The expression of the prion protein (PrP) in common cancers and its role in the metastasis of breast cancer and development of chemotherapeutic drug resistance in colon and breast cancer

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**Declaration by author**

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Dr Adrian Wiegmans (QIMR Berghofer) performed injection of cells (prepared by author) and took subsequent imaging of developed tumours as per Chapter 6, section 6.3.7. Additionally, Dr Wiegmans performed proliferation and migration imaging and data retrieval as per Chapter 6, Section 6.3.5 (experiment set up by author) and flow cytometry cell cycle analysis as per Chapter 5 section 5.3.7 (cells fixed and prepared by author). Dr Wiegmans assisted with immunofluorescence imaging as per Chapter 3, section 3.1.5 and provided the MDA-MB-231 human breast cancer cell lines and mouse metastatic cell lines. Dr Alan Munn provided editing assistance to entire thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

None

Published or submitted works by the author incorporated into the thesis


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Statement of Contributions to Jointly Authored Works Contained in the Thesis

Included in this thesis are papers in Chapters 2 and 4 which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. These chapters include co-authored paper(s). The bibliographic details of the published or submitted co-authored papers, including all authors and their contributions are:


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**Additional Published works by the Author Relevant to the Thesis but not Forming Part of it**

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To those who have also suffered the effects of cancer throughout your life, this is for us.
Abstract

Renowned for its role in a number of human and other animal neurodegenerative diseases known as prion diseases, the cellular prion protein (PrP\(^C\)) has recently been implicated in the development, progression and drug-resistance of numerous cancers. These cancers include gastric, breast, colon and pancreatic. While there has been considerable research into the role that PrP\(^C\) plays in the development and progression of cancer, there is still much to be elucidated, including (but not limited to) the mechanisms of action of PrP\(^C\) in drug-resistance and metastasis.

The work presented in this thesis aimed to further knowledge of the role that PrP\(^C\) plays in cancer development, metastasis, and drug-resistance. Firstly, we examined the expression of PrP\(^C\) at the gene and protein level in a range of cell lines derived from common cancers including colon, prostate, skin and breast cancer cell lines, comparing PrP\(^C\) expression levels in these cancer cell lines with the PrP\(^C\) expression level in a control non-cancerous cell line. Secondly, we began to explore the role of PrP\(^C\) in chemotherapeutic drug-resistance in colon cancer and identified a potential mechanism by which PrP\(^C\) confers cisplatin-resistance. Thirdly, we examined the expression and influence of PrP\(^C\) on metastasis of breast cancer and addressed the key question of whether PrP\(^C\) in conditioned media from highly metastatic breast cancer cells can transfer a metastatic phenotype to previously non-metastatic breast cancer cells. Lastly, we examined the influence of PrP\(^C\) on the sensitivity of breast cancer cells to doxorubicin treatment.

Our results show that PrP\(^C\) is highly expressed in cell lines derived from numerous cancer types, however we also show that PrP\(^C\) protein expression levels do not always correlate with PrP gene (PRNP) mRNA expression levels due to differences in the stability of PrP\(^C\) protein or export of PrP\(^C\) into the extracellular media in different cell lines. We further show that high PrP\(^C\) protein expression may influence cisplatin-resistance in colon cancer-derived cell lines through the inhibition of FOXO3a activation and subsequent translocation into the nucleus. Additionally,
we demonstrate how PrPC can influence the metastatic potential and doxorubicin sensitivity of breast cancer-derived cell lines.

Overall, the work presented in this thesis not only furthers knowledge of the role that PrPC plays in cancer development and progression, but also demonstrates the importance of research into the role of PrPC in cancer. We highlight how PrPC is a potential target for improving the effectiveness of established chemotherapeutic treatments such as doxorubicin and cisplatin. Furthermore, we demonstrate how PrPC expression at both the mRNA level and protein level may have a potential use as a prognostic marker for patient survival and disease progression in colon and breast cancers.

Keywords

Breast cancer, bovine spongiform encephalopathy, cisplatin, Creutzfeldt-Jakob Disease, doxorubicin, ectodomain shedding, epigenetic, exosomes, glycosylphosphatidylinositol anchor, protein folding, PRNP, scrapie.

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List of abbreviations

ADAM10- a disintegrin and metalloproteinase domain-containing protein 10
BFA- brefeldin A
BSE- bovine spongiform encephalopathy
cDNA- complementary deoxyribonucleic acid
CJD- Creutzfeldt-Jakob disease
CNS- central nervous system
DISC- death inducing signalling complex
DMEM- Dulbecco’s Modified Eagle Medium
DMSO- dimethyl sulfoxide
DNA- deoxyribonucleic acid
ER- endoplasmic reticulum
FBS- foetal bovine serum
fCJD- familial CJD
FFI- familial fatal insomnia
FOXO- forkhead/winged-helix box class O
GPI- glycosylphosphatidylinositol
GSH/GST- glutathione/glutathione S-transferase
GSS- Gerstmann-Straussler-Scheinker
HR- hazard ratio
HRP- horseradish peroxidase
IC- inhibitory concentration
IGF- insulin-like growth factor
MAPK- mitogen-activated protein kinase
MDR- multi-drug resistance
MMP1 - matrix metalloproteinase-1
mRNA - messenger ribonucleic acid
MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
PBS - phosphate buffered saline
PDAC - pancreatic ductal adenocarcinoma
P-gp - P-glycoprotein
PR - progesterone receptor
PrP - prion protein
PrP<sup>C</sup> - cellular prion protein
PrP<sup>Sc</sup> - prion protein scrapie
RNA - ribonucleic acid
RT qPCR - real-time quantitative polymerase chain reaction
sCJD - sporadic CJD
SATB1 - special AT-rich sequence-binding protein-1
Scr - scrambled
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA - small interfering RNA
SOD - superoxide dismutase
TNF - tumour necrosis factor
TRAIL - tumour-necrosis-factor-related-apoptosis-inducing ligand
TSEs - transmissible spongiform encephalopathy
vCJD - variant CJD
Chapter 1: Introduction
Cancer is fundamentally a disease of abnormal and uncontrolled cell proliferation that extends beyond natural boundaries [1]. Familial or inherited cancers, resulting from known or still unknown predisposing genes, make up only a small percentage (5-10%) of cancer incidence [2, 3]. The greater percentage of cancer is sporadic, meaning there is no apparent predisposing genetic cause. Sporadic cancers are thought to be initiated by an accumulation of oncogenic mutations at the somatic level as a result of exposure to carcinogens. Carcinogens comprise both external and internal factors such as environmental pollutants, chemicals, ionizing radiations, hormones and infectious diseases (e.g. those caused by viruses). Cell division allows progressive passage of a DNA mutation within one cell to new daughter cells, thus amplifying its effect at the somatic level. Accumulation of several such mutations may then complete the oncogenic transformation of a normal cell [1].

Epigenetics is emerging as an important contributing factor in human diseases, including cancer. Epigenetics encompasses a (mitotically or meiotically heritable) change in gene function that does not result from an alteration in the DNA sequence [4, 5]. Epigenetic effects are broad and not only restricted to alterations in gene expression (e.g. due to changes in DNA methylation) but also encompass post-translational changes in protein structure that are stable (persists long-term) and are inheritable (somatically) and can result in a change of function, e.g. the conversion of normal cellular proteins into prions. Prions are misfolded, aggregated and infectious proteins [6]. Prions are normal cellular proteins that have undergone a stable change in protein folding that can be acquired by infection, inheritance (at the somatic level) or spontaneously [7]. The prion protein (PrP), is well known for its causative role in many mammalian neurodegenerative diseases such as Creutzfeldt-Jakob disease, scrapie and bovine spongiform encephalopathy [8]. These prion diseases are caused by the conversion of normal cellular prion protein (PrP\textsuperscript{C}) into the pathogenic isoform
Prion Protein Scrapie (PrPSc). Although the normal biological function of PrPc remains elusive, its expression in non-neuronal tissues has suggested its role in multiple cellular pathways and signalling processes throughout the body [9]. It was also noted that the expression of the gene encoding PrPc (PRNP) is up-regulated in the mucosa of patients infected with *Helicobacter pylori* while it was absent or weak in uninfected gastric mucosa [10]. More recently, *H. pylori* infection has also been linked to carcinogenesis of gastric mucosa, prompting investigations into the relationship between PrPc and gastric cancer [11]. Current literature now suggests PrPc is involved in a number of cancers such as colorectal, gastric [11-13], pancreatic [14], breast [15, 16]. Furthermore, PrPc expression is associated with the multi-drug resistant (MDR) phenotypes of gastric [12, 17, 18] cancers. Surprisingly, most of these reports do not confirm the conformational state of PrP (i.e. whether PrPc or PrPSc) in the cancer tissues, with the exception of gastric cancer in which the over-expressed PrPc was sensitive to proteinase K digestion (consistent with the protein being in the PrPc form) [11, 13]. There has been little or no investigation of PrPc expression level in other types of cancer such as colon, skin and lung, which represent three of the top five most common cancers in Australia [19]. Furthermore, while there is evidence showing a potential role of PrPc in the development of drug resistance in cancer, the role of PrPc in other aspects of cancer progression, such as metastasis, has not begun.

Investigation of the expression level of PrPc in other types of cancers, and combining this approach with ongoing functional analysis of PrPc in gastric, pancreatic and breast cancers, will reveal the role PrPc plays in the progression of cancer. This may be by further elucidating the effects PrPc has on the signalling pathways of normal cells and how alteration of these pathways results in cancer development and/or progression.
If PrP\(^C\) were known to promote cancer development and/or progression and/or multi-drug resistance then possible therapeutic treatments could be developed in the future using PrP\(^C\) as a target. This might include, for example, inhibiting the effects of PrP\(^C\) on the normal signalling pathways of the cell and possibly thereby preventing the growth and progression or multi-drug resistance of the tumour. PrP\(^C\) treatments could then be incorporated with other established, cancer treatment options, to improve the success rate of these established treatments. For example, it might be possible to determine whether inhibition of PrP\(^C\) can be combined with chemotherapy, surgery and/or radiotherapy, to improve patient survival rates.

Not only can PrP\(^C\) be utilised as a potential target for cancer treatment, identifying whether PrP\(^C\) is over-expressed in other cancer types could enable the use of PrP\(^C\) as a diagnostic marker for cancer. If the level of PrP\(^C\) over-expression that is associated with cancer development were known, early detection of elevated levels of PrP\(^C\) could be used to diagnose cancer and enable early treatment before the cancer progresses.

The research presented in this thesis further describes a possible role of PrP\(^C\) in cancer development, metastasis and drug resistance, incorporating and building upon the research presented in the literature. There were seven key findings investigated in this study.

Firstly, the level or PrP\(^C\) at the mRNA and protein level was determined in a wide range of cancer cell lines and compared to a non-cancerous control. While the expression of PrP\(^C\) has been investigated in a number of cancers, such a range of cancer cells lines has never been investigated. One cancer type (colon) was then selected from this range of cell lines to undergo investigation into the differences in mRNA and protein levels of PrP\(^C\) by assessment of stability.
Secondly, it was determined whether knock down of PrP\textsuperscript{C} had an effect on the resistance of colon cancer cells to cisplatin, a common chemotherapeutic drug. One of the highest PrP\textsuperscript{C} expressing colon cancer cell lines was determined to be resistant to cisplatin while their lower expressing counterparts were sensitive. The transcription factor FOXO3a has been previously shown to mediate the cytotoxic effects of cisplatin, by being dephosphorylated and allowed to activate downstream genes. In resistant colon cancer cells, this process is limited [20]. Furthermore, it has been shown that FOXO3a is a negative regulator of PrP\textsuperscript{C}. Therefore, it was deduced that knock-down of the PrP\textsuperscript{C} protein in those colon cancer cell lines resistant to the common chemotherapeutic drug cisplatin, may have an effect on the dephosphorylation of FOXO3a and therefore resistance to cisplatin.

The third key finding was to determine the effect of \textit{PRNP} expression on survival of patients with varying types and grades of tumours was assessed through the use of an online database analysing gene expression in patient samples and their survival. Following this, the next key finding was to identify the differences in the expression of PrP\textsuperscript{C} at an mRNA and protein level in non-metastatic and metastatic breast cancer. Using a human and mouse metastatic series, the \textit{PRNP} expression level and PrP\textsuperscript{C} protein levels was determined.

The fifth key finding focussed on the differences in characteristics of the PrP\textsuperscript{C} in metastatic and non-metastatic breast cancer cells. We look at the differences in PrP\textsuperscript{C} proteinase K resistance and aggregation, in summary, two defining characteristics of prions to begin to determine if there is a potential change in folding.

The sixth key finding was to begin to identify a possible mechanism of PrP\textsuperscript{C} involvement in metastasis through assessment of the effect of PrP\textsuperscript{C} knockdown on proliferation and migration as well as metastasis \textit{in vivo} mouse model. Furthermore,
if the metastatic potential of highly metastatic breast cancer cells could be transferred onto non-metastatic breast cancer cells by PrP\textsuperscript{C}.

The last key finding was to establish whether PrP\textsuperscript{C} knock down could sensitize metastatic and non-metastatic breast cancer cells to doxorubicin treatment.
Chapter 2: Literature Review

This chapter includes co-authored paper(s). The bibliographic details of the published co-authored papers, including all authors and their contributions are:


(Signed) _________________________________ (Date) 24.11.2015
Student: Caroline Atkinson

(Countersigned) ___________________________ (Date) 24.11.2016
Corresponding author of paper/supervisor: Ming Wei

Atkinson, C; Zhang, K; Wiegmans, A; Munn, AL; Wei, MQ. (2015) Prion Protein Scrapie and the normal cellular prion protein. Prion. (In production). C. Atkinson was responsible for 70% of drafting and writing. K. Zhang was responsible for 10% drafting and writing (Chapter 2, section 2.2.2). A.L. Munn was responsible for 15% drafting and editing, and M.Q. Wei and A. Wiegmans responsible for 5% drafting and editing.

(Signed) _________________________________ (Date) 24.11.2016
Student: Caroline Atkinson

(Countersigned) ___________________________ (Date) 24.11.2016
Corresponding author of paper/supervisor: Ming Wei
2.1 INTRODUCTION

Prions, originally abbreviated from proteinaceous infectious particle [9], are renowned for their role in many neurodegenerative diseases in humans and other animals. In 1929, Creutzfeldt and Jakob were the first to describe a human neurodegenerative disease, now known as the prion disease; Creutzfeldt-Jakob disease (CJD). They initially described the disease as a progressive dementia associated with astrocytic gliosis, gait abnormalities and vacuolation within the brain [21, 22]. Since this time, many other prion diseases in both humans and other animals have been identified including Gerstmann-Straussler-Scheinker disease (GSS), familial fatal insomnia (FFI) and kuru in humans, scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle [8, 23]. This group of prion diseases is also known as transmissible spongiform encephalopathy (TSEs) due to the ability of these diseases to be transmitted between humans and between animals and the characteristic vacuolar degradation of gray matter neuropil in the brain [24].

2.2 PRION DISEASE

Prion diseases have a number of common histopathological characteristics and neurological symptoms. These include spongiform degeneration of the central nervous system (CNS), formation of amyloid plaques, reactive gliosis (enlarged glial cells appearing after CNS injury) and neuronal loss [25]. Other atypical properties characteristic of prion diseases are long incubation periods (which can extend from several months to several years), lack of inflammation and lack of disease-specific immune response [26].

While at first these neuronal degeneration diseases were thought to be caused by “slow” viruses due to their long incubation periods [27], neither viral particles nor nucleic acids could be detected to support the hypothesis that these are viral diseases [26]. The viral
hypothesis also failed to account for the finding that 95% of human neuronal degeneration disease cases are not linked to infection and lacked the typical histopathological features of viral encephalitis. Hence, the viral disease hypothesis failed to adequately account for the findings that emerged from studies of these diseases [24, 26]. It was also discovered that between 10 and 15% of these neuronal degeneration diseases are dominantly inherited, including all cases of GSS and FFI and 10% of cases of CJD. The latter are referred to as familial CJD (fCJD). In summary, the data showed that these neuronal degenerative diseases can be both infectious and inherited [24].

During the attempts to uncover the molecular basis of prion diseases over the past few decades, it was uncovered that the causative agent of scrapie, is a 27-30kDa protease-resistant protein designated as PrP 27-30 [28]. PrP 27-30 was found to be encoded by a single mammalian gene located on human chromosome 20 [29]. This gene was designated \(PRNP\). Other animals have a homolog of the human \(PRNP\) gene and this gene was named \(Prnp\). The PrP 27-30 protein in scrapie-infected animals was designated prion protein scrapie (PrP\(^{Sc}\)) and was discovered to be a derivative of a normal, protease-sensitive, glycosylphosphatidylinositol (GPI)-anchored form of PrP designated cell-surface glycoprotein or cellular PrP (PrP\(^{C}\)) [30]. Upon further investigation, it was found that all dominantly inherited forms of prion diseases are linked to mutations of or insertions in the \(PRNP\) gene [30].

### 2.2.1 Development of prion disease

Neurodegenerative prion diseases such as CJD, Kuru and BSE, result from the post-translational conversion of PrP\(^{C}\) to a misfolded, aggregated and pathogenic form, PrP\(^{Sc}\) [6]. This conversion is followed by an accumulation of PrP\(^{Sc}\) within the central nervous system resulting in disease [8] (Figure 2.1).
**Figure 2.1 PrP\textsuperscript{Sc} propagation in the central nervous system.** Schematic representation of the propagation of PrP\textsuperscript{Sc} in neuronal cells of the CNS. PrP\textsuperscript{Sc} acquired by infection, mutation or spontaneous conversion of cellular PrP (PrP\textsuperscript{C}) combines with PrP\textsuperscript{C} thereby converting it to PrP\textsuperscript{Sc}. Additional PrP\textsuperscript{C} is recruited and converted to PrP\textsuperscript{Sc}. As PrP\textsuperscript{Sc} levels increase, PrP\textsuperscript{C} recruitment and conversion become more efficient, leading to an accumulation of PrP\textsuperscript{Sc} in neuronal cells. PrP\textsuperscript{Sc} accumulation causes cell dysfunction followed by death.

As shown in Figure 2.1, the newly formed PrP\textsuperscript{Sc} acts as a template to facilitate conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, causing the accumulation of PrP\textsuperscript{Sc} [31]. This process takes place regardless of the origin of the PrP\textsuperscript{Sc}, i.e. whether it is from an external source or produced internally due to mutations in the PRNP gene (as is the case in fCJD and FFI) [24] or due to a spontaneous conversion of wild-type PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, which is referred to as ‘de novo generation/synthesis’ [32].

PrP\textsuperscript{Sc} is characterised by its resistance to protease digestion, insolubility and its high content of β-sheet structure [33, 34] (43% β-sheet compared to 30% α-helical). In contrast, PrP\textsuperscript{C} is an soluble protein high in α-helical content (42%), low in β-sheet content (3%) and highly susceptible to protease digestion [31]. PrP\textsuperscript{C} contains two different domains that play different roles in the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. The first is a stable and ordered ‘core’ domain which contains a GPI lipid anchor that tethers PrP\textsuperscript{C} to the plasma membrane, three α-helices (helices A, B and C) (Figure 2.2), two
asparagine-linked oligosaccharides and a protein binding site capable of lowering the energy barrier for conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} when PrP\textsuperscript{C} binds to protein X (a species specific cofactor necessary for conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}) [35, 36]. The second domain is a ‘variable’ or disordered domain which interacts with PrP\textsuperscript{Sc} and changes PrP\textsuperscript{C} conformation from the unstructured form to the β-sheet characteristic of PrP\textsuperscript{Sc} (Figure 2.2) [34]. During conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}, helix A of the core domain of PrP\textsuperscript{C} (Figure 2.2) also gets converted into a β-sheet [24].

![Figure 2.2 Comparison of the 3D protein structure of PrP\textsuperscript{C} and PrP\textsuperscript{Sc}](image)

Schematic representation of the tertiary and secondary structure of PrP\textsuperscript{C} in comparison to PrP\textsuperscript{Sc} inferred from Syrian hamster recombinant PrP 90-231. A, B and C indicate α-helices.

### 2.2.2 Types of prion disease

Prion diseases in humans and other animals have thus far been classified into three broad categories, based on the properties of the corresponding pathogenic PrP protein that accumulate in the brain and on the neuroanatomical features of the prion-infected brain [24] as summarised in Table 2.1.
Table 2.1 Summary of the three broad categories of prion diseases and the defining characteristics of each.

