised HMECs, MCF10A cells were co-transduced with the PP2A-Aα shRNA construct used to suppress PP2A-Aα expression in section 4.2.2.2, together with shRNA resistant PP2A-Aα mutation constructs.

Mutated PP2A-Aα sequences with resistance to the PP2A-Aα shRNA used in section 4.2.2.2 were subcloned from pcDNA5/TO constructs (provided by Stefan Strack (Strack et al. 2004)) into the pBABE puro vector as this is a retroviral plasmid and also allows selection by puromycin resistance (Figure 4.8A, Section 2.2.2). The PP2A-Aα mutants and exogenous wild-type sequences are resistant to the PP2A-Aα shRNA construct due to 4 silent base pair changes in the region that the shRNA is generated against (Figure 4.8B). These silent alterations do not change the amino acid sequence, but as RNA interference must be very specific, the mRNA cannot be degraded by the short interfering RNA encoded by the shRNA sequence. As no matching restriction sites were available for subcloning, the mutant PP2A-Aα sequences were amplified from the pcDNA5/TO vector with vector specific primers containing a HindIII restriction site (Figure 4.9A). The PCR product was then ligated into pBABE puro and transformed in DH5α competent E. Coli. Ampicillin resistant transformed E. Coli were selected for on ampicillin agar plates, with resulting colonies picked and expanded prior to DNA extraction and testing for successful PP2A-Aα mutant inserts by restriction digest with HindIII (Figure 4.9B). All positive clones were sequenced to ensure correct orientation of the PP2A-Aα sequence as only one restriction site was used for insertion, and also to check for any mutations gained during the PCR amplification (data not shown).

MCF10A cells were co-transduced with PP2A-Aα shRNA constructs along with 1 of 5 different PP2A-Aα mutants or wild-type PP2A-Aα (PP2A-Aα WT) (Figures 4.10-4.12). MCF10A cells were first selected for PP2A-Aα shRNA expression by GFP expression using FACS and then were selected for pBABE puro PP2A-Aα mutant constructs by growth in puromycin for 12 days until all untransduced control cells, treated equally, had died. Cells were then plated at a low density to select for
Figure 4.8 Subcloning PP2A-Aα mutants.

A) pcDNA5/TO constructs were used as a template for PCR amplification of PP2A-Aα wild-type, or one of five different PP2A-Aα mutations, with primers containing HindIII restriction sites. PCR products as well as the pBABE puro vector were digested with HindIII restriction enzyme and ligated together.

B) A representative figure of PP2A-Aα displaying the location of the 5 different mutations, as well as the shRNA resistance site and the C terminal EE tag.
Figure 4.9 Subcloning PP2A-Aα wild-type and mutations.
All figures are 1.5% agarose gels with ethidium bromide.
A) PCR of PP2A-Aα sequence from pcDNA5/TO template.
B) Example of successful cloning. Digested vector and E64D PCR product (lanes 2 and 3 respectively) were ligated and transformed in DH5α competent *E. Coli*. Colonies containing ligated vector were selected on ampicillin agar plates, cultured and DNA extracted. Undigested plasmid DNA from successful clones containing the 1800bp insert were ~8000bp (lane 4). Clones were also digested and electrophoresed to ensure correct size product (lanes 5 and 6). Clones containing insert were sent for sequencing to determine correct orientation of the insert as a single digestion site was used.
Figure 4.10 PP2A-Aα mutant clones.