<table>
<thead>
<tr>
<th>Category</th>
<th>Prion Disease</th>
<th>Defining characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Scrapie; sporadic, familial and iatrogenic CJD (sCJD, fCJD, iCJD); BSE; kuru and sporadic and familial fatal insomnia (sFI and fFI)</td>
<td>Vacuolar (spongiform) degeneration of gray matter, accumulation of protease resistant PrP&lt;sub&gt;Sc&lt;/sub&gt; in gray matter, little or no PrP amyloid plaque formation.</td>
<td>[24]</td>
</tr>
<tr>
<td>2</td>
<td>Dominantly inherited syndromes (GSS)</td>
<td>Deposition of numerous PrP immunopositive amyloid (abnormal protein) plaques in multiple cortical and subcortical brain regions. PRNP mutation.</td>
<td>[24, 37]</td>
</tr>
<tr>
<td>3</td>
<td>Variant CJD (vCJD)</td>
<td>PrP&lt;sub&gt;Sc&lt;/sub&gt; amyloid deposition, vacuolation of gray matter, accumulation of protease resistant PrP&lt;sub&gt;Sc&lt;/sub&gt; in neuropil (space between neuronal and glial cell bodies comprised of dendrites, axons, synapses microvasculature and glial cell processes).</td>
<td>[24, 37-40]</td>
</tr>
</tbody>
</table>

The oral route of infection is the major mode of transmission for most cases of acquired prion diseases in humans. The spread of prion diseases via this route, both naturally and experimentally, has been described in many species. Kuru, for example, was shown to be spread amongst the Fore people who populate the eastern highlands of Papua New Guinea through ingestion of infected brain tissue during cannibalistic rituals which resulted in a particularly high incidence of the disease [41-43].

sCJD accounts for more than 90% of all cases of sporadic prion disease. However, not all CJD is sporadic. Beginning in the 1980s, when a considerable number of cattle were killed by BSE, concerns about the spread of BSE to humans grew dramatically in the United Kingdom [44]. The concern was that BSE could spread to humans through
ingestion of infected beef [45], a fear which was realised in 1995 [46]. A new form of CJD was first reported by the UK National CJD Research and Surveillance Unit in 1996, and is now known as variant CJD (vCJD) [46]. It is believed that vCJD possesses distinct clinical and neuropathological characteristics from sporadic CJD (sCJD) and other forms of prion disease in human [47]. The main distinguishing neuropathological feature of vCJD is an extensive deposition of PrP\textsuperscript{Sc} amyloid in the brain in the form of large ‘florid’ plaques [48]. Other phenotypes include a younger average age of onset and gliosis of the thalamus. Epidemiological studies supported the possibility that the outbreak of BSE in cattle in the UK in the same period may have been responsible for the emergence of vCJD. Subsequently, experimental studies of vCJD and BSE transmission in mice proved that the vCJD agent, unlike the agent responsible for sCJD, had biological properties closely similar to those of the BSE agent [38, 48]. Therefore, vCJD has been confirmed as a novel prion disease and the only human prion disease acquired from another species. Subsequent studies suggested that the pathogen could be present in blood during the incubation period for vCJD [49, 50], and that exposure to such blood could result in the infection of humans or other animals. Therefore, the UK National CJD Surveillance Unit reported that vCJD is caused by exposure to BSE and that the primary source of exposure is the consumption of infected meat products [47].

Unlike vCJD, iatrogenic CJD is an entirely person-to-person transmitted disease, identified to be transmitted through a number of mechanisms including contaminated surgical instruments [51] and dura mater grafts [52], blood transfusions [53] and injection of products from cadaveric pituitary glands [54, 55]. The first case of iCJD was described in 1974 where a corneal graft recipient died after acquiring a dementia-like illness [56]. With improved screening and sterilisation techniques, rates of iCJD
continue to decrease with new cases that occur often the result of longer incubation periods following infection acquired in the 1980s [57, 58].

Whereas the causes of prion diseases that are genetically inherited or acquired by infection have been extensively studied and now quite well known, the cause of sporadic prion diseases is still a topic of speculation [59]. Among the many mechanisms that have been proposed to provoke sporadic prion disease, the mechanism that is supported by the most compelling evidence is a mechanism in which malfunction of the so called quality control system in cells is responsible for initiation of the disease [60]. The cellular quality control complex acts like a set of enzymes which assist newly synthesised polypeptides to “grow” to their proper conformation and also play a role in disposal of those polypeptides that fail to adopt the native structure. Therefore, a lowered efficiency of the quality control complex, perhaps due to ageing or if the quality control complex becomes overwhelmed by excessive protein production, may result in errors in protein folding occurring and the production of misfolded proteins.

One of the inherited prion diseases is Gerstmann–Sträussler–Scheinker disease (GSS). GSS is caused by a pathological mutation in the prion protein gene (PRNP) located on chromosome 20. It is a very rare disease with autosomal dominant inheritance. The age of onset of GSS is relatively early but the disease progresses slowly with an average illness duration of 49–57 months (until death) [61, 62]. The typical GSS syndrome includes prominent ataxia, gait disturbances, cognitive decline and spasticity in the lower extremities [63, 64]. Rarely the syndrome may also include painful dysesthesias and visual disturbances, dystonia and myoclonus and dementia [63, 65, 66]. A GSS case attributable to an A133V mutation in PRNP resulted in an uncommon phenotype similar to that of progressive supranuclear palsy [67]. Several other pathological variations of GSS have been described. The most common mutation in PRNP is
P102L, which is found in more than 80% of cases [68]. The P102L mutation was found in the original GSS pedigree [69]. Other mutations in PRNP known to cause GSS include P105L, P105S, A117V, G131V, Y145*, H187R, D202N, Q212P, Q217R, M232T, and base-pair insertions at codons 96, 192, or 216 [70].

Recently, several GSS cases with novel PRNP mutations were reported. A 61-year-old British-born woman with no history of neurodegenerative disorder among her first-degree relatives in Australia presented with a rapidly progressive dementia. Sequencing of the PRNP gene demonstrated a V176G mutation. Subsequent Western blot analysis resulted in the detection of an 8 kDa atypical protease-resistant PrP band [71]. Sequencing of the PRNP gene of another GSS patient (this one in North America) revealed a 24-nucleotide insertion that when translated would result in a protein product with an 8-amino acid insertion [72]. Independent neuropathological studies of two GSS pedigrees with the P102L mutation obtained divergent findings [73, 74]. The authors note that the variable clinical presentation of GSS patients (even those with the same PRNP mutation) makes diagnosis of GSS challenging and in some families the presence of GSS may be missed. Routine clinical and laboratory investigations, sequence analysis of the PRNP gene and post-mortem examination are recommended in all cases with a family history of any type of neuropsychiatric syndrome.

### 2.2.3 Treatment of prion disease

There are currently no treatments that have proven effective for curing prion diseases in humans or other animals. However, monoclonal antibodies that recognise PrPSc and PrPC have been shown to inhibit prion replication and delay prion disease development in animal models [75]. These antibodies block PrPSc replication by
accelerating the degradation of PrP\textsuperscript{C} (i.e. through reduction of the half-life of the PrP\textsuperscript{C} protein) [76].

Another strategy has been the use of low molecular weight compounds. Compounds that have been undergoing clinical investigation as a possible therapy for prion disease include heterocyclic compounds, e.g. various tricyclic derivatives of acridine and phenothiazine, particularly quinacrine and chlorpromazine [77, 78]. Treatments such as this have been shown to be effective in inhibiting the formation of nascent PrP\textsuperscript{Sc} from PrP\textsuperscript{C} in ScN2a (scrapie-infected mouse neuroblastoma) cells [77, 79]. These chemicals have been in clinical use for many years, quinacrine as an antimalarial drug and chlorpromazine as an antipsychotic drug and both are capable of crossing the blood-brain barrier. Treatments with these chemicals are therefore considered to be attractive options for use essentially “as is” in treatment of prion infections [77, 80].

Quinacrine has been shown to have the ability to inhibit the formation of PrP\textsuperscript{Sc} in ScN2a cells with an IC\textsubscript{50} of 0.3-0.4µM [77, 79]. A study by Barrett \textit{et al.} [80] re-evaluated the potential of quinacrine and chlorpromazine as treatments for prion diseases. The efficacies of these drugs were assessed \textit{in vitro} using two cell line models (ScN2a and ScGT1) and \textit{in vivo} using a mouse model. While the results obtained from the previous studies using the ScN2a cell line were replicated in this study for the ScN2a cell line, this study obtained different results from previous studies for the ScGT1 cell line. In contrast to the findings from the previous studies using ScGT1 cells, this study found that only higher doses of chlorpromazine and quinacrine (10 times the concentration previously described, i.e. 4µM) decreased PrP\textsuperscript{Sc} accumulation in ScGT1 cells in a single treatment. Lower doses of quinacrine (0.4µM) cured ScGT1 cells only over longer treatment times (i.e. daily treatment for three weeks) and the effect was not permanent as PrP\textsuperscript{Sc} infection was re-established after approximately three months. Furthermore, quinacrine and chlorpromazine failed to
inhibit PrP$^{\text{Sc}}$ accumulation in vivo (either individually or even in combination). It was also noted that quinacrine treatment did not affect the proteinase K-resistance or accumulation of PrP$^{\text{Sc}}$ in the spleens of mice inoculated with scrapie. Thus, overall the studies show that quinacrine and chlorpromazine, individually or in combination, do not have therapeutic anti-prion effects in animal models and highlight the need for new and more effective therapeutics.

Doxycycline has been used as a compassionate treatment for CJD patients and was observed to increase mean survival times by 4-7 months in comparison to historical controls. Furthermore, a patient with variably protease-sensitive prionopathy was treated from an early stage and for four years in total with doxycycline and not only lived one year longer than the longest surviving patient with that subgroup of prion disease, but also had less severe and widespread lesions [81]. However, while indeed promising these beneficial effects were not confirmed in randomised, double-blind trials [82]. In experimental rodent models of prion disease, treatment with doxycycline at early stages (i.e. pre-clinical onset) showed good efficacy, while there was little or no apparent effect once clinical signs had emerged [83]. This suggests that treatment with doxycycline may be most useful as a preventive measure, for example for those patients who are carriers of the PRNP mutation that causes Familial Fatal Insomnia [84] and this trial is currently underway [85].

A recent study has shown that treatment of prion disease in humans with non-human prion proteins may be a viable treatment option. This approach is based on the knowledge that conversion of PrP$^{\text{C}}$ to PrP$^{\text{Sc}}$ has a strong dependence on protein sequence homology between the prion inoculum and host PrP$^{\text{C}}$ [86-88]. Skinner et. al. [89] showed that animals treated with a heterologous prion protein (bacterially expressed and purified recombinant hamster prion protein), demonstrated reduced prion-disease-associated pathology, decreased accumulation of protease-resistant
disease associated prion protein and delayed onset of clinical symptoms (including
motor deficits), as well as significantly increased mean survival times in comparison
to mock-treated control animals.

2.3 THE NORMAL CELLULAR PRION PROTEIN

Due to its central role in the development of many neurodegenerative diseases in
humans and animals, the normal PrP\(^C\) has been extensively studied. The structure and
cellular localisations of PrP\(^C\) have been elucidated in various studies. Owing to these
studies and the significant level of expression of PrP\(^C\) in various tissues and organs,
many putative functions have been proposed for PrP\(^C\). Nevertheless, the true function of
PrP\(^C\) still remains a matter of some debate.

2.3.1 Structure and cellular processing of PrP\(^C\)

PrP\(^C\) is generally located on the cell membrane and associates with cholesterol-rich
microdomains (rafts) in cultured non-neuronal and neuronal cells [90]. It is also
associated with detergent-resistant microdomains with a basolateral localisation in
polarised cells such as epithelial cells [91]. The immature PrP\(^C\) protein is
approximately 253 amino acid residues long and 32-35 kDa in mass and comprises
an unstructured N-terminal region and a globular C-terminal domain. The C-terminal
domains consists of three \(\alpha\)-helices, (\(\alpha\)-1: aa 144-153, \(\alpha\)-2: aa 172-192, and \(\alpha\)-3: aa
200-225), \(\beta\)-sheet comprising two antiparallel \(\beta\)-stands [92] (\(\beta\)1: aa129-130, and \(\beta\)2:
aa162-163) [92] and a signal sequence for attachment of the GPI anchor (aa 231-
253) [93] as shown in Figure 2.3 panel A. In humans, the \(PRNP\) gene that encodes
PrP\(^C\) protein contains 2 exons and is 20 kb in length. The \(PRNP\) gene, along with two
other genes, \(PRND\) and \(PRNT\), makes up the \(PRNP\) locus which spans 55 kb in the
p12/p13 region of chromosome 20 (Figure 2.3) [94].
Figure 2.3 Domain structure of human PrP<sup>C</sup> and Doppel proteins. A) PrP<sup>C</sup> contains an N-terminal unstructured or ‘variable’ and C-terminal structured or ‘core’ domain. The unstructured domain consists of N-terminal signal peptide, octarepeat region, hydrophobic region and two cleavage sites labeled α and β. The structured domain of PrP<sup>C</sup> contains three α-helices and two β-sheets along with signal peptide, GPI anchor and disulfide bond. B) Domain structure of Dpl is similar to the structured domain of PrP<sup>C</sup>, however it has an additional disulfide bond and a split α2 helix.

In order to form a mature protein PrP<sup>C</sup> undergoes a number of posttranslational modifications and these are initiated by the removal of the N-terminal and C-terminal signal peptides which is coincident with import of the nascent chain into the endoplasmic reticulum and attachment of the GPI anchor respectively. Two N-linked glycans are also attached and this is followed by formation of a disulphide bond between Cys178 and Cys213 [95, 96]. This disulphide bond is important as it connects the C-terminal α-helices and serves to stabilise the fold of the PrP<sup>C</sup> protein [97]. PrP<sup>C</sup> (which is 210 amino acid residues in length [95]) is then targeted to the outer leaflet of the plasma membrane by the GPI-anchor [95, 96]. PrP<sup>C</sup> can also undergo two endoproteolytic cleavage events [98]. The normal constitutive cleavage, known as α-cleavage [99] (Figure 2.4B), occurs in the brain and in cultured cells between residues 110 and 111. This cleavage is stimulated by agonists of the protein kinase C pathway [100] and results in the formation of a 11kDa soluble N-terminal fragment and a 18kDa C-terminal fragment that remains attached to the cell membrane via the GPI anchor [101-103]. The second cleavage, known as β-cleavage [99] (Figure 2.4C), is mediated by reactive oxygen species (ROS) [99, 104] and leads...
to the formation of a 20kDa GPI-anchored C-terminal fragment and a 9kDa N-terminal fragment [102, 103, 105].

PrP^C can, furthermore, be recycled from the cellular membrane surface through the endocytic pathway, where once the PrP^C has reached the plasma membrane, it is internalized in an endocytic compartment [106]. Once internalized PrP^C may be recycled to the cell surface or undergo proteolytic cleavage (α-cleavage) [107]. While the exact mechanisms of then internalization remain in debate, multiple processes have been reported to be involved such as involvement of clathrin-coated pits, and caveolae/raft processes [108, 109].

PrP^C can be released or exported from the cell via differing pathways such as ectodomain shedding or exosomal release. Ectodomain shedding involves the
cleavage of membrane bound PrP<sup>C</sup>, at a site close to the GPI anchor thus releasing the nearly full-length PrP<sup>C</sup> protein from the plasma membrane into the extracellular medium (Figure 2.4D). This proteolytic cleavage has been shown to be performed by the sheddase ADAM10 [110-112]. Release of PrP<sup>C</sup> from the cell surface has not only been demonstrated in cell culture, but also in neuronal and lymphoid cells in vivo [110, 113-115]. Additionally, the cellular and infectious forms of PrP have been shown to be associated with exosomes (cell-derived vesicles) [116]. In scrapie infected cells, PrP is packaged into exosomes via a novel pathway that involves the selection of specific PrP glycoforms for incorporation in the exosomes. Infection of neighboring or distant cells then can take place via transport of infectious PrP by exosomes [117]. The cellular form of PrP (PrP<sup>C</sup>) has also been detected in exosomes isolated from ovine plasma [118], exosomes from macrophages [119] and in activated platelets [120], assisting in the transfer PrP<sup>C</sup> into cells devoid of PrP<sup>C</sup> [116, 120].

### 2.3.2 Expression of PrP<sup>C</sup> in normal human tissues

Human PrP<sup>C</sup> is expressed in early embryogenesis. In adults, the highest level of PrP<sup>C</sup> expression is observed in neurons of the brain and spinal cord [121], specifically in those of the cerebellum, cerebral cortex, thalamus, hippocampus and medulla oblongata [94, 122, 123]. It is primarily concentrated within the glia and neuronal synapses [124] and has also been found on the surface of the neuron [125]. However, PrP<sup>C</sup> is not only expressed in the nervous system. It is also expressed at low levels in many normal peripheral tissues including secondary lymphoid organs, and at even lower levels in the kidney and liver. PrP<sup>C</sup> expression has also been detected in muscle cells, nonciliated lung epithelial cells, endothelial cells, immature T cells and
dendritic cells [12]. Table 2.2 below lists the tissue or organ, and the cell types within these that are currently known to express PrP<sub>C</sub>.

Table 2.2 Human organs, tissues and cells known to express PrP<sub>C</sub> protein and PRNP mRNA.

<table>
<thead>
<tr>
<th>Tissue type/Organ</th>
<th>Cell type/Subcellular localisation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Presynaptic</td>
<td>[126]</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Presynaptic</td>
<td>[127]</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Neurons</td>
<td>[128]</td>
</tr>
<tr>
<td>Stomach</td>
<td>Secretory globules</td>
<td>[129]</td>
</tr>
<tr>
<td>Kidney</td>
<td>Secretory globules</td>
<td>[129]</td>
</tr>
<tr>
<td>Spleen</td>
<td>Secretory globules</td>
<td>[129]</td>
</tr>
<tr>
<td>Blood</td>
<td>Lymphocytes, lymphoid cell lines, monocytes, T cells, B cells, dendritic cells (DC), CD34+ cells, megakaryocytes, platelets.</td>
<td>[130-133]</td>
</tr>
<tr>
<td>Muscle</td>
<td>Subsynaptic sarcoplasm</td>
<td>[134]</td>
</tr>
<tr>
<td>Lung</td>
<td>Non-ciliated epithelial cells</td>
<td>[12]</td>
</tr>
</tbody>
</table>

2.4 PUTATIVE FUNCTIONS OF CELLULAR PRION PROTEIN

As mentioned above, the precise normal function of PrP<sub>C</sub> remains unknown [9]. Nevertheless, several lines of evidence suggest that it plays a role in the regulation of intracellular calcium and presynaptic copper concentrations, signal transduction, lymphocyte activation and has anti-apoptotic and anti-oxidant properties [12]. There is also increasing evidence that PrP<sub>C</sub> plays a role within the central nervous system (CNS), e.g. in neuronal differentiation and neuroprotection.

2.4.1 Copper regulation

PrP<sub>C</sub> is a metal ion-binding protein. It binds copper and zinc with high affinity and manganese and nickel cations with a lower affinity [135-138]. Copper binding involves the histidine residues located within the octarepeat region of the N-terminal domain [139, 140] although recent studies reveal additional copper-binding sites [141]. Since the N-terminal domain is also involved in the binding of PrP<sub>C</sub> to a
number of protein ligands, it has been hypothesised that copper binding may play a structural role and influence the binding of PrP\textsuperscript{C} to these other proteins [142].

In support of a possible physiological role for PrP\textsuperscript{C} in copper homeostasis, it has been shown that PrP\textsuperscript{C}-deficient (PRNP null) mice exhibit a 50% lower copper concentration in synaptosomal fractions in comparison to wild type mice. This suggests that PrP\textsuperscript{C} may be involved in the regulation of copper concentrations in the synaptic region of the neuron, e.g. by playing a role in the uptake of copper into presynaptic cells [142]. Furthermore, PrP\textsuperscript{C} endocytosis has been shown to be stimulated when copper is added to cultured neuroblastoma cells [143], suggesting that PrP\textsuperscript{C} internalisation may be involved in the transport of copper from extracellular to intracellular compartments. It may also indicate that PrP\textsuperscript{C} functions as a copper buffer, binding the copper and transferring it to another membrane transporter [144].

Qin and colleagues [145] reported that in murine neuro-2a and human HeLa cells, endogenous PrP\textsuperscript{C} rapidly reacts with Cu\textsuperscript{2+}. Cu\textsuperscript{2+} elevates PrP\textsuperscript{C} expression through transcriptional up-regulation mediated by the ataxia-telangiectasia mutated (ATM) transcription factor. Elevation of PrP\textsuperscript{C} expression protects the cell against copper-induced oxidative stress (and therefore prevents cell death) by playing a role in the modulation of intracellular copper concentrations.

Recently, PrP\textsuperscript{C} has further been shown to function as a modulator of heavy metal concentrations, protecting cells against heavy metal build-up and thus oxidative stress. It was shown that cells with full-length PrP\textsuperscript{C} were more resistant to chronic overload of heavy metals (copper, zinc, nickel and manganese) than their PRNP-knock out counterparts [146].
2.4.2 Signal transduction

PrP\textsuperscript{C} has been hypothesised to modulate various signalling pathway components involved in cell proliferation, cell adhesion, transmembrane signalling, differentiation, and membrane trafficking. For example, PrP\textsuperscript{C} has been shown to have a functional link to phosphatidylinositol 3 kinase (PI-3K), a protein kinase involved in cell survival, with \textit{in vitro} (cell line) and \textit{in vivo} (mouse) studies showing cells that express PrP\textsuperscript{C} have higher PI-3K activity levels than those without PrP\textsuperscript{C} [16]. PrP\textsuperscript{C} has further been shown to transduce neuroprotective signals through the cyclic AMP-dependent protein kinase/protein kinase A (PKA) pathway [147] as well as Fyn non-receptor tyrosine kinase-dependent signalling pathway(s) [148] and many other pathways.

2.4.3 Immune system

PrP\textsuperscript{C} has recently been reported to play a role in the development, activation and proliferation of T lymphocytes [149]. While PrP\textsuperscript{C} is widely expressed in the immune system including in human T and B lymphocytes, natural killer cells, platelets, monocytes, and dendritic cells, it has been found to be up-regulated during T-lymphocyte activation and even more up-regulated during natural killer cell differentiation [150]. Follicular dendritic cells show high expression of PrP\textsuperscript{C}, however, mice with follicular-dendritic-cell-specific \textit{PRNP} knock down, showed no alteration in on maturation or function of follicular dendritic cells [151], indicating the high expression or PrP\textsuperscript{C} is non-essential. In contrast, PrP\textsuperscript{C} expression has been shown to be important for macrophage function, modulating phagocytosis \textit{in vitro} and \textit{in vivo} [152]. PrP\textsuperscript{C} has been found to physically interact with a signal transduction protein with an important role in T lymphocyte activation and proliferation: zeta-chain-associated
protein (ZAP)-70 [149]. In addition, the expression of interleukin-2 is increased when PrP<sup>C</sup> is expressed [153]. These observations support PrP<sup>C</sup> being involved in activation and proliferation of T-lymphocytes. Furthermore, a recent study showed a soluble recombinant form of PrP<sup>C</sup> activates human natural killer cells via the ERK and JNK signalling pathways, as well as inducing phosphorylation of ERK1/2 and JNK, facilitating IL-15-induced proliferation of natural killer cells [154].

### 2.4.4 Protection from programmed cell death

When <i>PRNP</i> was knocked out in mice, there was no alteration of life span or observed change in the phenotype of mice [155], indicating that PrP<sup>C</sup> has a non-critical function or its function is taken over by another protein in its absence. However, further research into the function of PrP<sup>C</sup> in the CNS demonstrated that its absence in hippocampal neurons results in apoptotic (programmed) cell death [156]. PrP<sup>C</sup> also has a structural similarity to the BH2 domain of B-cell lymphoma (Bcl)-2 family members, resulting in the suggestion that PrP<sup>C</sup> may also function as a member of this family of proteins [157]. It was demonstrated that in vitro, PrP<sup>C</sup> protects human neurons against Bcl-2-associated X protein (Bax)-mediated cell death [158]. Bax is a pro-apoptotic protein that accelerates cell death by initiating the release of apoptogenic factors by mitochondria [159]. When Bcl-2 and Bax are co-expressed, hyperactivation of Bax-induced apoptosis is prevented. Similarly, co-expression of PrP<sup>C</sup> with Bax so prevented Bax-induced cell death, implying that PrP<sup>C</sup> may play a role in protection of neurons against Bax-induced cell death [157, 160].

### 2.4.5 Role of PrP<sup>C</sup> in central nervous system functions

Electrophysiological <i>in vivo</i> studies on <i>PRNP</i>-null mice have found that PrP<sup>C</sup> influences a number of processes within the CNS. These studies demonstrated a
number of functional abnormalities in the hippocampus. First, there was reduced gamma-aminobutyric acid receptor type α/GABAa receptor-mediated synaptic transmission (GABAa is a ligand-gated ion channel and voltage-dependent calcium channel that plays a role in synaptic transmission and regulation of neuronal excitability). Another defect observed in PrP\(^C\)-deficient neurons was attenuation of long-term potentiation. Long-term potentiation is the long-lasting signal transmission between neurons involved in memory. Furthermore, the PrP\(^C\)-deficient neurons exhibited slow after polarisation (i.e. prolonged phase of an action potential in which the membrane potential of a neuron falls below resting potential). Other defects in PRNP-null neurons included: disruption of calcium currents, activation of potassium currents, reduction in the amplitude of inhibitory postsynaptic potentials [161-165] and abnormal reorganisation of the mossy fibre circuitry in the hippocampus [162].

Impairment in hippocampus-dependent spatial learning and altered excitatory and inhibitory neurotransmission in PRNP-null mice further support the proposed role of PrP\(^C\) in synaptic function [166]. Studies have also found changes in mobility, anxiety and equilibrium in adult mice that were attributable to reduced levels of PrP\(^C\) [167].

In addition, PrP\(^C\) has been shown to bind to the neural cell adhesion molecule (NCAM) which is a signalling receptor [168] in the nervous system that takes part in a number of developmental processes including cell migration, synaptic plasticity and neurite outgrowth [169-171]. In summary, these various studies suggest that PrP\(^C\) may play a role in CNS development [168, 172] through effects on directed cell migration of neural progenitor cells and the spatial coordination of the outgrowth of neurites as well as playing a role in neuronal survival [173].
2.5. CANCER DEVELOPMENT AND AUSTRALIAN CANCER STATISTICS

Cancer is characterised by the abnormal growth and development of cells, and has become the most second common cause of death in Australia. With more than 46,000 who were expected to die in 2015 alone [19] the importance of research into the causes of, possible therapies for and early diagnosis of cancer, is becoming increasingly significant.

2.5.1 Incidence rates

In 2015, 126,800 new cases of cancer are projected with and over 46,570 projected deaths from cancer, making cancer the second most common cause of death. The 5 projected most common types of diagnosed cancer during 2015 (Figure 2.5) were prostate cancer (17,250 cases), bowel cancer (17,070 cases), breast cancer (15,740 cases), melanoma of the skin (over 12,960 cases) and lung cancer (11,880 cases) [19].

![Projected five most commonly diagnosed cancers in Australia in 2015](image)

Figure 2.5 The projected five most commonly diagnosed cancers in Australia in 2015 [19].

In 2011, there were 118,711 new cases of cancer diagnosed in Australia (67,117 males, 51,594 females) with the age standardised incidence rate at 484 cases per
100,000 persons (580 for males, 404 for females). Based on incidence rates from 2002 to 2011, it is expected that by 2015, the number of new cases will increase to over 126,800 (69,790 males and 57,010 females) (Figure 2.6) while the age standardised incidence rate is estimated to decrease to 466 cases per 100,000 persons (537 for males and 407 for females) [174].

Figure 2.6: Summary of new cases of all cancer types in Australia. The number of new cases of all cancer types (excluding non-melanoma skin cancers) between the years 1982 and 2011 with projection to 2014 including age standardized, combined incidence rate per 100,000 persons. Rates are standardized to the Australian population as of 30 June 2001. □ Actual number; □ estimated number; ----- actual rate; ---- estimated rate. Figure adapted from [174].

2.5.2. Cancer definition

Cancer is a disease consisting of the abnormal and uncontrolled proliferation of cells beyond their natural boundaries. Current theories of cancer development suggest that the uncontrolled proliferation of cells begins when the DNA of a cell undergoes mutation as a result of exposure to a carcinogen. A carcinogen may include a number
of external or internal factors such as hormones, immune response or viral infection, chemicals or radiation. When the cell divides, each new cell contains the altered DNA. Accumulation of several DNA mutations may then complete the transformation of a normal cell into a cancer cell [175]. Cancer formation (carcinogenesis) is theorised to consist of 3 distinct phases including initiation, promotion and progression [176]. During the initiation phase, cells undergo constant and heritable genetic alteration and may become malignant if promotion and progression phases follow. The initiation phase may result from an amplification, mutation or translocation of the genes of a target cell and is irreversible [177]. The next stage, promotion, involves the selective proliferation of initiated cells resulting in a benign lesion. This stage can be reversible and arrested using anti-cancer agents [178, 179]. The final phase, progression, involves the development of a malignant, fast-growing neoplasm from the benign lesion [177, 180].

Complications of primary tumour development may arise including development of metastatic tumour sites and/or chemotherapeutic drug resistance. Metastasis is the detachment of tumour cells from a primary tumour site [181] and their migration to distant organs after entering circulation (e.g. blood vessels). Tumour cells then invade and proliferate at the new site, forming new tumour colonies [182].

Resistance to chemotherapeutic drugs can be either intrinsic or acquired. Cells within tumours can undergo genetic alterations as the tumour progresses, with selection for those with a growth advantage and when under therapeutic treatment, those cells most adaptive or resistant to treatment will be selected for [183].
2.6. INVOLVEMENT OF PRP\textsuperscript{C} IN CANCER

2.6.1. Role of PrP\textsuperscript{C} in multi-drug resistant gastric cancer

Multi-drug resistance in cancer has a significant influence on the effectiveness of chemotherapeutic treatment and therefore patient survival rates [184]. This makes the discovery of potential causes of such multi-drug-resistance a high priority. Primary studies of differential gene expression profiles revealed that the PrP\textsuperscript{C} gene is up-regulated in adriamycin-resistant gastric carcinoma cell line (SGC7901/ADR) when compared to its parental cell line SGC7901 [185]. This indicated that PrP\textsuperscript{C} may have a role in the development of multi-drug resistant (MDR) phenotypes in gastric carcinomas and led to a focus on PrP\textsuperscript{C} expression levels in gastric cancer and the mechanisms of PrP\textsuperscript{C} action within MDR gastric carcinoma [12]. Using northern and western blot techniques, PrP\textsuperscript{C} was found to be highly expressed and promoted the MDR phenotype via up-regulation of P-gp (P-glycoprotein) expression and suppression of apoptosis [12]. P-gp is a plasma membrane glycoprotein that functions as an energy dependent multi-drug efflux pump that exports drugs out of cells [186]. The up-regulation of P-gp was inferred from the results of \textit{in vitro} drug sensitivity assays performed on the SGC7901/ADR cells. A greater increase in resistance to P-gp substrates such as ADR, vincristine (VRC) and etoposide (VP-16) was observed compared to only a slight increase in resistance to non-P-gp substrates like 5-fluorouracil and cisplatin (CDDP) [12]. More importantly, this study also demonstrated that the suppression of PrP\textsuperscript{C} expression can partially reverse ADR resistance in SGC7901/ADR cells and that PrP\textsuperscript{C} can, in an indirect manner, promote expulsion of drugs from cells [12].

The AKT1 pathway (an intracellular signalling pathway involved in cell survival) has also been demonstrated to play a role in the MDR phenotype of gastric cancer via regulating P-gp and cooperating with Bcl-2/Bax. Down-regulation of the AKT1
pathway using AKT1-specific siRNA (small interfering RNA) reversed the MDR phenotype of gastric cancer [18]. Further investigation into the role of the PI3K/Akt pathway in PrP-induced MDR in gastric cancer demonstrated that the inhibition of the PI3K/Akt signalling pathway using LY294002 (an AKT-specific inhibitor), or AKT-specific siRNA, inhibited the PrP-induced drug resistance as well as the up-regulation of P-gp expression in gastric cancer cells [17]. Using *in vitro* experiments, it was inferred that PrP<sup>C</sup> over-expression led to drug resistance by decreasing apoptosis in gastric cancer cells through synergistic effects with Bcl-2. Furthermore, those patients with gastric cancers that were shown to over-express PrP<sup>C</sup> displayed a poor response to chemotherapy and decreased 2-year survival rates. On the other hand, those patients with gastric cancers that were shown to be negative for PrP<sup>C</sup> expression showed high sensitivity to chemotherapy and therefore an increased 2-year survival rate [187]. These findings thus confirm the earlier results that first identified the involvement of PrP<sup>C</sup> in the development of MDR in gastric cancer.

The octarepeat region (aa51-91) of PrP (Figure 2.4A), is known to be functionally important for Cu<sup>2+</sup>/Zn<sup>2+</sup> transport and antioxidant mechanisms, as discussed earlier. This domain consists of a histidine rich nonapeptide (R1) and four octapeptide repeats (R2-R4) [188]. Owing to the importance of antioxidative mechanisms in MDR development, a recent study investigated the role of the octarepeat peptides in gastric cancer MDR. This study found that PrP<sup>C</sup> octarepeat peptides play a role in drug tolerance and stress response in gastric cancer cells. They appear to mediate these through a regulatory effect on the superoxide dismutase (SOD) and glutathione/glutathione S-transferase (GSH/GST) protein families (reactive oxygen species scavengers) [184]. These results indicate that the octapeptide repeat of PrP<sup>C</sup> may be an important contributing factor in the MDR phenotype of gastric cancer and therefore a potential target for therapeutic treatment.
It has been shown that small drug-like molecules can antagonise some prion strains of \( \text{PrP}^{\text{Sc}} \) while simultaneously selecting for drug resistant prions [189]. Upon treatment of mice with a high dose of quinacrine, it was observed that though there was an initial reduction of proteinase K resistant PrP, quinacrine-resistant prion forms subsequently emerged, rendering quinacrine ineffective as a treatment [190]. Together with the above findings of PrP\(^{\text{C}}\) involvement in MDR gastric cancer, it may be concluded that PrP\(^{\text{C}}\) may be useful as a marker for following the development and prognosis of MDR in gastric cancers, including the possible use of PrP\(^{\text{C}}\) to monitor patient response to chemotherapy.

### 2.6.2 Expression level of PrP\(^{\text{C}}\) in gastric cancer

Over-expression of PrP\(^{\text{C}}\) has been reported in a number of human gastric cancer cell lines including SCG7901 and AGS [11, 12, 191]. Du et al. [12], using northern blot and western blot analysis, confirmed the expression of PrP\(^{\text{C}}\) in gastric carcinoma cell lines SGC7901 and SCG7901/ADR. Through the use of immunofluorescence staining, it was found that PrP\(^{\text{C}}\) is clustered within the cytoplasm or plasma membrane of SGC7901 and AGS cells [13, 191]. It was also confirmed that the over-expressed PrP\(^{\text{C}}\) in these cell lines was the proteinase K-sensitive form (PrP\(^{\text{C}}\)) [11]. Further western blot analysis revealed that PrP\(^{\text{C}}\) is widely expressed in several other gastric cancer cell lines including MGC803 and KATOIII [191]. PrP was also found to be expressed in gastric carcinoma tissues using immunohistochemical staining [12, 191]. Du et al. [12] discovered that PrP\(^{\text{C}}\) is expressed more strongly in gastric adenocarcinoma tissues than in adjacent non-tumorous tissues and is weakly, or not expressed, in normal gastric mucosa. Liang et al. [191] then examined clinical parameters including age, sex, tumor, node, metastasis (TNM) stage and histological differentiation. While there was no
correlation between PrP$^C$ expression level and patient sex or age, there was a significant correlation with histological type and TNM stage [191], a result similar to that of Comincini et al. [192] in relation to the prion-like Dopple gene (PRND) in human gliomas. The mechanism(s) that underlie the biological actions of PrP$^C$ was further investigated by Liang et al [11]. When poorly differentiated cancer cell line AGS was transfected with PRNP cDNA, the release of reactive oxygen species (ROS) was significantly reduced and apoptosis was decreased in the transfectants. Anti-apoptosis protein Bcl-2 was also expressed at a higher level, while the expression of apoptosis promoter proteins p53, Bax and cytochrome c was lower. The opposite effect was observed when cells were transfected with a PRNP siRNA (small interfering RNA), further suggesting that PrP$^C$ may play an anti-apoptotic role, like the Bcl-2 protein by interfering with apoptotic pathways in gastric cancer cells [191].

PrP$^C$ was also further shown to stimulate cell proliferation by promoting the transition from G1 to S phase in the cell cycle and by elevating the transcription of cyclin D1. Levels of cyclin D1 were found to be up regulated in cells transfected with PRNP cDNA at both the mRNA and protein levels as assessed by microarray and western blot analysis. Furthermore, the authors demonstrated that PrP$^C$ increases the level of phosphorylated Akt. This suggests that PI3K/Akt signalling may mediate the transactivation of cyclin D1 gene transcription induced by PrP$^C$. When Akt was blocked by LY294002, an Akt specific inhibitor, the pro-proliferation effect of PrP$^C$ was inhibited. This further indicates that the PI3K/Akt pathways play a role in transducing the proliferation-promoting signal of PrP$^C$ in gastric cancer. Additionally, the N-terminal octarepeat region of PrP$^C$ was found to be critical yet again, this time for promoting the proliferation of PRNP-transfected gastric cancer cells [11].
2.6.3 PrP<sup>C</sup> involvement in pancreatic cancer

<sup>PRNP</sup> was found to be over-expressed in pancreatic ductal adenocarcinoma (PDAC) in a microarray study by Han <i>et al.</i> [193]. Another study investigated seven human PDAC cell lines to determine whether PrP<sup>C</sup> is over-expressed. While PrP<sup>C</sup> is over-expressed in the PDAC cell lines, it exists in the form of a pro-protein (Pro-PrP) and is neither glycosylated nor GPI-anchored. It was further determined that the Pro-PrP binds filamin A (FLNa), which is a cytoplasmic linker protein that links cell surface receptors and integrates cell mechanics and signal transduction pathways to effect changes in cell morphology. Interaction of Pro-PrP with FLNa was shown to interfere with the normal function of FLNa, increasing PDAC cell aggressiveness and therefore resulting in a growth advantage for the PDAC. This was confirmed by the reduction in proliferation and invasiveness of PDAC upon down-regulation of PrP<sup>C</sup> [14]. More importantly, the expression of PrP was shown to be closely associated with a poorer clinical outcome independent of other factors, such as age and gender of the patients, as well as the size or differentiation stage of the tumor. This is because, as in the PDAC cell lines <i>in vitro</i>, PrP<sup>C</sup> expression confers a growth advantage and aggressiveness to PDAC tumors <i>in vivo</i> [194]. Thus, Pro-PrP could serve as a marker for early detection of PDAC. Moreover, the physical interaction between Pro-PrP and FLNa could serve as a target for therapeutic intervention in PDAC.

2.6.4 PrP<sup>C</sup> involvement in breast cancer

Studies comparing the tumour necrosis factor-α (TNF)-sensitive breast cancer cell line MCF7 and its TNF-α-resistant clone showed that PrP<sup>C</sup> is relatively over-expressed in the TNF-α-resistant clone. It was demonstrated that PrP<sup>C</sup> over-expression in MCF-7 cells protects these cells from induced cell death, thus
converting TNF-α-sensitive cells into TNF-α-resistant cells. This occurs, in part, via alterations in the apoptosis signalling pathways that induces cytochrome c release from mitochondria and nuclear chromatin condensation [15]. Further research showed that resistance to adriamycin and tumor-necrosis-factor-related-apoptosis-inducing ligand (TRAIL)-induced apoptosis is associated with over-expression of PrP<sup>C</sup>. Silencing of PrP<sup>C</sup> expression by transfection of adriamycin-resistant and TRAIL-resistant breast carcinoma cell lines with PRNP-specific siRNA, was shown to sensitize the cells to TRAIL-induced apoptosis. Furthermore, this increased sensitivity was shown not to be associated with increased recruitment of receptors and other signalling molecules to the DISC (death-inducing signalling complex) [195]. DISC is essential for the initiation of death-receptor-mediated apoptosis [196].

Death receptors are members of the TNF family and are activated by complementary cytokines known as cognate ligands [197, 198]. These findings indicate that interference with DISC formation by TRAIL is not essential for the acquisition of resistance to TRAIL-induced apoptosis [195]. Li et al [199] demonstrated that not only is PRNP over-expressed in MCF7/Adr cells in comparison to MCF7 cells, but at the protein level PrP<sup>C</sup> interacts with P-glycoprotein (P-gp) playing a role in the resistance to Paclitaxel (P-gp substrate) and the anti-apoptotic properties of MCF7/Adr cells.