PP2A-Aα shRNA constructs and shRNA resistant PP2A-Aα mutations were transduced into MCF10A cells by retroviral gene transfer. MCF10A cells expressing the shRNA construct were selected for by GFP expression using FACS (top graph, green bars). Expression of PP2A-Aα mutations were selected for by puromycin resistance, and verified by western blotting with an anti-EE antibody directed against a C-terminal EE tag (EE expression by densitometry, middle graphs, purple bars). Overall PP2A-Aα expression was determined by western blotting with an anti-PP2A-A antibody, normalised to actin and compared to untransduced MCF10A cells (lower graphs, blue bars). * indicates clones selected for further analysis. BC = Bulk culture before clonal selection.
Figure 4.11 PP2A-α mutant clones.
PP2A-α shRNA constructs and shRNA resistant PP2A-α mutations were transduced into MCF10A cells by retroviral gene transfer. MCF10A cells expressing the shRNA construct were selected for by GFP expression using FACS (top graph, green bars). Expression of PP2A-α mutations were selected for by puromycin resistance, and verified by western blotting with an anti-EE antibody directed against a C-terminal EE tag (EE expression by densitometry, middle graphs, purple bars). Overall PP2A-α expression was determined by western blotting with an anti-PP2A-A antibody, normalised to actin and compared to untransduced MCF10A cells (lower graphs, blue bars). * indicates clones selected for further analysis. BC = Bulk culture before clonal selection.
Figure 4.12 PP2A-Aα mutant clones.
PP2A-Aα shRNA constructs and shRNA resistant PP2A-Aα mutations were transduced into MCF10A cells by retroviral gene transfer. MCF10A cells expressing the shRNA construct were selected for by GFP expression using FACS (top graph, green bars). Expression of PP2A-Aα mutations were selected for by puromycin resistance, and verified by western blotting with an anti-EE antibody directed against a C-terminal EE tag (EE expression by densitometry, middle graphs, purple bars). Overall PP2A-Aα expression was determined by western blotting with an anti-PP2A-A antibody, normalised to actin and compared to untransduced MCF10A cells (lower graphs, blue bars). * indicates clones selected for further analysis. BC = Bulk culture before clonal selection.
individual clones. Single cells were grown into colonies until visible without microscopy, picked using a pipette tip and expanded into a cell line. Following selection, expression of PP2A-Aα shRNA was assessed by GFP fluorescence using flow cytometry (Figures 4.10-4.12 top graphs). The PP2A-Aα sequences contained a C-terminal EE tag, and expression of exogenous proteins was determined by western blotting with an anti-EE antibody (Figures 4.10-4.12 middle graphs). Overall PP2A-Aα expression, the combined total of reduced endogenous PP2A-Aα expression and exogenous expression of PP2A-Aα mutants, was also determined by western blotting with an anti-PP2A-A antibody that recognises both endogenous and exogenous PP2A-Aα (Figures 4.10-4.12, lower graphs).

Western blotting for the EE tag revealed 7 successful clones expressing exogenous shRNA resistant PP2A-Aα WT (Figure 4.10A). Of these 4 demonstrated high GFP expression. Clones 5 and 6 were chosen as wild-type controls for comparison with MCF10A cells expressing mutant PP2A-Aα as they demonstrated high EE tag and also GFP expression. The sum total of PP2A-Aα expression in clones 5 and 6 was 117% and 140% of untransduced. A great variety of EE and GFP expression was observed in the mutant clones, thus for each mutant, 2 clones were selected that demonstrated both high GFP expression and good EE tag expression. Repeated attempts were made to generate more clones expressing the R418W mutant (Figure 4.13A). None of the initial 12 colonies picked from low density plating of GFP sorted, puromycin treated cells, survived expansion into a clonal cell line, and despite 10 colonies growing after a second round of low density plating, only 1 expressed the EE tag (data not shown). This clone did not have very efficient GFP expression, and so was sorted again by FACS to generate two populations of cells, one with higher GFP expression, and one with low GFP expression, used for further analysis in this thesis (Figure 4.12A). Only 6 D492G mutant colonies grew out of the low density plating (approximately 100 cells were added to 2x 6 well plates to allow colony formation) and were picked for expansion. Of these only 3 grew into an expanded population expressing the EE tag. Like the R418W clones, those expressing the mutation had low GFP expression (data not shown) and so were sorted again by FACS to generate a more enriched shRNA expressing population. After this round of sorting all 3 clones
Figure 4.13 Comparison of PP2A-α mutants in MCF10A.

All figures are western blotting on whole cell lysates with antibodies as indicated. Error bars are SEM for three independent experiments. A representative blot for each graph is shown above the graph.

UTD = Untransduced MCF10A cells. Scr = scrambled shRNA.

A) EE tag expression in PP2A-α mutants and exogenous WT PP2A-α expressing MCF10A cells. Note PP2A-α shRNA cells were co-infected with pBABE puro empty vector. MCF10A untransduced or shRNA expressing cells do not express the EE tag. For this reason expression is compared to PP2A-α WT #5 for this graph.

B) PP2A-A expression normalised to actin and compared to untransduced MCF10A cells.

C) PP2A-C expression normalised to actin and compared to untransduced MCF10A cells.

* p<0.05, ** p<0.01, ***p<0.001 using a students t-test compared to cell lines indicated in A-C above.
demonstrated good GFP and EE tag expression (Figure 4.12B), and thus no further attempts were made to generate more clones expressing this mutation.