**Figure 2.7** PRNP mRNA expression in MCF-7 sensitive and MCF-7/Adr (adriamycin-resistant) cell lines. MCF-7/Adr demonstrates 5.6-fold increase in PRNP mRNA expression compared to MCF-7, normalised to glyceraldehyde phosphate dehydrogenase (GAPDH). Figure adapted from [164].
In another study, Roucou and colleagues [200] have shown that PrPC expression prevents Bax-mediated cell death in MCF-7 cells by inhibiting the pro-apoptotic Bax conformational change. This mechanism appears to be functionally analogous to the interactions of PrPC with the Akt pathway that affect the functions of Bcl-2 and Bax in gastric cancer. In both MCF-7/ADR breast cancer cells and SCG7901/ADR gastric cancer cells, PrPC acts by up-regulating the anti-apoptotic signaling properties of cells (i.e. inducing Bcl-2 up-regulation to increase anti-apoptotic functions and Bax down-regulation to inhibit pro-apoptotic functions).

### 2.6.5 PrPC involvement in colorectal cancer

RT-PCR analysis conducted on surgically removed colorectal cancer specimens revealed that PRNP mRNA expression is up-regulated in colorectal carcinoma when compared to normal colorectal tissue. This suggests a role for PrPC in the development of colorectal cancer. The expression level of PRNP was found to be unrelated to age, gender, or either the grade or stage of the carcinomas. However, it was found that PRNP mRNA expression levels are associated with the site of the primary tumor with higher PRNP mRNA expression in the rectum and left colon in comparison to the right colon. Furthermore, it was noted that PRNP mRNA expression levels may be an independent prognostic factor [201].

Examination of PrPC expression in colorectal cancer was conducted using formalin-fixed paraffin-embedded colonic neoplastic tissue samples from 110 patients. This study also found that PrPC protein expression increased in cancerous colorectal tissues in comparison to normal colorectal tissues [202] and, moreover, that the differential expression in these tissues was even greater than that observed for PRNP mRNA levels in the previous study [201]. This over-expression of PrPC correlates with what was found in gastric carcinomas [191] as discussed previously.
Furthermore, PrP\textsuperscript{C} expression was found to correlate with recurrence of disease, such that patients with high PrP\textsuperscript{C} expression levels relapsed earlier than those with low PrP\textsuperscript{C} expression levels [202]. Overall, the investigation of PrP\textsuperscript{C} expression in colorectal cancer has provided further support for the idea that PrP\textsuperscript{C} has potential as a new target for therapeutic and prognostic strategies. In fact, an in vitro study demonstrated that anti-PrP\textsuperscript{C} antibodies could be used as an effective anticancer therapy. Different anti-PrP\textsuperscript{C} antibodies were shown to exhibit varying degrees of anti-proliferative activity and to have an increased efficiency when used in combination chemotherapy [203]. Another recent study has identified that the glycosylation state of PrP\textsuperscript{C} is critical for its anti-apoptotic functions in colon cancer cells and therefore the glycosyl transferases that modify PrP\textsuperscript{C} could be another potential therapeutic target [204]. However, further studies are required to evaluate the state of PrP\textsuperscript{C} (i.e. whether PrP\textsuperscript{c} or prion-form) and the role of PrP\textsuperscript{C} in cancer development.

### 2.6.6 PrP\textsuperscript{C} involvement in prostate cancer

PrP\textsuperscript{C} expression has been detected in the androgen-independent prostate cancer cell line Du-145. A role for PrP\textsuperscript{C} in the oxidative defense system is also evident from the correlation between increased PrP\textsuperscript{C} expression and an elevated intracellular redox level in the prostate tumor spheroids [205]. This suggests that PrP\textsuperscript{C} potentially acts as a sensor molecule and/or free radical scavenger for oxidative stress in tumor cells. In vitro studies performed on these tumour spheroids revealed that the level of ROS correlated with an increase in expression of PrP\textsuperscript{C}, Cu/Zn SOD-1 and catalase in small spheroids relative to the larger spheroids. This suggests that not only does PrP\textsuperscript{C} expression correlate with redox state, but also with tumour size [205]. However, further research is required to confirm this role of PrP in prostate cancer.
2.6.7 PrP<sup>C</sup> involvement in colon and gastric cancer metastasis

PrP<sup>C</sup> has been shown to promote metastasis of colorectal cancer by mediating epithelial-mesenchymal transition. It was shown that PrP<sup>C</sup> expression is associated with the invasive front of colorectal cancer where cells gain characteristics of EMT and facilitate tumour invasion. Furthermore, functional assays showed that ectopic PrP<sup>C</sup> expression promotes in vitro metastatic potential while inhibition of PrP<sup>C</sup> expression reduces the motility of cells. Additionally, knock down of PrP<sup>C</sup> in implanted colorectal cancer cells in orthotopic xenograft mouse model reduced the number of distant metastases. It was shown that PrP<sup>C</sup> accelerates tumour metastasis by up-regulation of special AT-rich sequence binding protein 1 (SATB1) expression via the Fyn-SP1 pathway [206]. SATB1, a matrix attachment region-binding protein that mediates the attachment of chromatin to the nuclear matrix [207], has been shown to alter the gene expression profile of cancer cells inducing an aggressive phenotype and metastasis. Depletion of SATB1 reduces cancer progression and results in a normal phenotype of metastatic cells [208]. The molecular events downstream of PrP<sup>C</sup> were abolished when the Fyn pathway was inhibited, indicating its requirement for PrP<sup>C</sup>-mediated SATB1 expression. Furthermore, SP1 inhibition also reduced SATB1 expression and the metastatic potential of colorectal cancer cells. Overall, this study showed the first link between PrP<sup>C</sup> expression and the metastasis of colorectal cancer through the PrP<sup>C</sup>-Fyn-SP1-SATB1 axis, therefore providing a potential target for inhibition [206].

Once the PrP<sup>C</sup> over-expression in gastric cancer tissues relative to adjacent non-tumorous tissues and normal gastric mucosa was confirmed [12], it was assessed whether PrP<sup>C</sup> expression influenced the invasive and metastatic properties of gastric cancer. While PrP<sup>C</sup> was found to be highly expressed in metastatic cancer compared to non-metastatic cancer, there was no significant correlation in gene expression
profile between the primary site and the metastatic site of the same metastatic gastric cancer. This suggests that an increase in PrP$^C$ expression is an early determinant of metastasis and may be useful as a prognostic factor. PrP$^C$ was also shown to promote adhesive, invasive and in vivo metastatic properties of gastric cancer cells. The N-terminal region of PrP exhibited an invasion-promoting effect through the activation of the mitogen-activated protein kinase (MAPK)/ extracellular-signal-regulated kinase (ERK) pathway (MEK/ERK pathway) [11]. The MEK/ERK pathway controls cellular processes such as proliferation, apoptosis, survival and differentiation [209] and consequently transactivates MMP11 (matrix metalloproteinase 11) expression [11], which in turn promotes matrix degradation, inflammation and tissue remodeling [210].

2.7 PRION-LIKE PROTEINS IN DISEASE

The tumour suppressor protein p53 is a transcription factor [211] involved in the regulation of the cell cycle and apoptosis [212]. Approximately 50% of tumours harbour a mutation in the gene that encodes p53 [212] with the remaining 50% possessing a faulty component in either the machinery responsible for post-translational modification of p53 or in a component of the signalling pathway downstream of p53 [213]. Recently, it has been shown through studies of mutant p53 that cancer may be considered as a protein aggregation-associated disease. It was shown that oncogenic p53 has an increased aggregation propensity that is acquired by the exposure on the surface of the folded p53 protein of an aggregation-nucleation sequence. Exposure of this aggregation-nucleation sequence on the protein surface is induced by structural destabilisation of the p53 DNA-binding domain and results in the co-aggregation of mutant p53 with wild-type p53 to form cellular inclusion thus eliminating wild-type p53 activity. Mutant p53 was also demonstrated to co-aggregate with p63 and p73, which are other members of
the p53 protein family. Furthermore, aggregated p53 was shown to increase the expression of a number of heat-shock proteins, including Hsp70 which is an anti-apoptotic agent [212]. Further research on this topic set out to determine if wild-type p53 and the hot-spot mutant (R248Q) p53 protein have the ability to aggregate and form amyloid fibres under physiological conditions and, furthermore, if the mutant p53 protein can seed aggregates of the wild type p53 protein. They found that the R248Q mutant p53 protein had a greater tendency to aggregate and form amyloid fibres than the wild-type p53 protein. Furthermore, they found that full length p53 aggregated to form amyloid-like species in a similar pattern to that of the p53 core domain [214] which had been previously shown to form β-sheet rich aggregates under increased hydrostatic pressure [215]. The R243Q mutant p53 was further found to seed wild-type p53 aggregation in a prion-like manner.

2.8 SUMMARY

Cancer is one of the leading causes of death worldwide and with such a low percentage being attributed to familial causes (inheritance of predisposing genes), determining sporadic causes such as epigenetic causes is becoming increasingly more important. PrP\(^C\) has already been shown to have a potential role in the development of a number of cancers including breast, prostate, gastric, pancreatic and colorectal cancer, as well as in metastasis of gastric and colorectal cancer and in the MDR phenotype of breast and gastric cancers. However, the molecular mechanisms of PrP\(^C\) involvement have yet to be precisely identified. Furthermore, in many cases, the investigation into folding of PrP in these cancers has yet to be performed. With PrP\(^C\) being a potential target for therapeutic intervention as well as an indicator of progression and prognosis, further investigation into the role of PrP\(^C\) in cancer development and progression has become essential.
The objective of this thesis is to further the knowledge of PrP$^C$ involvement in a number of cancer types, in particular, colorectal and breast cancer and the influence of PrP$^C$ on drug resistance and metastasis. We first look at expression of PrP$^C$ in a range of cancer types, before focussing on the role of PrP$^C$ in the resistance of colorectal cancer to cisplatin. We then investigate the expression of PrP$^C$ in breast cancer, looking at correlation of the PrP$^C$ expression level with prognosis, in particular, expression and influence on metastasis. We also assess the state of PrP. Finally we determine the effect of PrP$^C$ expression on the effectiveness of doxorubicin treatment of metastatic and non-metastatic breast cancer.
Chapter 3: Materials and Methods
3.1 GENERAL METHODS

3.1.1 Cell culture

3.1.1.1 Culture media and conditions

Media for various cell lines was prepared to specifications as shown in Table 3.1. Unless otherwise specified, cells were cultured at 37°C and 5% CO₂ in high glucose DMEM (Invitrogen) containing foetal bovine serum (Invitrogen) to final concentration of 10% (v/v) and penicillin and streptomycin solution (Life Technologies) at a final concentration of 1% (v/v) to prevent bacterial contamination (a major limitation of cell culture). Each cell line was subjected to mycoplasma testing to ensure there was no contamination (Lonza MycoAlert™ Mycoplasma detection kit).

3.1.1.2 Reviving and subculturing

Preparation of frozen stocks

Cells are stored at -80°C or liquid nitrogen in prepared freezing media (10% DMSO, 30% FBS and 60% complete growth media as described above). Cells were dispersed with 0.25% EDTA trypsin (Invitrogen) when 80% confluent. Cells were centrifuged at 500rpm for 5 minutes, after which trypsin was decanted. Cell pellet was resuspended in required volume of freezing media, and aliquoted into 1ml cryotubes (Sigma Aldrich). Aliquots were placed into -20°C freezer for 2 hours, then transferred to -80°C overnight before being transferred to liquid nitrogen.

Reviving cell lines from liquid nitrogen

Each cultured cell line (see Table 3.1) was lifted from liquid nitrogen cryostorage, defrosted for no more than 5 minutes at 37°C, and then suspended in 2ml of freshly prepared media. Cell suspensions were centrifuged at 500rpm for 5 minutes and supernatant removed in order to remove freezing media. Cell pellets were
resuspended in 2 ml of culture medium and transferred to a prepared t25 flask containing 2 ml of culture medium and cultured over night.

**Subculture**

Subcultivation was performed at ratios recommended by the ATCC recommendations (Table 3.1) every 3-4 day when cells in culture reached at least 80% confluence. Subculturing was performed using 0.25% EDTA trypsin (Invitrogen) to disperse cells (approximately 5-10 minutes). Cells were centrifuged at 500rpm for 5 minutes and the resulting cell pellets washed in phosphate buffered saline (PBS). When required, cells were counted using trypan blue staining method and counted using a Neubauer haemocytometer chamber (Figure 3.1).

![Figure 3.1 Neubauer improved haemocytometer chamber. Cells in boxes 1 to 4 are included in the cell count](image)

The number of cells in the cell suspension can then be calculated using the following equation:

$$\text{#cells/ml} = \frac{\sum \text{cells in boxes 1-4 (as indicated in figure 3.1)}}{4 \times 2 \times 10 \times 10^4}$$

The result will be divided by 1000µL to yield the number of cells per µL.

**3.1.1.3 Cell lines**

Various human cancer and control cell lines are used throughout this study. Cell lines were chosen not only according to the prevalence of the corresponding cancer type in
Australia (Chapter 2, section 2.5.1), but also to represent a diverse range of cancer types. Normal cell lines were also chosen as controls. While it was not possible to obtain normal cell lines that corresponded exactly with the cancer cell lines, an overall non-cancerous epithelial cell line was used.

Contamination of cell lines is a major issue in biomedical research with a number of articles being retracted due to contaminations leading researchers to incorrect conclusions. For example, a study showed that cell lines derived from human breast cancer tissue (HBC and BrCa5) were contaminated with rat and HeLa cells, respectively [216]. Contamination with fast growing cell lines such as HeLa, can create misleading results as these cell lines may react differently to treatments and have differing expression levels of proteins or be a completely different tissue/cell type [217]. It is therefore best to authenticate tissues as well as performing testing for bacterial contamination. While mycoplasma testing was conducted (see below section 3.1.1.4), authenticity of all cell lines was not conducted although cell lines were originally purchased from ATCC.

3.1.1.4 Mycoplasma testing of cell lines

Each cell line was subjected to mycoplasma testing using the MycoAlert™ Mycoplasma Detection Kit following the manufacturer’s protocol. Briefly, 2 ml of media from each cell sample was centrifuged at 200 x g for 5 min, then 100 µl of cleared supernatant was removed to a fresh tube. 100 µl of reconstituted MycoAlert™ reagent was added to sample and incubated for 5 mins. Luminescence was measured. 100 µl of MycoAlert™ substrate was then added to sample and incubated for 10 mins before measuring luminescence. Ratio of initial reading to secondary reading was used to determine whether a cell culture was contaminated by mycoplasma.
Table 3.1 Summary of cell lines. Description of all cell lines used throughout study.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Description</th>
<th>Media</th>
<th>Media Renewal</th>
<th>Sub-culture Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>Colorectal adenocarcinoma, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:3</td>
</tr>
<tr>
<td>SW620</td>
<td>Duke’s type C colorectal adenocarcinoma Derived from metastatic site in the bone, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:2-1:10</td>
</tr>
<tr>
<td>T84</td>
<td>Colorectal carcinoma derived from metastatic site in the lung, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:2-1:4</td>
</tr>
<tr>
<td>PC3</td>
<td>Prostate adenocarcinoma derived from metastatic site in the bone, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:3-1:6</td>
</tr>
<tr>
<td>DU145</td>
<td>Prostate carcinoma derived from metastatic site in the brain, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:4-1:6</td>
</tr>
<tr>
<td>ALVA</td>
<td>Prostate carcinoma derived from metastatic site in the bone, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:3-1:8</td>
</tr>
<tr>
<td>A549</td>
<td>Lung Carcinoma, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:3-1:8</td>
</tr>
<tr>
<td>A431</td>
<td>Epidermoid carcinoma, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:3</td>
</tr>
<tr>
<td>FHC</td>
<td>Normal fetal colon, epithelial</td>
<td>DMEM with: 10ng/ml cholera toxin, 5µg/ml insulin, 5µg/ml transferring, 25mM HEPES, 100ng/ml hydrocortisone, FBS to 10%</td>
<td>Every 3-4 days</td>
<td>1:2</td>
</tr>
<tr>
<td>293T</td>
<td>Embryonic kidney, epithelial</td>
<td>DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:4-1:10</td>
</tr>
<tr>
<td>MCF10a</td>
<td>Mammary gland/breast of patient with fibrocystic disease, epithelial</td>
<td>DMEM/F12 with 5% horse serum, 100ng/ml cholera toxin, 20ng/ml EGF, 0.5mg/ml hydrocortisone, 10µg/ml insulin</td>
<td>Every 3-4 days</td>
<td>1:2</td>
</tr>
<tr>
<td>4T1 Series: 67NR 168FARn 66C14 4T07 4T1.2</td>
<td>Epithelial, mouse mammary gland carcinoma Non-metastatic</td>
<td>All DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:4-1:8</td>
</tr>
<tr>
<td>MDA-MB-231 Series: PAR NI PAR LM IA LM HM LNM5</td>
<td>Epithelial, mammary gland carcinoma Parental (Non-metastatic) Non-Invasive parental Lung metastasis Invasive lung metastasis Lymph node metastasis</td>
<td>All DMEM with 10% FBS</td>
<td>Every 3 days</td>
<td>1:4-1:8</td>
</tr>
</tbody>
</table>
3.1.2 Sample preparation for real-time quantitative PCR

3.1.2.1 RNA extraction method

Unless otherwise specified, RNA was isolated from each cultured cell line using TRIzol® Reagent (Invitrogen). This method of RNA extraction was used due to the time efficiency of the protocol and amount of RNA yield acquired in comparison to the Invitrogen RNA mini-prep. The following description is the protocol that was used throughout this study (adapted from TRIzol® reagent protocol obtained from Invitrogen) [189].

Stage 1: Homogenisation

Cell numbers were controlled (as described in Section 3.1.4 above) to 600,000 cells/10 cm² culture plate and incubated until cell layers were 95% confluent. Culture medium was removed from the cells in culture and then the cells were washed with 3 ml PBS.

At room temperature, 1 ml of TRIzol® reagent was directly applied to the cells in culture. The cells were then dislodged from the culture plate using a sterile cell scraper and then transferred to 1.5 ml eppendorf tubes. TRIzol/cell mix was then subjected to gentle pipetting up and down to ensure homogeneity.

Stage 2: Phase separation

Samples were then incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Chloroform was then added (ratio of 1:5 Chloroform:Trizol), the samples were shaken vigorously for 15 seconds and then each sample was incubated for 2 minutes at room temperature. Each sample was then subjected to centrifugation at 12,000 x g for 15 minutes.

Stage 3: RNA precipitation

The aqueous phase was then transferred to a new 1.5 ml eppendorf tube. 0.5 ml of isopropanol was then added to the aqueous phase. Then each sample was mixed and
incubated at room temperature for 10 minutes. Each sample was then subjected to centrifugation for 10 minutes at 12,000 x g. The formation of gel-like pellets was observed after centrifugation.

Stage 4: RNA wash
Each supernatant was removed and the RNA pellets washed in RNAse/DNAse-free 70% ethanol. Each sample was again subjected to centrifugation for 5 minutes at 7,500 x g.

Stage 5: Redissolving RNA
RNA pellets were allowed to slightly dry before being dissolved in 50 µl of RNAse/DNAse-free Ultrapure™ water (Invitrogen).

3.1.2.2 Synthesis of cDNA
Unless otherwise specified, cDNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad) following the manufacturer’s protocol. Briefly, 1µg total RNA was combined with 4µl iScript reaction mix, 1µl reverse transcriptase and nuclease-free water to total volume of 20µl. Reaction mix was incubated at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Samples were stored at -20°C until use.

3.1.3 Quantitative real-time polymerase chain reaction
Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to determine the level of transcription of genes of interest. There are 3 phases of PCR: exponential, linear and plateau (Figure 3.2A).
RT-qPCR provides accurate data for the quantification of transcripts by collecting data from the exponential growth phase when the quantity of the PCR product is directly proportional to the amount of template nucleic acid. In comparison, traditional RT-PCR is not quantitative and only measures the amount of PCR product in the plateau phase of the PCR cycle [218]. Figure 3.2B shows the threshold line, i.e. the level of detection. This is where the reaction reaches a fluorescence intensity that is above background [218, 219]. It also shows the cycle threshold, i.e. the PCR cycle at which the sample reaches the threshold level. By comparing the cycle threshold line of a sample with an unknown concentration of template nucleic acid against a standard curve, the level of template nucleic acid in the unknown sample can be determined [218].