A direct comparison of all clones chosen for further analysis in Chapter 6 is presented in Figure 4.13. MCF10A-Aα WT #5 and -Aα WT #6 expressed 117% and 140% PP2A-A respectively compared to untransduced cells. Following the assessment of PP2A-A levels it was decided that instead of using both PP2A-Aα WT cell lines as controls it would be more beneficial to use vector control cells as the primary control to determine if over expression of the exogenous wild-type PP2A had any effect on cellular phenotype. Thus only PP2A-Aα WT#5 and not PP2A-Aα WT #6 was used in Chapter 6. All mutants express the EE tag by western blotting, whereas untransduced MCF10A cells and cells expressing shRNA constructs alone do not (Figure 4.13A). In the PP2A-Aα WT cells, two bands are observed which correspond to endogenous PP2A-Aα which is not completely knocked out by the shRNA construct (lower band) and the higher molecular weight exogenous PP2A-Aα with the EE tag (upper band) (Figure 4.13B). The endogenous wild-type band can also be seen in P179A #1 very faintly. The single bands in the other mutants have migrated at the same rate as the higher exogenous band suggesting successful knockdown of the endogenous PP2A-Aα in these cells by the shRNA constructs. The PP2A-Aα shRNA #1 clone demonstrated significant reduction in PP2A-A expression as expected. A number of clones demonstrated increased total PP2A-A expression, due to efficient expression of the exogenous mutant PP2A-Aα proteins (Figure 4.13B). PP2A-C expression levels remained constant except for three clones (Figure 4.13C). PP2A-Aα WT #5 and P179A #1 had reduced PP2A-C expression, whereas E64G #4 demonstrated increased expression.
4.3. Discussion

To investigate the functional effects of altered PP2A expression in breast cancer, the immortalised, non-tumourigenic MCF10A cell line was transduced with a number of constructs that alter PP2A holoenzyme composition. The SV40 ST antigen was expressed as a general mechanism of PP2A inhibition through altered regulatory B subunit binding. A truncation mutant incapable of binding PP2A, Mut3, and the H-Ras-V12 oncoprotein were also expressed as negative and positive controls respectively. To determine if specific regulatory B subunits are involved in the development of breast cancer, PP2A-Bα, -B’α and -B’γ were knocked down with shRNA constructs. In addition, PP2A-A expression is altered in breast cancer through both reduced expression and also mutation. Thus, PP2A-A expression was knocked down in MCF10A cells by shRNA, and PP2A-A mutations identified in human cancers were also expressed in MCF10A cells.

Despite high levels of GFP expression, ST and Mut3 expression in MCF10A cells was low, as demonstrated by the need for 30 cycles to generate a visible PCR product on a gel and poor protein expression on western blots (Figure 4.1). This demonstrates a potential pitfall of relying on selection markers alone to assume expression of the gene of interest. ST expression in a breast tissue specific inducible mouse model was varied in a number of strains, with one strain expressing ST at a low rate while two other strains expressed ST at a high rate (Goetz et al. 2001). Importantly, the level of ST expression affected the induced phenotype, with high expression of ST preventing mammary duct and subsequently milk production, but low expression did not affect milk production as normal ducts were formed. In addition, while high ST expression led to breast tumour formation in 6-12% of animals, mice with low ST expression did not form any tumours (Goetz et al. 2001). These results demonstrate dose dependent effects of ST that needs to be taken into consideration when assessing MCF10A cells expressing low amounts of ST. Future studies could employ increasing viral titres of retrovirus containing the ST genes in order to assess the dose dependent effects of ST expression on human breast cell transformation. Potential transformation of MCF10A cells by ST was assessed by cellular proliferation, anchorage independent growth, and acini formation in 3D cultures as presented in Chapter 5.
RNA interference is a powerful tool that selectively knocks down expression of specific proteins of interest. Short hairpin RNA (shRNA) structures utilise the cellular machinery that processes microRNAs (miRNAs) that regulate human gene expression. The human genome encodes over 200 miRNAs, which are transcribed as single RNA strands from the human genome and are folded and processed by a number of cellular proteins including Drosha and Dicer into short double stranded RNA molecules of about 21 nucleotides. The shRNA constructs mimic these miRNAs. The short double stranded RNA sequences are then loaded into a RISC protein complex that degrades one of the strands, but uses the other strand as a template to specifically cleave messenger RNAs within the cell and hence effectively knockdown protein expression (Cullen 2006; Kim et al. 2008). The PP2A-Bα, -B’α and -B’γ subunits were knocked down in MCF10A cells by shRNA constructs (Figure 4.3) to assess the role of individual regulatory B subunits in the transformation of breast cells. These shRNA sequences have been used in previous studies to target the gene of interest (Chen et al. 2004; Chen et al. 2005; Kranias et al. 2010; Sablina et al. 2010). In addition, the specificity of the shRNA constructs was tested by western blotting in this chapter (Figure 4.7), which shows efficient knockdown of the subunit of interest. However, expression of other PP2A subunits were also affected by the shRNA constructs. Whether this is due to off target effects of the shRNA constructs or cellular responses to knockdown of the target subunits was not determined. For example, PP2A-Bα shRNA resulted in reduced expression of not only PP2A-Bα, but also the PP2A-B’β subunit (Figure 4.8). Further, shRNA constructs targeted against PP2A-B’α and -B’γ reduced expression of all the regulatory PP2A-B and -B’ subunits examined, although to varying extents. PP2A-B’”β expression was not affected by any of the regulatory B subunit shRNA constructs. However, it must be noted that PP2A-Bα knockdown was not as great (79%) compared to -B’α (47%) or B’γ (46%), thus the lack of effect on other subunits could be simply due to inefficient PP2A-Bα knockdown. Potential transformation of MCF10A cells by knockdown of these specific regulatory subunits was assessed by cellular proliferation, anchorage independent growth, and acini formation in 3D cultures as presented in Chapter 5.