### 3.1.3.1 Primers

Standard primer sequences were obtained from Origene™. Synthesis of primers was performed by Sigma-Aldrich® (Australia).
Table 3.2 List of human forward and reverse primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>GC%</th>
<th>Tm(°C)</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRNP Forward</td>
<td>22</td>
<td>55</td>
<td>67.4</td>
<td>TTCGGCAGTGACTATGAGGACC</td>
</tr>
<tr>
<td>PRNP Reverse</td>
<td>22</td>
<td>55</td>
<td>72.1</td>
<td>TTGTGGTGACCGTGTGCTGCTT</td>
</tr>
<tr>
<td>GAPDH Forward</td>
<td>22</td>
<td>54.6</td>
<td>65.3</td>
<td>GTCTCCTCTGACTTCAACAGCG</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>22</td>
<td>54.6</td>
<td>69.7</td>
<td>ACCACCCCTTCTGACTGAGCCAA</td>
</tr>
<tr>
<td>β- actin Forward</td>
<td>21</td>
<td>52.4</td>
<td>66.4</td>
<td>GCATGGGTAGAGGATTCTCT</td>
</tr>
<tr>
<td>β- actin Reverse</td>
<td>20</td>
<td>55</td>
<td>68</td>
<td>TCGTCCCAGTTGACGTAG</td>
</tr>
<tr>
<td>RPLP0 Forward</td>
<td>22</td>
<td>54.6</td>
<td>72.2</td>
<td>TGGTCATCCACAGGTTGTTGCA</td>
</tr>
<tr>
<td>RPLP0 Reverse</td>
<td>22</td>
<td>54.6</td>
<td>71.4</td>
<td>ACAGACACTGGCACAATTGCGG</td>
</tr>
<tr>
<td>CRYAB Forward</td>
<td>22</td>
<td>54.6</td>
<td>63.6</td>
<td>ACCTCCCTGACTCCCTTCTACC</td>
</tr>
<tr>
<td>CRYAB Reverse</td>
<td>22</td>
<td>54.6</td>
<td>67.1</td>
<td>GGAGAAAGTGACTCCACATCCAGG</td>
</tr>
</tbody>
</table>

Table 3.3 List of mouse forward and reverse primer sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Length</th>
<th>GC%</th>
<th>Tm(°C)</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prnp Forward</td>
<td>23</td>
<td>52.2</td>
<td>69.9</td>
<td>CAGCAACCAGAACAACTTCGTGC</td>
</tr>
<tr>
<td>Prnp Reverse</td>
<td>22</td>
<td>54.6</td>
<td>70.9</td>
<td>CGCTCCATCATCTTCAACTCGG</td>
</tr>
<tr>
<td>GAPDH forward</td>
<td>23</td>
<td>56.6</td>
<td>70.1</td>
<td>CATCACTGCCACCCGAGAAGACTG</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>23</td>
<td>56.6</td>
<td>72.4</td>
<td>ATGCCAGTGGAGCTCCCTGTCAG</td>
</tr>
<tr>
<td>β-actin Forward</td>
<td>23</td>
<td>52.2</td>
<td>70</td>
<td>CATTTGTCGACAGGATCGAAGAG</td>
</tr>
<tr>
<td>β-actin Reverse</td>
<td>22</td>
<td>59.1</td>
<td>70.9</td>
<td>TGCTGAAAGTGGACAGTGAGG</td>
</tr>
</tbody>
</table>

3.1.3.2 Quantitative real-time PCR

Unless otherwise specified, real-time qPCR was performed on Bio-Rad iCycler iQ™ Real-Time PCR detection system using Bio-Rad IQ™SYBR® Green supermix, following manufacturer’s protocol. Briefly, samples were prepared into 96 well plates (on ice) as followed: 200nM of each primer, 20ng of cDNA, 10µl SYBR® green master mix and nuclease-free water up to total volume of 20µl.

Table 3.4 Number of cycles, temperatures and timing used for the stages of the RT-qPCR reaction.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature (°C)</th>
<th>Time (Min)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initialization</td>
<td>95</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>0.10</td>
<td>40</td>
</tr>
<tr>
<td>Annealing</td>
<td>60</td>
<td>0.30</td>
<td>40</td>
</tr>
<tr>
<td>Elongation</td>
<td>72</td>
<td>0.30</td>
<td>40</td>
</tr>
<tr>
<td>Melt Curve</td>
<td>55</td>
<td>0.30</td>
<td>81</td>
</tr>
</tbody>
</table>
3.1.3.3 Statistical analysis of quantitative real-time PCR data

Statistical analysis of RT-qPCR results was performed using Microsoft Excel and GraphPad Prism 5.1. To compare the expression level of target genes in normal and cancer cell lines, the comparative Ct method was used. This method expresses the change in gene expression as a relative fold difference between a sample of interest (e.g. from a cancer cell line) and a calibrator (e.g. from a normal cell lines). The Ct values of the calibrator and the sample of interest are first normalised to that of a house-keeping (which acts as a reference) [219, 220] (e.g. RPLP0, GAPDH or β-actin) and the difference in Ct values is the ΔCt.

The mean and standard deviations of triplicate data were obtained after the removal of any outliers. Outliers were therefore not included in all calculations.

Calculation of ΔCt for each cell line was as follows:

\[ ΔCt = Ct_{\text{target}} - Ct_{\text{reference}} \]

\[ SD = \sqrt{(SD_{\text{target}})^2 + (SD_{\text{calibrator}})^2} \]

The relative expression level of gene of interest between the control sample (e.g. sample from normal cell line) and the sample of interest (e.g. sample from cancer cell line) is given by:

\[ ΔΔCt = ΔCt (\text{target}) - ΔCt (\text{control}) \]

Expression level of gene of interest in sample of interest relative to control sample =

\[ 2^{-ΔΔCt} \]

Each calculated fold change in expression from triplicate experiments was then entered into GraphPad 5.2.1. Statistical analysis was then performed to calculate (from the triplicate data) the mean relative change in expression, standard error and significance of the difference of the means. Student’s T Test was applied to determine the significance of any differences in gene expression levels observed between controls and cancer samples.
3.1.4 Western blot analysis of protein expression

To detect the expression of proteins of interest, SDS-PAGE and western blot analysis were conducted.

3.1.4.1 Whole cell lysate preparation

Protein Extraction

Cells were trypsanised at 90% confluency, transferred to 15 ml falcon tube and pelleted via centrifugation at 1,400 rpm for 4 minutes. Cells were washed 3 times in ice-cold PBS. Supernatants were removed and cell in the pellets were subjected to lysis for 1 hour on ice in freshly prepared lysis buffer (25 mM Tris-HCl (pH7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1% Halt™ protease inhibitor cocktail containing the following protease inhibitors; AEBSF, aprotinin, bestatin, E-64, leupeptin and pepstatin A in DMSO) with occasional vortexing. The solution was centrifuged for 10 minutes at 13,000 rpm at 4°C to sediment cell debris. The supernatant was transferred to a new 1.5 ml eppendorf tube and stored at -80°C until use.

Protein extraction: Phosphorylated Proteins

To ensure the retention of the phosphate group on potentially phosphorylated PrP proteins, cells were lysed with RIPA buffer (150mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, 1% Phosphatase inhibitor cocktail 2 (Sigma Aldrich) containing the following inhibitors; sodium orthovanadate, sodium molybdite, sodium tartrate and imidazole, 1% Halt™ protease inhibitor cocktail).

3.1.4.2 Protein Quantification

Protein concentrations were determined with Polarstar Omega (BMG Labtech) using a Bio-Rad Protein DC Protein Assay Kit against a BSA standard curve generated by
a 2-fold serial dilution beginning at 5mg/µL BSA and finishing at 0.015625mg/mL BSA in lysis buffer.

3.1.4.3 Antibodies

Numerous primary antibodies were used throughout this project.

Primary antibodies:

- 12F10 (Santa Cruz Biotechnology): Mouse monoclonal IgG that reacts with PrP in human cells. This antibody recognises an epitope located within the amino acid residues 142-160. Used at 1:1,000 dilution in western blot.

- C20 (Santa Cruz Biotechnology): Goat polyclonal IgG, for detection of the C-terminus of PrP in mouse, rat and human cells. Used at 1:1,000 dilution in western blot.

- 6D11 (Santa Cruz Biotechnology): Mouse monoclonal IgG2a for detection of PrP in mouse and humans cells. Used at 1:1,000 dilution in western blot.

- 3F4 (Merck Millipore): Mouse monoclonal IgG2a for detection of PrP aa 109-112 in human and hamster. Used at 1:5,000 dilution for western blot and 1:1,000 dilution for Immunofluorescence.

- 7D9 (ABCAM): Mouse monoclonal IgG reacting to PrP aa23-237 in mouse, rat, sheep, hamster, cow, cat human, deer and elk cells. Used at 1:1,000 dilution in western blot.

- Calnexin H-70 (Santa Cruz Biotechnology): Rabbit polyclonal IgG for detection of calnexin in mouse, rat and human cells. Used at 1:3,000 dilution for western blot.

- β-actin (Merck Millipore): Clone EP1123Y, rabbit polyclonal IgG, reacting with C-terminus of β-actin. Used at 1:3,000 dilution for western blot.
- FOXO3a (Santa Cruz Biotechnology): Goat polyclonal IgG, reacting with N-terminus of FKHRL1 (N-15) in human and mouse cells. Used at 1:200 dilution for western blot.

- P-FOXO3a (Santa Cruz Biotechnology): Goat polyclonal IgG reacting with p-FKHRL1 antibody (Thr 32) in human and mouse cells. Used at 1:200 dilution for western blot.

- Bax (ABCAM): Rabbit polyclonal IgG reacting with BAX in mouse, rat, human cells. Used at 1:1,000 dilution in western blot.


- Histone H3 (Cell Signalling Technologies): Mouse monoclonal IgG1 antibody reacting with histone H3 protein of human, mouse and rat cells. Used at 1:1,000 dilution for western blot.

**Secondary Antibodies:**

- Goat- anti-mouse IgG HRP-conjugated antibody (Santa-Cruz Biotechnology) used at 1:5,000 for western blot.

- Mouse anti-goat IgG HRP-conjugated antibody (Santa Cruz Biotechnology) used at 1:5,000 for western blot.

- Goat anti-rabbit IgG HRP-conjugated antibody (Santa-Cruz Biotechnology) used at 1:5,000 for western blot.

**3.1.4.4. SDS PAGE and Western Blot**

To resolve proteins, equal concentrations of prepared lysates were loaded onto Bio-Rad mini-PROTEAN® TGX™ pre-cast 4-15% gradient gels or 10% gels prepared using Bio-Rad TGX™ FastCast™ Acrylamide Kit and electrophoresed at 100V using Bio-Rad Mini-PROTEAN Tetra system and SDS running buffer (25 mM
Tris-HCl, 40 mM glycine, 1% SDS (v/v), adjusted to pH 8.3). Resolved proteins were transferred to a PVDF membrane (Bio-Rad) using Bio-Rad Trans-Blot® Turbo™ Transfer system at 25 V for 7 minutes, in transfer buffer (50 mM Tris-HCl, 40 mM glycine, pH adjusted to 8.3, 20% methanol added fresh). Following transfer, membranes were blocked in 5% milk or BSA in 10 ml TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween-20) for 1 hour at room temperature. Membranes were incubated at 4°C overnight in appropriate primary antibody (diluted in blocking buffer), washed 3 x 5 min in TBS-T and incubated in secondary antibody (diluted in TBS-T) for 2 hours at room temperature. After washing, (TBS-T, 3 x 5 min), target proteins were detected using Pierce™ ECL Western Blotting Substrate (Thermo-Fisher-Scientific) and Bio-Rad ChemiDoc system (Aus). Target protein bands were quantified and normalised to a loading control using ImageJ and Microsoft Excel software.

3.1.5 Immunofluorescence

Reagents:
- 1x phosphate-buffered saline (Cold) (Life Technologies)
- Fixing Solution: paraformaldehyde (4% in PBS) (Sigma Aldrich)
- Permeabilising solution (200 ml): 400 µl Triton-100 (Sigma Aldrich) diluted to 200ml with PBS
- Blocking buffer (200 ml): 10 mM Tris-HCl, 5% FBS (Life Technologies), 1%BSA, 0.05% Tween-20 (Sigma Aldrich), 100 mM MgCl₂ (Sigma Aldrich), 1xPBS to 200 ml

Primary antibodies:
- 3F4 anti-PrP (See section 3.1.4.3)
- A11 anti-oligomer (Merck Millipore): an antibody that detects all types of amyloid oligomers [221].
Secondary antibodies

- anti-mouse IgG (Texas Red-conjugated) (Invitrogen)
- anti-rabbit IgG (IRDye 680-- conjugated) (Thermo-Fisher)

Cover slips were coated in poly-L-lysine for 10 mins at room temperature, washed in ethanol then (when dry) placed into wells of 24-well plates (Corning) and washed 2 x with 1ml PBS. Wells were inoculated with 7.5x10⁴ cells in 1 ml media and allowed in incubate at 37°C overnight. Media was decanted and cells washed 2x in PBS before being fixed with 4% PFA for 15 mins. After washing in 1 ml PBS (2x), cells were permeabilised with 0.5% Triton x-100 in PBS for 15 mins at 4°C. Permeabilised cells were then washed with 1 ml ice cold PBS (3x). Proteins were blocked in 1 ml blocking buffer for 30mins at room temperature. Cells were incubated for 1 hour at room temperature in primary antibody diluted in FBT. After washing in ice cold PBS (3x), cells were incubated in secondary antibody diluted in blocking buffer and DAPI for 30minutes in the dark at room temperature. Cells were washed (3x ice cold PBS) and coverslips mounted onto slides with Gold antifade reagent (Life Technologies) and allowed to set for 1 hour at 37°C. Proteins were visualized at 60 x magnification using the DeltaVision personal dv deconvolution microscope (GE Healthcare Life Sciences, WA, USA).

3.1.6 MTS assay of cell viability

MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was used to determine the effect of treatments on cell proliferation/death. 5 x 10³ cells per well were plated out into 96-well plates (in triplicate) and cultured overnight. Appropriate treatments were applied, and the cells incubated for 72 hours. 100 µl of MTS solution (10% MTS reagent (Promega), 10% FBS (Bivogen), 80% media (DMEM, Invitrogen)) was added and the cells incubated
at 37°C for 2 hours. Absorbance at 480 nm was measured using a POLARSTAR Omega (BMG Labtech, Ger) spectrophotometer. Data were analysed using Graphpad Prism 5.1.

3.1.7 siRNA transfection

Knock down of PRNP was performed using Thermo-Fisher-Scientific ON-TARGET plus Human PRNP (5621) siRNA- SMART pool (5nmol) alongside a universal negative control siRNA (Invitrogen Universal negative control). Redissolving of siRNA was performed according to the manufacturer’s protocol. To perform siRNA transfection into cells, $1.5 \times 10^5$ cells were plated in each well of 6-well plates and incubated overnight. Media was removed and 1 ml Opti-MEM® (Thermo-Fisher-Scientific) without serum was applied to the cells while siRNA complex (5 nM siRNA, 10 ul/ml Invitrogen Lipofectamine 2000 reagent in Opti-MEM®) was prepared. The siRNA complex was incubated for a minimum of 15 mins before application to cells. 1 ml of complex was applied to cells, and the cells were incubated for 6 hours at 37°C 5% CO₂. The siRNA complex was then removed and complete culture media applied. Cells were incubated for 48 hours. Knock-down efficiency was assessed via western blot analysis. PRNP-specific siRNA remains effective between 5-7 days following transfection.
Chapter 4: Prion protein (PrP) influences cisplatin response of colorectal cancer via regulation of FOXO3a phosphorylation.

This chapter include co-authored paper(s). The bibliographic details of the submitted (Nov 2015) co-authored papers, including all authors and their contributions are:

Manuscript submitted for publication November 2015: Atkinson, CJ; Munn AL; Wei, MQ; Wiegmans, AP. Prion protein (PrP) influences cisplatin response of colorectal cancer via regulation of FOXO3a phosphorylation. C. Atkinson was responsible for 70% conception and design, 80% data analysis and interpretation and 80% drafting and writing and 70% editing. AL. Munn was responsible for 10% editing. AP. Wiegmans was responsible for 30% conception and design, 20% data analysis and interpretation and 10% drafting and writing.

(Signed) _______________________________ (Date) __24.11.2016__
Student: Caroline Atkinson

(Countersigned) ___________________________ (Date) __24.11.2016__
Corresponding author of paper/supervisor: Ming Wei
4.1 ABSTRACT

While the misfolded scrapie form of this protein (PrP$^{Sc}$), is renowned for its role as the causative agent of a number of human and other mammalian neurodegenerative diseases, the function of the cellular form of prion protein (PrP$^{C}$) has yet to be fully elucidated. However, the potential role of PrP$^{C}$ in cancer development and progression is becoming increasingly more significant. Previous research has demonstrated PrP$^{C}$ over-expression in cancers such as gastric, pancreatic and breast with functions that result in enhanced drug resistance. Here, we delineate a role for PrP$^{C}$ in colorectal cancer after observing contrasting levels of mRNA and protein expression of PrP$^{C}$ in a screen of ‘normal-like’ cell lines and human cell lines isolated from a broad range of common cancers. Intriguingly, we further determined that significantly higher levels of PRNP transcript did not correlate with PRNP mRNA expression, however it did correlate with cisplatin resistance. Knockdown of PrP$^{C}$ enhanced the cisplatin-sensitivity of a cisplatin-resistant colon cancer cell line HT29 and this could be further enhanced when combined with p38MAPK inhibition. We hypothesised that PrP$^{C}$ activity may be regulated by the phosphorylation status of FOXO3a, a member of the family of forkhead transcription factors which can be regulated by p38MAPK. We show that PrP$^{C}$ depletion in cisplatin-resistant cell line HT29 decreased levels of phosphorylated FOXO3a and increased the expression levels of FOXO3a, with retention of FOXO3a in the nucleus upon PrP$^{C}$ depletion. These results indicate a role for PrP$^{C}$ in drug resistance that could contribute to poor patient prognosis, in particular in colorectal cancer. The mechanisms by which PrP$^{C}$ is able to regulate cell metabolism in the cancer setting still require further examination to fully realise the potential of PrP$^{C}$ as a target for overcoming drug resistance in cancer.
**4.2 INTRODUCTION**

The cellular prion protein (PrP\(^C\)), encoded by the *PRNP* gene, is a GPI-anchored, plasma membrane bound protein of unknown precise function [90, 93]. In humans PrP\(^C\) is expressed in early embryogenesis and in adults PrP\(^C\) is ubiquitously expressed with highest expression in neurons of the brain and spinal cord while lower expression is observed in a range of peripheral tissues [12, 121]. The misfolded form of PrP\(^C\) (PrP\(^Sc\)) is well known as the causative agent in a number of mammalian neurodegenerative diseases such as Scrapie in sheep, bovine spongiform encephalopathy in cattle and Creuzefeldt-Jakob Disease in humans. Known as prion diseases or transmissible spongiform encephalopathies, these mammalian neurodegenerative diseases are the result of the post-translational conversion of PrP\(^C\) into the misfolded form, prion protein scrapie (PrP\(^Sc\)), an proteinaceous infection particle (prion) [8]. Although the precise physiological function(s) of PrP\(^C\) remain undetermined, several lines of evidence suggest its involvement in the regulation of intracellular calcium and copper concentrations; signal transduction [148], lymphocyte activation [133] suppression of apoptosis, oxidative stress response [12] and cell proliferation [222]. In addition, there is evidence suggesting PrP\(^C\) involvement in the progression of a number of cancers, including colorectal [9, 191], breast [15, 16], gastric [11, 13, 191], prostate and pancreatic [14], as well as potentially supporting drug resistance in gastric and breast cancer [12, 199]. Resistance to chemotherapeutic drugs is a leading complication in the effective treatment of cancer. It has been demonstrated that PrP\(^C\) over-expression increases the resistance of gastric cancer cells to adriamycin, while PrP\(^C\) knock-down renders them sensitive [12]. Similar drug resistance is associated with PrP\(^C\) expression in breast cancer [199]. In order to understand the mechanism for PrP\(^C\)-mediated drug-resistance there is a requirement to understand the regulation of PrP\(^C\) expression levels.
A member of the forkhead/winged-helix box class O (FOXO) transcription factors, FOXO3a is a negative regulator of PRNP expression in response to insulin-like growth factor (IGF-1) signalling [223]. The FOXO family of transcription factors is regulated by phosphorylation, ubiquitination and/or acetylation, which affect subcellular localisation and stability. As such they are involved in a number of cellular processes including those observed to involve PrP\textsuperscript{C} [224-226]. For example, phosphorylation of FOXO by protein kinase B represses transcriptional activity by means of chaperone-dependent FOXO sequestration in the cytoplasm. When dephosphorylated, FOXO is able to migrate to the nucleus and regulate gene expression, inducing cell cycle arrest and apoptosis by the mediating oncogenic signals [224, 227, 228]. Interestingly, inactivation of the FOXO3a pathway in colorectal cancer is associated with drug-resistance (specifically to the drug cisplatin) [20, 229]. Cisplatin is a platinum based therapy causes in cell death by binding to genomic DNA and inhibiting DNA replication [230-232] and is commonly used for treatment of a number of cancer types, including bladder, cervical, non-small cell lung, ovarian, squamous cell carcinoma of the head and neck and testicular cancers [232]. Similar platinum-based therapies are commonly used for the treatment of colorectal cancer, however colorectal cancer cells can acquire resistance, reducing the efficacy of the treatment. FOXO3a has been demonstrated to be a key mediator of the cytotoxic effect of cisplatin [20, 229]. In cisplatin-sensitive colorectal cancer cells, FOXO3a is dephosphorylated, undergoes nuclear translocation and target genes are expressed. However, this mechanism is compromised in those cell lines resistant to cisplatin [20]. Of note, in colorectal cancer cells, signalling via P38MAPK represses FOXO3a activity and inhibition of p38MAPK has been shown to increase the effect of cisplatin by inducing FOXO3a dephosphorylation in colorectal cancer cells [229]. We speculate that FOXO3a
dephosphorylation may result in down-regulation of PrP<sup>C</sup> expression and this may directly contribute to enhancing the effectiveness of cisplatin.

4.3 METHODS

4.3.1 Cell culture

Cancer cell lines used throughout this study include: A431 (skin); A549 (lung); HT29, SW620 and T84 (colorectal); DU145, PC3 and ALVA (prostate); and a non-cancerous control: 293T (embryonic kidney) (ATCC, USA). Each cell line was cultured at 37°C with 5% CO<sub>2</sub> in high glucose DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and a 1% penicillin/streptomycin cocktail (Invitrogen).

4.3.2 PRNP gene silencing

HT29, SW620 and T84 cells were seeded at 1.5x10<sup>5</sup> cells per 35 mm well, and incubated overnight. The cells were then transfected with siRNAs (Dharmacon On-TARGETplus human PrP (5621) and universal negative control (Sigma-Aldrich), used at a final concentration of 10nM each) as described in Chapter 3, section 3.1.7.

4.3.3 Protein stability/measurement of protein half life in cultured cells

HT29, SW620 and T84 cells were seeded at 0.3x10<sup>6</sup> cells per 35 mm well and treated with cycloheximide (100 µg/ml) with or without proteosome inhibitor MG132 for 3, 6, 9 or 18 hours. Cells were harvested via trypsanisation and used to prepare cell lysis. The growth rate of HT29, SW620 and T84 cells with or without cycloheximide or MG132 treatment was also assessed. 2.5x10<sup>4</sup> cells were seeded into each well of a 12 well plate. The cells in one well were harvested at each time point (24, 48, 72, 96 hours) and number of viable cells was assessed using the trypan blue exclusion method. Exponential growth rate of cell lines with and without cycloheximide were calculated using non-linear regression (GraphPad Prism 5.2.1).
4.3.4 Assessing cisplatin resistance

Cisplatin was dissolved in DMSO to a concentration of 333mM to create a stock solution. The IC50 was established using a dose response curve (0, 1, 3, 10, 30, 100 and 300 uM) and analysed for effects on cell viability via MTS assay. siRNA-transfected cells were seeded at 5x10³ cells per well in a 96-well plate and then treated with cisplatin at a final concentration of 20 µM for 48 hours, after which cell viability was assessed using MTS assay. Absorbance of samples was measured at 500 nm, and the percentage of viable cells was calculated using Graphpad prism 5.2.1.

4.3.5 Quantitative real-time polymerase chain reaction.

RNA was extracted from cells using Trizol® (Life Technologies) following the manufacturer’s protocol (see Chapter 3, section 3.1.2). cDNA was synthesised using an iScript cDNA synthesis kit (Bio-Rad), performed according to the manufacturer’s protocol. An IQ SYBR green supermix (Bio-Rad) kit was used according to the manufacturer’s instructions to perform RT-qPCR. PRNP mRNA expression was compared to that of three commonly used housekeeping genes β-actin, GAPDH (data not shown) and RPLP0. PRNP mRNA expression in each cancer cell line relative to non-cancerous 293T cells was calculated after normalisation of the expression levels to those of the house-keeping gene RPLPO (see Chapter 3, section 3.1.1.3). The statistical significance of any observed differences was assessed using Student’s t test. Experiments were performed in experimental duplicates and biological triplicates. Primers were purchased from Sigma Aldrich. See Chapter 3, section 3.1.3 for protocol and primer sequences.
4.3.6 SDS-PAGE and western immunoblot analysis

Samples were prepared as described in Chapter 3, section 3.1.4.1. The following commercially available antibodies were used: monoclonal 3F4 anti-PrP\textsuperscript{109-112} anti-TP53, anti-FKHRL1, anti-phospho-FKHRL1 (Thr-32), anti-calnexin, anti-histone 3, anti-\(\beta\)-actin, anti-Histone H3, HRP conjugated anti-rabbit IgG, HRP conjugated anti-goat and HRP conjugated anti mouse IgG. See Chapter 3, section 3.1.4.3. for dilutions. Samples were subjected to SDS-PAGE and western blot analysis as per Chapter 3, section 3.1.4.4.

4.3.7 Statistical analysis

Graphpad Prism 5.2.1 was used to perform statistical analysis with a \(p\) value of <0.05 considered significant. Means of duplicate or triplicate experiments and statistically significant differences were calculated using Student’s \(t\) test.

4.4 RESULTS

4.4.1 High expression levels of prion protein in cancer cell lines

To examine the expression profile of PrP\textsuperscript{C} across a spectrum of different cancers we evaluated \(PRNP\) mRNA levels in a range of cancer-derived cell lines and compared them to \(PRNP\) mRNA levels in a non-cancerous, epithelial cell line (293T). We determined that SW620, A431, PC3 and ALVA cell lines displayed significantly (\(p<0.05\)) higher \(PRNP\) mRNA expression levels than the 293T cell line (Figure 4.1A). We validated the expression levels with a brain pool sample known to have high \(PRNP\) mRNA levels (Supplementary Figure 4.1). Of note, \(PRNP\) mRNA expression levels were not consistent within the cancer types as seen with the colorectal cancer samples HT29, T84 and SW620 (Figure 4.1A). This was also evident with PrP\textsuperscript{C} protein expression (Figure 4.1B), for example, the SW620 cell line displayed higher mRNA levels than the HT29 cell line but had lower protein levels.
Chapter 4: Prion Protein (PrP) Influences Cisplatin Response

(Figure 4.1B). In contrast, the SW620 cell line showed less than half the level of mRNA expression of the A431 cell line but only slightly less protein expression, while the PC3 cell line showed one quarter the level of mRNA expression compared to the ALVA cell line but higher protein expression levels (Figure 4.1C). This suggests a significant role in stability/turnover, secretion or differential translation for PrP\(^\text{C}\) protein in determining steady-state levels of protein expression.

Figure 4.1 PrP\(^\text{C}\) expression in a plethora of cancer cell lines relative to non-cancerous cell line 293T. A) Comparison of PRNP mRNA expression levels in cancerous cell lines and the non-cancerous 293T cell line normalised to the expression of the house-keeping gene RPLPO. Error bars indicate standard error of the mean. *Statistically significant difference (p<0.05) B) Whole cell lysates were subjected to SDS-PAGE and western blot analysis, probing for PrP\(^\text{C}\) and \(\beta\)-actin as a loading control. The figure shown is a representation of three independent experiments. C) \(\beta\)-actin-adjusted PrP\(^\text{C}\) protein band density in the various cancer cell lines relative to the PrP\(^\text{C}\) protein band density of the non-cancerous control (of three independent experiments).
4.4.2 PrP\textsuperscript{C} protein stability varies in colon cancer cell lines

To determine if differences in the rate of protein turnover in different cancer cell lines could account for the observed lack of correlation between \textit{PRNP} mRNA and PrP\textsuperscript{C} protein expression level, cells were treated with cyclohexamide (CHX), a protein biosynthesis inhibitor. PrP\textsuperscript{C} stability in the colorectal cancer cell lines HT29 (Figure 4.2A- high PrP\textsuperscript{C} protein expression), SW620 (Figure 4.2B- moderate PrP\textsuperscript{C} protein expression) and T84 (Figure 4.2C- low PrP\textsuperscript{C} protein expression) was assessed. Cell lines were treated with cycloheximide for 0, 3, 6, 9 or 18 hours (with or without proteasome inhibitor MG132). The SW620 cell line demonstrated lowered PrP\textsuperscript{C} protein stability compared to the other cell lines tested, with levels declining between 3 and 6 hours and the majority of PrP\textsuperscript{C} being degraded between 6 and 9 hours after CHX treatment (Figure 4.2B). In concordance with this observation, SW620 cell line displayed the highest growth rate of the three cell lines (Figure 4.2D) and a moderate reduction in cell growth in the presence of CHX (Figure 4.2E). Taken together, these observations appear consistent with the disparity observed between high \textit{PRNP} mRNA expression levels and low protein levels in SW620 cells observed. T84 cells displayed slower turnover of PrP\textsuperscript{C} protein compared to SW620 cells (Figure 4.2C), with depletion of PrP\textsuperscript{C} starting between 6-9 hours, and a similar inhibition of growth in the presence of CHX (Figure 4.2E). However, the T84 cell line displayed a much slower overall growth rate and this may account for the less obvious disparity between \textit{PRNP} mRNA levels and PrP\textsuperscript{C} protein levels than that observed for SW60 cells (Figure 4.2D). In contrast to the other two cell lines, the HT29 cell line displayed stable PrP\textsuperscript{C} protein levels beyond 9 hours after CHX treatment (Figure 4.2A) and its growth rate was not as adversely affected by the presence of CHX (Figure 4.2D vs 4.2E). This may account for why the HT29 cell line displayed the highest levels of PrP\textsuperscript{C} protein expression even with low \textit{PRNP}
mRNA levels. Thus PrP\textsuperscript{C} protein expression level appears to depend on differences in protein stability/turnover and cell growth rate which are independent of PRNP mRNA levels.

![Figure 4.2 Protein stability of PrP\textsuperscript{C} in colon cancer cell lines.](image)

A) HT29
B) Sw620
C) T84
D) Growth rate of untreated HT29, T84 and SW620 cell lines. Exponential growth equations: HT29 Y= 18127*e\textsuperscript{0.02235*time}; T84 Y= 29788*e\textsuperscript{0.031230*time}; SW620 Y= 16158*e\textsuperscript{0.03151*time}. Error bars indicate standard error of the mean.

E) Growth rate of cyclohexamide-treated HT29, T84, and SW620 cells. Exponential growth equations: HT29 Y= 24801*e\textsuperscript{0.00318*time}; T84 Y=25003*e\textsuperscript{0.005673*time}; SW620 Y= 27311*e\textsuperscript{-0.001320*time}. Error bars indicate standard error of the mean.

### 4.4.3 PRNP knock-down increases cisplatin sensitivity in cisplatin resistant colon cancer cells

Up-regulated PrP\textsuperscript{C} protein expression has been previously shown to correlate with acquisition of drug resistance in colorectal cancer. The colon cancer cell lines HT29,
SW620 and T84 have been shown to have varying degrees of cisplatin sensitivity (Table 4.1).

**Table 4.1 Site of isolation, p53 status and drug sensitivity of colon cancer cell lines used in this study**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell line isolation site</th>
<th>p53 status</th>
<th>Drug sensitivity</th>
<th>Cisplatin resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT29</td>
<td>Primary Tumour</td>
<td>+, R273H</td>
<td>Increased resistance</td>
<td>Resistant</td>
</tr>
<tr>
<td>SW620</td>
<td>Metastatic site (lymph node)</td>
<td>+, R273H, P309S</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
<tr>
<td>T84</td>
<td>Metastatic site (lung)</td>
<td>+, WT</td>
<td>Sensitive</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

HT29 is highly resistant [233], which interestingly correlates with high stability of PrP格外. To verify this, each cell line was subjected to treatment with varying concentrations of cisplatin for 96 hours. We confirmed that the HT29 cell line is resistant to cisplatin treatment, while SW620 is approximately four times more sensitive to cisplatin than the T84 cell line, with IC50 values of 36.45 µM and 159.9 µM respectively (Figure 4.3A). Thus we find that resistance to cisplatin correlates with high PrP格外 stability. To determine if changes in PrP格外 expression are able to affect the sensitivity of each cell line to cisplatin treatment, we depleted PrP格外 expression with an siRNA specific to *PRNP* (Supplementary Figure 4.2) in the presence of cisplatin (SW620 IC25). In HT29 cells depletion of PrP格外 expression significantly enhanced sensitivity to cisplatin, which was not dramatically enhanced by targeting p38MAPK (Figure 4.3B). In the moderate- and low-PrP格外 expressing T84 and SW620 cell lines depletion of *PRNP* did not affect sensitivity to cisplatin or p38MAPK inhibitor alone or in combination (Figure 4.3C/D). Thus, we can speculate that over-expression of PrP格外 may influence cellular signalling pathways that regulate cisplatin sensitivity, such as the FOXO3a pathway, however this is unlikely mediated via p38MAPK.
Figure 4.3 Cell death analysis of cisplatin- and p38-inhibitor-treated colon cancer cells (PRNP siRNA+/−). A) Cisplatin dose response curve analysis of HT29, SW620, T84 cell lines. B) HT29 treatment with PrP<sup>C</sup> knockdown. C) SW620 treatment with PrP<sup>C</sup> knockdown. D) T84 treatment with PrP<sup>C</sup> knockdown. * Indicates statistical significance (p<0.05) versus Scr negative control siRNA with the same treatment. DMSO control for both PRNP- and Scr-siRNA-transfected cells was set at 100%. Error bars indicate standard error of the mean.

4.4.4 PRNP knock-down effects FOXO3a activation

It has previously been shown that FOXO3a dephosphorylation in response to cisplatin treatment is incomplete in HT29 cells in comparison to SW620 cells [20]. To determine if the increase in sensitivity of colon cancer cells to cisplatin treatment is due to an increase in the dephosphorylation and activation of FOXO3a, PRNP was knocked down using siRNA transfection and the cells were then treated with cisplatin. In HT29 cells, phosphorylated-FOXO3a was less abundant in PRNP
knock-down cells (Figure 4.4- lane 2). We further observed that total FOXO3a protein levels were increased in cisplatin treated PRNP knock down HT29 cells also treated with a p38MAPK inhibitor, however this did not correlate with enhanced signalling with reduced FOXO3a phosphorylation. SW620 cells exhibited no significant differences in total FOXO3a expression. While decreased with treated with cisplatin, particularly in the presence of a p38MAPK inhibitor, SW620 cells displayed no difference in phosphorylated-FOXO3a expression in PRNP knockdown or Scr-siRNA-transfected control cells. Taken together these findings support our hypothesis that PrP\textsuperscript{C} plays a role in modulating the cisplatin sensitivity response, although independently of the p38MAPK signalling. To evaluate this further we examined whether cisplatin-induced ER stress is affected by PrP\textsuperscript{C} levels by probing for ER marker calnexin, which increases in expression when cells undergo ER stress [234]. We observed an increase in calnexin in cisplatin treated SW620 cells, but not in HT29 cells however, calnexin expression levels are increased when in both in HT29 cells following cisplatin treatment when cells are depleted of PRNP.
Figure 4.4 Analysis of FOXO3a activation in response to cisplatin treatment with and without PRNP knock-down. HT29 and SW620 cells were transfected with 10 nmol PRNP siRNA or scrambled (Scr) control siRNA. 72 hours following transfection, cells were treated with 20 µM cisplatin with or without 5 µM p38 MAPK inhibitor Ly22 for 48 hours. Equal concentrations of whole cell lysates were subjected to SDS-PAGE and western blot analysis, probing for total FOXO3a (FOXO3a\(^T\)), phosphorylated FOXO3a (FOXO3a\(^P\)), calnexin and β-actin. Values below each lane indicate relative protein density normalised to the density of the β-actin protein band. Protein band densities of the controls (Scr-siRNA, -cisplatin/p38 MAPK inhibitor) were defined as 1. The data shown is a representation of two separate experiments.

Phosphorylated FOXO3a is a measure of signalling activation however, nuclear localization of dephosphorylated FOXO3a is required for transcriptional activity that correlates with chemosensitivity in colorectal cancer [20, 229]. To further confirm a role for PrP\(^C\) in chemoresistance via FOXO3a, we isolated the nuclear proteins from HT29 cells transfected with PRNP or SCR control siRNA and treated with cisplatin in the presence or absence of p38MAPK inhibitor. We observed that total FOXO3a levels were stabilized in the nucleus in response to not only depletion of PRNP but was enhanced in the presence of cisplatin and further enhanced with the addition of...
p38MAPK inhibitor (Figure 4.5). This shows a direct correlation between depletion of PRNP and chemosensitivity. Thus we suggest that PrP<sup>C</sup> regulates cisplatin response via inhibition of FOXO3a translocation to the nucleus and inhibition of downstream effectors.

![Figure 4.5 Total FOXO3a increases in nuclear fraction of HT29 cells upon PRNP knock-down and cisplatin treatment.](image)

To further assess increased FOXO3a translocation to the nucleus upon treatment with cisplatin, nuclear fractions were prepared from of HT29 cells following transfection with PRNP siRNA or Scr siRNA and treatment with 20 µM cisplatin with or without 5 µM p38 inhibitor. Western blot analysis of the nuclear fractions from each treatment group indicated probing for FOXO3a<sup>T</sup> and Histone 3 (nuclear loading control). Numbers indicate FOXO3a protein band density (of 2 independent experiments) relative to SCR siRNA transfected, -cisplatin, -p38i HT29 nucleus fractions.

4.4.5 PRNP knock-down up-regulates Bax and p53 expression in SW620, but not in the HT29 colorectal cancer cell line

In gastric cancer cells, both Bax and p53 have been shown to be up-regulated when PRNP expression is knocked down [191]. Bax and p53 are key components of the apoptotic cell death program induced in response to chemotherapies. Thus, we speculate that PRNP expression could support chemoresistance via reducing the expression of key apoptotic proteins and that knockdown of PRNP could prevent this. We assessed the effect of PRNP depletion on expression of p53 and Bax in HT29 and SW620 cell lines. We confirmed that p53 and Bax were up-regulated only in SW620 cells following PRNP depletion or inhibition of p38 MAPK (Figure 4.6).
No up-regulation of Bax or p53 were observed in HT29 cells when transfected with PRNP-specific siRNA, however a down-regulation in Bax in HT29 cells was observed.

In SW620 cells, cisplatin treatment alone did not result in a strong up-regulation of Bax or p53 expression. Interestingly, the combination of PRNP knock down and p38 MAPK inhibition did not further increase the up-regulation of p53 compared to the knock down of PRNP alone. Furthermore, we showed while the combination of PRNP knock down and cisplatin treatment did not increase the up-regulation of Bax expression compared to knockdown of PRNP alone, the combination of either PRNP knockdown and p38 MAP kinase inhibition or p38 MAP kinase inhibition and cisplatin treatment did result in stronger up-regulation of Bax expression compared to PRNP knockdown alone, p38 MAP kinase inhibition alone, or cisplatin treatment alone.

We therefore suggest that PrPC is acting to suppress Bax expression and apoptosis via a pathway other than activation of the p38 MAPK kinase signaling and this PRNP-dependent p38-MAPK-independent pathway can partially suppress Bax expression following cisplatin treatment. Furthermore, cisplatin works independently of PRNP knock-down to increase Bax expression only when p38 MAPK is also inhibited. When p38 MAPK is not inhibited cisplatin has no additional effect on Bax up-regulation compared to PRNP knock-down alone. When p38 MAPK is not inhibited the effect of cisplatin could be entirely through inhibition or knock-down of PRNP (i.e. not independent of PRNP knockdown). This may indicate why the triple combination (low PrP expression, p38 MAPK inhibition and cisplatin treatment) is required for the maximal up-regulation of BAX.
### 4.5 DISCUSSION

It has been shown that PrP is expressed at low levels in normal tissues but is expressed at high levels in a number of cancer tissues such as colon cancer [201, 202] and gastric cancer [191]. Expression of PrP at the mRNA and protein levels in normal cell lines has not been examined previously. While 293T is not a normal cell line as it is immortalised and transformed with an SV40 virus [233], it is a kidney cell line originating from the non-cancerous HEK293 and would thus be expected to have low expression of PrP at both the mRNA and protein levels (as shown here), albeit higher than expression in a normal kidney cell line. Primary cell lines would need to be examined in future studies to examine the exact difference in expression of PrP between normal cell types and cancerous cell types.