A reduction of functional PP2A-A structural subunit may be important in breast cancer, either through reduced endogenous protein expression (Section 3.3.9, (Suzuki et al. 2005)) or mutation in the PP2A-A gene (Calin et al. 2000; Esplin et al. 2006).
To determine the functional effects of altered PP2A-A expression in breast cancer, PP2A-Aα expression was knocked down in MCF10A cells by shRNA (Figure 4.6A). This also caused knockdown of the PP2A-B’α, -B’β, -B’γ and -B’’β subunits, but has a lesser effect on PP2A-Bα or PP2A-C (Figure 4.7). Similar results were observed in HEK-TER cells with increasing PP2A-Aα shRNA viral titers, where reduced expression of the PP2A-B’ subunits PP2A-B’γ, -B’δ and -B’ε was observed (PP2A-B’α and -B’β were not examined) at all concentrations of PP2A-Aα shRNA titer, while only the highest concentrations of PP2A-Aα shRNA reduced PP2A-Bα expression (Chen et al. 2005). Despite nearly complete ablation of PP2A-Aα expression in MCF10A cells, PP2A-Bα expression was reduced by less than 10% (Figure 4.7, PP2A-Aα shRNA #1). However, in HEK-TER cells, knockdown of PP2A-Aα caused a corresponding reduction in PP2A-C, while no reductions in PP2A-C were observed in MCF10A cells with PP2A-Aα knockdown. This indicates the response to altered PP2A subunit expression may differ between different cell lines. The functional effects of reduced PP2A-Aα expression in MCF10A cells was investigated with the MCF10A cells with reduced regulatory B subunit expression in chapter 5.

In addition, 5 PP2A-Aα mutants were expressed in MCF10A cells along with the PP2A-Aα shRNA to mimic haploinsufficiency. Even during the generation of these cell lines, some interesting observations about PP2A-Aα function in breast cells were made. Firstly, suppression of PP2A-Aα levels to below 20% of untransduced MCF10A cells in clone 5 resulted in reduced proliferation and ultimately cellular death (Figure 4.6A and Appendix 15). This corresponds with previous reports that suppression of PP2A-Aα expression below approximately 1/3 of original expression results in cellular death (Strack et al. 2004; Chen et al. 2005; Ruediger et al. 2011). In addition, while a number of clones expressing exogenous PP2A-Aα WT, E64D, E64G and P179A mutations were successfully generated, clones expressing the D492G and particularly the R418W mutations were far more difficult to establish (Figures 4.10-4.12). As these mutations prevent PP2A-C subunit binding (Ruediger et al. 2001a), cells expressing these mutations may not have sufficient functional PP2A to survive stressful conditions associated with clonal selection. That is, cells are initially plated at a very low density to form individual colonies, before being mechanically scraped from the dish and mixed vigorously in a new well to break up the small colony of
cells to allow further growth of the clone. Despite these limitations, two clones expressing each mutation were chosen for further analysis (Figure 4.13), with the exception of the scrambled shRNA clones, where further experimentation was abandoned due to altered cellular morphology. EE tag expression across the panel of clones was varied, likely due to different transduction efficiencies. PP2A-A expression was also varied, due to combined efficiencies of mutant and also shRNA expression. PP2A-C expression remained fairly constant across the panel of mutant MCF10A cell lines. These clones were assessed for cellular transformation using cellular proliferation and migration assays, anchorage independent growth, and also acini formation in 3D cultures as described in Chapter 6.