Previous work has demonstrated that while PRNP is up-regulated in colorectal cancer compared to normal tissues [201], PrP<sup>C</sup> protein levels correlate poorly with PRNP...
mRNA levels [202] and indeed it was often the case that PrP\(^\text{C}\) protein level was up-regulated to a greater extent than \(PRNP\) mRNA level. Here, we observed a poor correlation between \(PRNP\) mRNA levels and PrP\(^\text{C}\) protein levels in each colon cancer cell line tested as well as in cell lines derived from other types of cancer such as skin and prostate. These results support our suggestion that \(PRNP\) mRNA expression levels are not predictive of the strength of phenotypes attributable to PrP\(^\text{C}\) over-expression. Cheng \textit{et al} [235] showed a correlation between CD44 expression and PrP\(^\text{C}\) expression levels in post-neoadjuvant (treatment prior to main treatment) chemotherapy breast cancer cases with high expression of PrP\(^\text{C}\) and CD44 in unresponsive patients and low expression of PrP\(^\text{C}\) and CD44 in responsive patients. This indicates that in combination CD44 and PrP\(^\text{C}\) over-expression enhances the response to chemotherapy [235]. We observed a similar response in colon cancer cells, where stable high-level PrP\(^\text{C}\) expression correlated with resistance to drug treatment. Additionally, PrP\(^\text{C}\) has been found to be over-expressed in drug-resistant breast cancer cells [195, 199, 235] and gastric cancer cells [185] and when \(PRNP\) was silenced, sensitivity to treatment was restored [195]. We observed that sensitivity to cisplatin treatment was not further enhanced by \(PRNP\) knock-down in already sensitive cell lines, indicating that PrP\(^\text{C}\) may not influence DNA damage pathways induced by cisplatin. However, we showed that depletion of PrP\(^\text{C}\) (by approximately 50\%) can restore cisplatin sensitivity to the cisplatin-resistant cell line HT29 making it responsive to cisplatin treatment. This indicates a specific role for PrP\(^\text{C}\) in chemoresistance. The restoration of cisplatin sensitivity is particularly striking when PrP\(^\text{C}\) depletion is combined with treatment with a p38MAPK inhibitor. While this restoration of cisplatin sensitivity is minor (but nonetheless significant), complete and stable depletion of PrP\(^\text{C}\) may further enhance the sensitivity of HT29 to cisplatin treatment, and that a higher dosage of cisplatin would result in greater cancer cell death. Further experiments would therefore be required to
examine whether or not complete and/or stable knockdown of PRNP would enhance cisplatin sensitivity in HT29 cells.

Previous studies have shown variable amounts of PrP\(^C\) being retained in the cytosol following proteosome inhibition [95]. For example, it was shown using treatment with proteosome inhibitors that only 10% of PrP\(^C\) was to be degraded through the proteosomal pathway [236]. The ineffectiveness of proteosome inhibitor MG132 treatment suggests PrP\(^C\) is not completely degraded through the proteosome but degraded through other mechanisms that would not be affected by a proteosome inhibitor. For example, PrP\(^C\) may be taken up into the lysosome by chaperone-mediated autophagy then degraded in the lumen of the lysosome [237-239]. Additionally, PrP\(^C\) may be secreted into the extracellular medium rather than being proteolytically degraded. Export of PrP via exosomes are utilised methods of transferring misfolded PrP (PrP\(^{Sc}\)) to uninfected cells in prion diseases [240, 241] which would not be affected by proteosome inhibitors. As we are unsure of the specific folding of PrP\(^C\) here, we cannot be certain if a switch of PrP\(^C\) to a prion form followed by secretion of the prion form (perhaps via exosomes) into the extracellular medium has also taken place thus accounting for the difference between the PrP\(^C\) mRNA and protein expression levels.

In cisplatin-sensitive cancers FOXO3a is dephosphorylated and translocated into the nucleus upon cisplatin treatment [20, 242]. In cisplatin resistant colorectal cancer, incomplete FOXO3a dephosphorylation and nuclear retention is observed [20]. It was suggested that the dephosphorylation of FOXO3a was being inhibited by an unknown mechanism in these cells [20]. Our results indicate that high expression levels of PrP\(^C\) in cells may interfere with the complete dephosphorylation of FOXO3a upon cisplatin treatment. This in turn would be expected to result in the retention of p-FOXO3a in the cytoplasm and lower levels of total FOXO3a in the nucleus. We demonstrated that the cisplatin-resistant cell line HT29 has the highest level of PrP\(^C\) expression. Furthermore,
we showed that cisplatin treatment of HT29 cells does not result in FOXO3a activation, but when PrP\(^C\) levels are reduced in HT29 cells using siRNA the ability of cisplatin treatment to activate FOXO3a by dephosphorylation is restored. The activated FOXO3a then translocates from the cytoplasm to the nucleus (Figure 4.7) where it may potentially resume transactivation of target genes and induce apoptotic cell death.

**Figure 4.7 Proposed effect of high PrP\(^C\) expression levels on the effectiveness of cisplatin.** High PrP\(^C\) levels inhibit complete dephosphorylation of FOXO3a in cisplatin-resistant colon cancer cells. FOXO3a remains phosphorylated and unable to translocate to the nucleus. FOXO3a is therefore unable to transactivate genes whose expression is required for cell cycle arrest and cell death and is unable to negatively regulate PRNP expression. Up-regulation of PrP\(^C\) expression therefore continues. Initial up-regulation may be due to an external factor, as indicated by the observation that silencing of PRNP allows dephosphorylation and activation of FOXO3a.

Bcl-2 associated protein X (Bax) expression induces cell cycle arrest and apoptotic cell death and has previously shown to be connected to PrP\(^C\) [243, 244]. PrP\(^C\) inhibits Bax-mediated cell death by inhibition of the conformational change of Bax into a pro-apoptotic protein [244]. In the current study, we observed an increase in Bax expression in SW620 when undergoing treatment with cisplatin however we did not see a
significant change in expression in HT29 cells, regardless of PrPC expression. Additionally, active FOXO3a, has been shown to promote up-regulation of the Fas ligand gene (*FasL*), triggering the Fas signalling cascade [224], and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-mediated apoptosis [245]. Interestingly, regulation of *PRNP* transcription by FOXO3a has been demonstrated in SH-SH5Y cells where *PRNP* transcription is negatively regulated by FOXO3a binding to the *PRNP* promoter [223] and therefore accounts for the increase in FOXO3a when *PRNP* is depleted. We therefore suggest that high PrPC expression is able to inhibit cisplatin activity by repressing activity of FOXO3a and expression of downstream factors including pro-apoptotic mediators *FasL* and TRAIL but not via Bax (Figure 4.7).

Treatment with antibodies directed against PrPC has been shown to increase the efficacy of cisplatin treatment of colorectal cancer cells, suggesting immune-blockade of PrPC function may be as effective as PrPC depletion using siRNA [203]. While PrPC and FOXO3a have individually been demonstrated to play a role in resistance to chemotherapeutic drugs, a definite link between them has yet to be demonstrated. Our study demonstrates the importance of PrPC in the development of resistance to chemotherapeutic drugs and thus further underscores the potential of PrPC as a future target for the development of new drug therapies for colorectal cancer.

**4.7 CONCLUSION**

We have defined PrPC as a key regulator of chemoresistance via regulation of the FOXO3a transcription factor in colorectal cancer. The full elucidation of this mechanism is still required, with questions into whether PrPC directly binds FOXO3a or one of its phosphatases yet to be answered. However we have shown that PrPC has a role in the cancer setting which may translate to becoming a potential biomarker to
stratify patients for treatment. This discovery further supports the view that PrP$^C$ is a promising target for the future development of therapeutic strategies aimed at overcoming drug resistance in colorectal cancer. Such therapeutic strategies may involve, for example, depleting PrP$^C$ (e.g. using anti-PrP$^C$ antibodies) or blocking PrP$^C$ expression (e.g. using siRNA directed against the PRNP gene that encodes PrP$^C$) to increase the sensitivity of cancer cells to chemotherapeutic agents in combination with the use of current chemotherapeutic agents.
Chapter 5: The role of expression and export of the prion protein (PrP$^C$) in breast cancer metastasis
5.1 ABSTRACT

High expression of PrP\textsuperscript{C} in breast cancer has been observed in numerous studies and furthermore is linked the multi-drug resistance in breast cancer. However, a role for PrP\textsuperscript{C} in the development of breast cancer metastasis has yet to be determined. Additionally, demonstration of the state of PrP (whether in the cellular PrP\textsuperscript{C} or a misfolded PrP\textsuperscript{Sc} form) has yet to be achieved. This chapter provides the first indication of the involvement of PrP\textsuperscript{C} in breast cancer metastasis through expression studies using online \textit{PRNP} expression data obtained from patient samples and expression studies using an established metastatic breast cancer series model. Furthermore, this study looks at the state of PrP in highly metastatic and non-metastatic breast cancer cells, to begin to determine if the state of PrP changes when cells become highly metastatic. Overall, we found that high \textit{PRNP} expression (using a Kaplan Meier plotter) is correlated with high grade and metastatic breast cancer. Using a breast cancer metastatic series cell line, we further determined that with increasing metastatic capabilities, \textit{PRNP} mRNA expression increases, however, PrP\textsuperscript{C} protein levels decrease. We show that the decrease in protein levels in highly metastatic cells is due to the increased export of PrP from the cell membrane into the extracellular media, observing that the highly metastatic cell line has lower total PrP\textsuperscript{C} protein expression and membrane-bound PrP\textsuperscript{C} but higher PrP\textsuperscript{C} protein levels in the growth medium. However, we observe that PrP\textsuperscript{C} within these cells does not display the characteristics of PrP\textsuperscript{Sc} (scrapie prion being resistant to proteinase K digestion, and increased aggregation within cells) indicating that PrP may be in the cellular PrP\textsuperscript{C} form. Overall, we further confirm that PrP\textsuperscript{C} has a potential role in the development and progression of breast cancer.
5.2 INTRODUCTION

Metastasis in breast cancer is typically linked with mortality [246, 247]. In Australia alone, over 60% of patients diagnosed with metastatic breast cancer will not survive past 5 years [248]. While a role of PrP\textsuperscript{C} in breast cancer metastasis has yet to be observed, PrP\textsuperscript{C} has been shown to promote the metastatic potential of colorectal [206] and gastric cancer cells [13]. PrP\textsuperscript{C} expression was shown to correlate with metastatic potential of colorectal carcinoma, with higher PrP\textsuperscript{C} levels observed in high grade tumours, tumours with incomplete tumour encapsulation and tumours with microvascular invasion. Furthermore, PrP\textsuperscript{C} expression was shown to be associated with metastatic potential by regulating SATB1 expression by activation of the Fyn-SP1 signalling pathway. When PrP\textsuperscript{C} was depleted, metastasis of colorectal cancer \textit{in vivo} and \textit{in vitro} was inhibited, further indicating a major role of PrP\textsuperscript{C} in metastasis of colorectal cancer [206].

In samples isolated from patients, PrP\textsuperscript{C} was found by immunohistological staining to be over-expressed in metastatic gastric cancer tissues in comparison to non-metastatic gastric cancer tissues. Additionally, as seen in colorectal cancer cells, PrP\textsuperscript{C} expression significantly promoted metastatic potential \textit{in vitro} and \textit{in vivo} [13]. It was shown that PrP\textsuperscript{C} increased promoter activation and expression of MMP11 via activation of Erk1/2. When MEK inhibitor and MMP11 antibodies were applied, the in vitro invasive and in vivo metastatic abilities induced by PrP\textsuperscript{C} expression were inhibited, overall indicating that PrP\textsuperscript{C} promotes the metastatic abilities of gastric cancer cells via activation of the MEK/ERK pathway [13].

Breast cancer may be classes in numerous ways, one of these methods may be by their molecular classification. These molecular subtypes may be Luminal (A and B) which are hormone-receptor positive, and the hormone-receptor negative basal [249]. Luminal subtypes have high expression of hormone receptors and associated genes, are estrogen
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receptor (ER) and progesterone receptor (PR) positive, express cytokeratins 8 and 18 and typically respond to endocrine therapy. On the other hand, basal subtypes have high expression of basal epithelial genes, basal cytokeratins (cytokeratin 5/6 and cytokeratin 17) and low expression of ER and associated genes, mostly triple negative (ER/PR/HER2 negative) and do not respond to endocrine therapy. Additionally, basal subtypes are invasive ductal carcinomas that have high histological grades and are associated with a poor prognosis in comparison to luminal [250-253].

To begin to determine if PrP<sup>C</sup> has a potential role in the development of breast cancer metastasis, indicating it as a target for further examination, we look at the correlation between PRNP expression with 5 year patient survival rates in high grade, lymph-node-positive breast cancer. Using an established breast cancer metastatic series, we determine the expression of PrP<sup>C</sup> at the protein and mRNA level and investigate possible correlations with metastatic potential. Additionally, we compare the transport of PrP<sup>C</sup> to the cell membrane and into the extracellular medium in metastatic and non-metastatic breast cancer cells. Finally, we establish if PrP within these cells has the same characteristics displayed by the scrapie form of PrP (PrP<sup>Sc</sup>), indicating whether or not PrP in metastatic breast cancer cells is in a misfolded (possibly amyloid prion) form.

In view of the results of previous studies on the expression of PrP<sup>C</sup> in colorectal and gastric cancer, we expect to see a higher expression of PrP<sup>C</sup> at the protein and mRNA level in metastatic breast cancer cells, in comparison to non-metastatic breast cancer cells. While we see a significant over-expression of PRNP mRNA, we see an under-expression of PrP<sup>C</sup> protein, which we shown to be potentially attributable to the export of PrP<sup>C</sup> from the cell membrane into the extracellular medium. Overall, this chapter describes the first indication of the involvement of PrP<sup>C</sup> in the development of metastatic breast cancer and identifies PrP<sup>C</sup> as a target for further analysis of the mechanisms that underlie its involvement in breast cancer metastasis.
5.3 METHODS

5.3.1 Kaplan-Meier Plotter

The Kaplan-Meier Plotter is an online analysis tool that was used to assess the effect of PRNP mRNA over-expression on the long-term patient on the survival probability [254] using data obtained from over 4,000 samples from breast cancer patients [255]. Gene expression data (established by quantitative real-time PCR) as well as relapse free and overall survival information are downloaded from GEO, EGA and TCGA into a database handled by a PostgreSQL server capable of integrating gene expression and clinical data simultaneously. To assess the prognostic value of PRNP, patient samples were split into two groups according to the quantile expressions (i.e. high vs. low PRNP expression). The two patient cohorts were then compared by a Kaplan-Meier survival plot, and the hazard ratio with 95% confidence intervals and logrank P value was calculated. The following cohorts were analysed and subsequent plots retrieved from [254]:

Relapse-free survival:
1) All grades, all subtypes
2) All grades, basal subtype
3) Grade 3, basal subtype
4) Grade 3, basal subtype, lymph-node-positive
5) Post-progression survival, all grades, all subtypes

5.3.2 Cell culture

The cancer cell lines used throughout this study include: MDA-MB-231 metastatic series, mouse 4T1 breast cancer metastatic series and non-cancerous control: MCF10a (see Chapter 3, section 3.1.1 for specific culture methods). Each cell line
was cultured at 37°C with 5% CO₂ in appropriate medium (Table 3.1, Chapter 3, section 3.1.1.3).

5.3.3 Quantitative real-time polymerase chain reaction

RNA was isolated from cells using Trizol® (Life Technologies) following the manufacturer’s protocol (Chapter 3, section 3.1.2.1). cDNA was synthesised using an iScript cDNA synthesis kit (Bio-Rad), performed according to the manufacturer’s protocol. An IQ SYBR green supermix (Bio-Rad) kit was used according to the manufacturer’s instructions to perform RT-qPCR. PRNP mRNA expression level was compared to the mRNA expression level of two commonly used housekeeping genes β-actin and RPLP0 (see Chapter 3, section 3.1.3.1 for primer sequences). PRNP mRNA expression level in cancer cells relative to expression level in non-cancerous MCF10a was calculated after normalisation to the expression level of house-keeping genes RPLPO or β-actin. PRNP mRNA expression levels of metastatic cancer cells were relative to non-metastatic cells after normalization with a house-keeping gene. Statistically significant differences were determined using Student’s T test (see Chapter 3, section 3.1.3.3 for calculation methods). Experiments were performed in experimental duplicates and biological triplicates.

5.3.4. SDS-PAGE and western immunoblot analysis.

Whole cell lysates from were prepared as described in Chapter 3, section 3.1.4.1. Equal amounts of cell lysate or concentrated media samples were diluted in 4x sample buffer (50 mM Tris-Hcl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.002% bromophenol blue), heated for 10 minutes at 90°C using a heating block then loaded onto a 10% polyacrylamide gel (Bio-Rad). Samples were then subjected to SDS-PAGE and western blot analysis as
described in Chapter 3 section 3.1.4.4. The following commercially available antibodies were used; monoclonal 3F4 anti-PrP<sup>109-112</sup>, β-actin 1, HRP-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG (see Chapter 3, section 3.1.4.3 for dilutions).

### 5.3.5 Brefeldin A treatment

Treatment with brefeldin A (BFA) has been established to increase PrP<sub>C</sub> expression in breast cancer cell lines MDA-MB-231 and MCF7 [256]. PrP<sub>C</sub> expression has been suggested to increase as a consequence of the induction of endoplasmic reticulum (ER) stress. Furthermore, BFA treatment has been shown to inhibit the transport of PrP<sub>C</sub> to the cell surface in cultured cells [105] as well as blocking exosome secretion from cells [257]. Therefore, treatment of metastatic and non-metastatic breast cancer cells with BFA will begin to identify if PrP<sub>C</sub> is being transported differently in metastatic breast cancer cells in comparison to non-metastatic. The knowledge that BFA increases PrP<sub>C</sub> expression can guide further experimentation to determine the effect of PrP<sub>C</sub> over-expression on cancer growth and progression, including metastasis.

The maximum concentration of BFA that does not cause significant cell death was determined for each cell line. The optimal concentration of BFA was determined using western blot analysis to assess PrP<sub>C</sub> expression level and MTS assay to assess IC25. A dose response curve of BFA in complete media with concentrations of 100 ng/ml, 75 ng/ml, 50 ng/ml, 25 ng/ml, 10 ng/ml and 0 ng/ml was used for both western blot and MTS assays. Cells were incubated in BFA for no more than 24 hours. The optimal BFA concentration was determined to be 50 ng/ml. Where indicated, cells were incubated in complete medium with 50 ng/ml BFA over night.
5.3.6 Measurement of cell growth

2.5x10^4 cells were seeded in each well of a 12 well plate. The cells in one well were harvested at each time point (24, 48, 72 hours), and cell number calculated via the trypan blue exclusion method (see Chapter 3, section 3.1.1.2 for protocol). The exponential phase (growth rate of cell lines) growing with and without treatment were calculated using non-linear regression (GraphPad Prism 5.2.1). Statistically significant differences were calculated using ANOVA (GraphPad Prisim 5.2.1)

5.3.7 Flow Cytometry

To assess changes in the cell cycle of brefeldin A treated MDA-MB-231 HM LNM5 cells, flow cytometry cell cycle analysis using propium iodide DNA staining was conducted. Flow cytometry was performed at QIMR berghofer by specialists. Cells were fixed and prepared by author. Propidium iodide binds in proportion to the amount of DNA present inside fixed cells. Cells will have differing amount of DNA depending on the cell cycle phase they are in. Cells in S phase (DNA replication phase), will have more than those in G1 (growth) phase and G2 more than G1. Cells that have lost DNA due to DNA fragmentation (characteristic of apoptotic cells) will appear in a subG1 peak.

To fix cells for analysis, 1x10^6 BFA treated and un-treated MDA-MB-231 HM LNM5 cells were harvested by trypsinisation and washed in PBS. Cells were then fixed in 1 ml cold 70% ethanol for 30 mins at 4°C. Cells were then centrifuged and wash twice in cold PBS. Cell pellet was then treated with 50 µg of a 100µg/ml stock of ribonuclease (Thermo Fisher). 200 µl of a 50 µg/ml stock of propidium iodide was also added to cells. Cell cycle was then analysed using a LSR 4 Laser fortessa (BD Biosciences) following manufacturer’s protocol and under collaboration with QIMR
berghofer specialists. Data was graphed with GraphPad 5.2.1 by Dr Adrian Weigmans of QIMR berghofer.

5.3.8 Concentration of culture media

To assess the concentration of PrP<sup>C</sup> in the cell culture media of MDA-MB-231 Par and HM LNM5 cells 8 x T175 flasks for each cell line were seeded with an equal number of cells and the cells were then grown until 80% confluent. Complete media was then removed and cells washed three times with 20 ml of PBS to remove all traces of FBS. 20 ml of serum free media (with or without 50 ng/ml BFA) was applied to the cells and cultured for 6 hours. The culture media was then decanted into Merck Millipore Centricon® Plus-70 centrifugal filter units and concentrated according to manufacturer’s protocol. Briefly, up to 70 ml of conditioned media per centrifugal filter was subjected to centrifugation for 20 min in fixed rotor centrifuge at 3,500 x g, then retentate recovered by centrifugation at 1,000 x g for 2 minutes. Equal volumes of the culture media concentrate for each treatment group were subjected to SDS-PAGE and western blot analysis and probed for PrP<sup>C</sup> and β-actin (loading control).

5.3.9. Isolation of cell surface proteins

Cell surface proteins were isolated from MDA-MB-231 Par and HM LNM5 cells (following BFA treatment when indicated) cells using Pierce™ Cell Surface Protein isolation kit according to manufacturer’s protocol. Pierce™ Cell Surface Isolation Kit uses biotinylation reagent (EZ-Link™ Sulfo-NHS-SS Biotin) to label cell-surface proteins of mammalian cells. These labeled proteins can then be isolated for subsequent subjecting to SDS-PAGE and western blot.
Briefly, for each sample, 4 x T75 flasks of 90-95% confluent cells were washed with ice cold PBS, then incubated, rocking, with 10 ml of Sulfo-NHS-SS-Biotin (dissolved in ice cold PBS) per flask, for 30 min at 4°C. The reactions were then quenched with a quenching solution and then cells scraped into solution and transferred to a 50ml falcon tube. Cells were centrifuged, washed with TBS, then resulting cell pellet was lysed with lysis buffer for 30 min. Biotinylated proteins were isolated with NeutrAvidin Resin then eluted with SDS-PAGE buffer with 50 mM DTT. Equal volumes of samples were subjected to SDS-PAGE and western blot analysis, blotting for PrP^C and β-actin (loading/contamination control).

5.3.10 Immunofluorescence

MDA-MB-231 Par and HM LNM5 cells were fixed and processed for immunofluorescence and stained with 3F4 anti-PrP\textsuperscript{C} and or anti-amyloid A11 antibodies followed by fluorophore-conjugated secondary antibodies. See Chapter 3, section 3.1.5 for protocol.

5.3.11. Assessing proteinase k-sensitivity of PrP

Whole cell lysates were prepared as described in Chapter 3, section 3.1.4.1 with the absence of protease inhibitors for those samples to be treated with proteinase K. 80 mg of the Par cell lysate was treated at 37°C for one hour with a series of serial dilutions of proteinase K, starting at 50 µg/ml and going to 3.125 µg/ml and starting at 1 µg/ml and going to 0.07 µg/ml. PrP digestion was assessed using SDS-PAGE and western blot. PrP\textsubscript{Sc} is commonly detected in ScN2a cells (PrP\textsubscript{Sc}-infected neuroblastoma cells) by digestion of 120 of protein with 20 ug/ml PK for 30-60 min at 37°C [258, 259]. The cell line was not available for use as a control in this study due to the restrictions on its import and use.
5.3.12 Statistical analysis.

Graphpad prism 5.2.1 was used to perform statistical analysis with a p value of <0.05 considered statistically significant. The means of duplicate or triplicate data, error bars and the statistical significance of any differences observed in the means of different treatment groups were calculated using Student’s T test.

5.4 RESULTS

5.4.1 PrP\textsuperscript{C} expression in metastatic breast cancer

5.4.1.1 PRNP mRNA expression correlates with poor patient survival

The correlation between PRNP expression and the probability of breast cancer patient survival was assessed using the Kaplan Meier (KM) plotter. The KM plotter assesses the effect of gene expression on survival using data obtained from (up to) 4,142 breast cancer patients. The effect of high PRNP gene expression on the survival of 4 cohorts of breast cancer patients, including the following cancer categories: all grades, all subtypes; all grades, basal subtype; grade 3, basal subtype; and grade 3, basal subtype, lymph-node-positive. It was shown that high PRNP expression significantly correlated with lower survival probability over 200 months (P<0.05) in all grades and all subtypes of breast cancer (Figure 5.1 A). High PRNP expression particularly strongly correlated with grade 3, lymph-node-positive, basal subtypes of breast cancer (P=0.0016), with no patients surviving past 100 months (Figure 5.1 D). High PRNP expression further correlated with post-progression survival probability in patients with all grades, all types of breast cancer.
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Figure 5.1 The effect of PRNP expression on survival probability in various cohorts of breast cancer patients as assessed using the Kaplan-Meier plotter. A) All grades, all subtypes. B) All grades, basal subtype. C) Grade 3, basal subtype. D) Basal subtype and lymph-node-positive. E) Post-progression survival, all grades, all subtypes. P indicates statistical significance, HR indicates hazard ratio. Numbers below indicate number of patient samples assessed (high PRNP = red, low PRNP expression = black). Ticks indicate patient deaths.
Additionally, basal-like subtype invasive breast carcinoma patient samples display 1.7-fold higher PRNP expression in comparison to luminal-like subtype invasive breast carcinoma patient samples (Figure 5.2), indicating that basal subtype breast carcinomas have a higher expression of PRNP than luminal the subtype.

**Figure 5.2 PRNP expression is higher in basal-subtype breast tumours.** PRNP expression levels in basal-like and luminal-like subtypes of invasive breast cancer based on the analysis of patient samples. Statistical significance of the differences in mean expression level is shown (top right).

### 5.4.1.2 Expression levels of PrP<sup>C</sup> in a human breast cancer metastatic series

To examine the expression profile of PrP<sup>C</sup> in human metastatic and non-metastatic breast cancer cells, we evaluated PRNP mRNA levels in a range of breast cancer cell lines ranging from non-metastatic (e.g. Par) to highly metastatic (e.g. HM LNM5), comparing the metastatic cell lines to the non-metastatic Par cell line. We found that PRNP mRNA expression levels were significantly increased (p<0.05) in the highly metastatic cell line HM LNM5 with more than 2-fold higher expression. To determine if PRNP mRNA expression levels correlate with PrP<sup>C</sup> protein expression levels, we analysed the PrP<sup>C</sup> protein level in each cell line shown in Figure 5.3A. We determined that PrP<sup>C</sup> protein expression levels decrease with increasing metastatic potential (Figure 5.3B).
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**Figure 5.3 PRNP mRNA expression levels and PrP<sup>C</sup> protein expression levels in human breast cancer metastatic series.** A) PRNP mRNA expression level relative to parental, normalised to the expression level of house-keeping gene RPLP0. B) PrP<sup>C</sup> total protein expression level. C) β-actin-adjusted protein band density in metastatic cancer relative to non-metastatic control protein band density of three independent experiments. Error bars indicate standard error of the mean of triplicate experiments. * indicates statistical significant difference compared to the parental (p<0.05).

Furthermore, we determined that PRNP mRNA expression level are significantly increased in the Parental cell line in comparison to the non-cancerous control MCF10a (Figure 5.4A). Additionally PrP<sup>C</sup> protein levels were significantly increased in Parental cell line in comparison to the non-cancerous MCF10a cell line (Figure 5.4 B/C).
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Figure 5.4 PRNP mRNA and PrP\textsuperscript{C} protein expression levels in non-cancerous and cancerous breast cells A) PRNP mRNA expression level in cancer cell line MDA-MB-231 relative to non-cancerous cell line MCF10a normalised to the expression level of the housekeeping gene RPLP0. B) PrP\textsuperscript{C} total protein expression level expressed by western blot. C) β-actin-adjusted protein band density in cancerous MDA-MB-231 Par cell line relative to protein band density of non-cancerous cell line MCF10a control of three independent experiments. Error bar s indicate standard error of triplicate experiments. * indicates a statistical significant difference to MCF10a (p<0.05)

5.4.1.3 Expression of PrP\textsuperscript{C} in mouse metastatic breast cancer series

To examine the expression of PrP\textsuperscript{C} in a mouse breast cancer metastatic series, we evaluated the Prnp mRNA levels in a 4T1-derived metastatic cell line series, ranging from the non-metastatic 67NR cell line to the highly metastatic cell line 4T1.2 cell line. We determined that Prnp mRNA expression was significantly increased in the metastatic 4T1.2 cell line in comparison to the non-metastatic cell line 67NR (Figure 5.5A). To determine if Prnp mRNA expression levels correlate with PrP\textsuperscript{C} protein expression levels, the protein levels were assessed (Figure 5.5B, C). A mouse brain protein sample was included as a positive control (Supplementary figure 5.1). Furthermore, a significant difference in PrP\textsuperscript{C} protein expression level between the non-metastatic cell line and the metastatic cell line 4T1.2 was observed.
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Figure 5.5 *Prnp* mRNA and PrP<sup>C</sup> expression levels in a mouse breast cancer metastatic series. A) *Prnp* mRNA expression levels in metastatic cancer cells relative to non-metastatic 67NR cell line normalised to β-actin. B) Representational image of PrP<sup>C</sup> protein and β-actin expression levels assessed by western blot. C) β-actin adjusted PrP<sup>C</sup> protein band density in metastatic cancer cell lines relative to non-metastatic 67NR based on means of data from three independent experiments. Error bars indicate standard error of the mean of data from triplicate experiments. * indicates statistically significant difference from 67NR (<0.05).

5.4.2 Inhibiting PrP<sup>C</sup> export to the plasma membrane

5.4.2.1 PrP<sup>C</sup> expression levels increase with brefeldin A treatment

Brefeldin A (BFA) inhibits the export of PrP<sup>C</sup> from the golgi to the cellular membrane, increasing the cellular PrP<sup>C</sup> level. To determine the optimal concentration of BFA to block PrP<sup>C</sup> export, the low PrP<sup>C</sup> protein expressing cell line HM LNM5 was treated for 24 hours with varying doses of BFA. PrP<sup>C</sup> protein expression levels
were assessed by western blot and cell death assessed by MTS assay. We demonstrated that PrP<sup>C</sup> protein levels in the cell were increased when HM LNM5 cells were treated with 50 ng/ml, 75 ng/ml and 100 ng/ml BFA (Figure 5.6A). A shift to lower molecular weight was also observed, possibly due to altered glycosylation. Of the concentrations that were demonstrated to increase PrP<sup>C</sup> cellular protein levels, the MTS assay showed the lowest percentage of cell death occurred following treatment with a BFA concentration of 50 ng/ml (Figure 5.6B) which caused a decrease in cell viability of 25% while treatment with 75 ng/ml and 100 ng/ml BFA caused a decrease in cell viability of 60-75%. A BFA concentration of 50 ng/ml was therefore used for all subsequent experiments.

Figure 5.6 Identifying the optimal concentration of BFA to block PrP<sup>C</sup> export. A) PrP<sup>C</sup> steady state protein levels in HM LNM5 cells following treatment various concentrations of BFA to create a dose response curve. B) MTS assay of HM LNM5 cells following treatment with various concentrations of BFA to create a dose response curve. Error bars indicate standard error of the mean of triplicate experiments.
PrP\textsuperscript{C} steady state protein levels in the parental (Par) cell line were not significantly affected by BFA treatment (Figure 5.7).

Figure 5.7 BFA does not significantly affect PrP\textsuperscript{C} expression levels in MDA-MB-231 Parental cells  A) PrP\textsuperscript{C} expression in BFA treated and non-treated Parental cells  B) B-actin adjusted relative density

To examine the effect of 50 ng/ml BFA on cell cycle progression, populations of unsynchronised cycling HM LNMS cells with or without treatment with BFA was subjected to flow cytometry analysis. To assess possible effects of BFA treatment on cell growth rates, growth assays were also performed. A shift in G1 (growth) phase, shortened S (DNA synthesis) phase and prolonged G2 (second growth) phase were observed (Figure 5.8A). A decrease in growth rate over a 72 hour period was also observed (Figure 5.8B).
Figure 5.8 Treatment HM LNM5 cells with 50ng/ml BFA affects cell growth. A) Effect of BFA treatment on cell cycle profile assessed by flow cytometry. B) Growth rate of HM LNM5 cells with and without BFA treatment. Solid lines indicate observed growth rate. Dashed lines represent calculated (non-linear) growth. Growth equation for HM LNM5 NT is $Y=169010\times\exp(0.02565\times X)$ with a doubling time of 27.02 hours. Growth equation for HM LNM5 Bref A is $Y=134717\times\exp(0.01782\times X)$ with a doubling time of 38.9 hours. * indicates statistically significant difference (P<0.05) as calculated by ANOVA.

5.4.2.2 Membrane-surface-bound PrP<sub>C</sub> is higher in non-metastatic breast cancer cells

Membrane-bound proteins were isolated from Parental (Par) and HM LNM5 cells (Figure 5.9) and were resolved by SDS-PAGE, then subjected to western blot analysis, probing for PrP<sub>C</sub> and β-actin. The latter was a lysate contamination control to measure the amount of internal protein contaminating the surface membrane protein samples. We determined that the Parental cell line had a higher amount of PrP<sub>C</sub> attached to the plasma membrane than the HM LNM5 cell line (Figure 5.9A, B). We further analysed PrP<sub>C</sub> protein expression in fixed Par and HM LNM5 cells using immunofluorescence, probing for PrP<sub>C</sub>. We showed a higher intensity of
staining of PrP\textsuperscript{C} in the Parental cell line in comparison to the HM LNM5 cells (Figure 5.9C).

**Figure 5.9** PrP\textsuperscript{C} protein levels on the plasma membrane surface of Parental (Par) is higher than the surface of HM LNM5 cells. A) Representative image of western blot analysis of plasma membrane-enriched fractions and total cell lysates prepared from Parental and HM LNM5 cells, probed for PrP\textsuperscript{C} and \(\beta\)-actin control for cytoplasmic contamination. B) Optical protein band density of PrP\textsuperscript{C} plasma-membrane-enriched fractions of Par and HM LNM5. C) Optical protein band density of Par and HM LNM5 normalised with protein band density of \(\beta\)-actin, relative to Par. D) Representative field view of PrP\textsuperscript{C} in Parental (Par) and HM LNM5 cells. PrP\textsuperscript{C} was detected using anti-PrP\textsuperscript{C} antibody 3F4 and an anti-mouse IgG (IRDye 680-T-conjugated). DAPI was included in the mounting medium to stain nuclear DNA Lower panel represents merge.
We further determined the effect of BFA treatment on surface membrane bound PrP\(_C\). We determined that following treatment with 50 ng/ml BFA, plasma membrane surface PrP\(_C\) levels increased in both the Parental (Par) and HM LNM5 cell lines (Figure 5.10A and B(ii)). A higher concentration of untreated HM LNM5 whole cell lysate was loaded in order to be clearly seen on the blot.

**Figure 5.10** PrP\(_C\) protein levels in the cellular membrane of Parental and HM LNM5 cells increase following treatment with 50 ng/ml BFA. A) Western blot analysis of whole cell lysates and plasma membrane surface protein fractions of BFA treated and untreated Par and HMLAN5 cells, probing for PrP\(_C\) and \(\beta\)-actin as a loading or contamination control. Image represents two independent experiments. B) i) \(\beta\)-actin-adjusted protein band density of whole cell lysates (treated and untreated with BFA) relative to untreated Par. ii) Protein band density of surface protein...
5.4.2.3 Export of PrP\textsuperscript{C} is up-regulated in metastatic breast cancer cells in comparison to non-metastatic breast cancer cells

To verify the difference in export of PrP\textsuperscript{C}, 140 mls of conditioned cell growth media was concentrated and analysed by western blot, probing for PrP\textsuperscript{C} and β-actin as a negative control. We showed that there was a higher amount of PrP\textsuperscript{C} in the growth medium of HM LNM5 cells than Parental cells, confirming a greater degree of PrP\textsuperscript{C} export is taking place in the HM LNM5 cells, while independently confirming previous findings of low amount of cell surface PrP\textsuperscript{C} in HM LNM5 cells. The presence of β-actin in the conditioned media of Par and HM LNM5 indicates the presence of lysed cells, most likely due to the absence of FBS in growth media for an extended time.

We further treated each cell line with 50 ng/ml BFA and assessed the PrP\textsuperscript{C} protein levels in the conditioned growth medium. A lower level of PrP\textsuperscript{C} protein was observed in the media of BFA-treated Par cells (Figure 5.11B and C), in comparison to Par untreated cells while a higher level of PrP\textsuperscript{C} was observed in the media of the BFA-treated HM LNM5 cells in comparison to all samples. However, a high concentration of β-actin was observed in the BFA-treated HMLNM5 media sample, indicating that the media had high number of lysed cells under BFA treatment therefore contaminating the medium.
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5.4.3 PrP$^C$ detected in breast cancer cells do not display PrP$^C$-like characteristics

5.4.3.1 PrP$^C$ in breast cancer cells is not resistant to proteinase K digestion

Whole cell lysate prepared from parental (Par) cell line was digested with serial dilution of proteinase K, starting at 50 µg/ml and finishing at 3.125µg/ml (Figure...
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5.12Ai) and then starting at 1 µg/ml and finishing at 0.07 µg/ml (Figure 5.12Aii). PrP was entirely digested at all concentrations in the whole cell lysates prepared from Par. Whole cell lysate prepared from HM LNM5 cell line was then subjected to PK digestion at 0.07µg/ml. PrP was also fully digested at this concentration. This shows that PrPC is not resistant to proteinase K digestion like the PrPSc form which resists digestion with 20 µg/ml proteinase K. Furthermore, we found no difference in proteinase K resistance between the PrPC found in Parental and HM LNM5 whole cell lysates (Figure 5.12B).

**Figure 5.12 PrPC in metastatic and non-metastatic cells are not resistant to proteinase K digestion.** A) Whole cells lysate prepared from parental (Par) cell line was digested with various concentrations of proteinase K i) 50 µg/ml–0 µg/ml proteinase K. ii) 1 µg/ml-0 µg/ml proteinase K. B) Whole cell lysate prepared from Par and HM LNM5 cells was subjected to digestion with 0.07 µg/ml proteinase K to assess resistance of PrPC in each cell line.
5.4.3.2 Aggregation of PrP\textsuperscript{C} is not up-regulated in metastatic breast cancer cells

Potential formation of amyloid aggregates in parental (Par) and HM LNM5 cells was assessed by immunofluorescence staining, probing with an A11 antibody that recognises all types of amyloid oligomers. The antibody appears to recognise a peptide backbone epitope that is common to all amyloid oligomers, but is not found in native proteins or amyloidogenic monomer. We found there was no increase in aggregation in HM in comparison to Par but higher green fluorescent intensity (Figure 5.13B).

![Figure 5.13](image-url)

**Figure 5.13** Protein amyloid aggregates are not increased in HM LNM5 cells in comparison to Parental.

A) Representative field view (stacked) of PrP\textsuperscript{C} and amyloid aggregates for each cells line. PrP\textsuperscript{C} was detected using antibody 3F4 and amyloid aggregates were detected using antibody A11. DAPI was included in the mounting medium to stain nuclei. The lower panels represent a merge of the three panels. Scale bar = 20\mu m

B) Graphical representation of the quantification of the ratio green (PrP\textsuperscript{C}) over red (amyloid) of parental and HM LNM5.
5.5 DISCUSSION

In our study, we began to examine the role of PrP$^C$ in breast cancer metastasis. While PrP$^C$ expression in breast cancer has been studied over the past decade, particularly in relation to drug resistance [15, 195, 200, 260], PrP$^C$ expression in breast cancer metastasis and correlation with long-term survival has not yet thoroughly been examined. Furthermore, the state of PrP$^C$ (whether in a cellular or misfolded form) has yet to be determined. We examined the correlation between high PRNP expression level and reduced long-term patient survival, expression of PrP$^C$ at the mRNA and protein level in metastatic and non-metastatic breast cancer cells, as well as the physical state of PrP by examining whether it has characteristics typical of misfolded PrP such as proteinase K resistance in metastatic and non-metastatic breast cancer cells. Overall, we have begun to demonstrate the importance of PrP$^C$ in breast cancer development and progression.

In the present study, we examined if high PRNP mRNA expression correlated with poor patient prognosis in breast cancer under various conditions. The correlation of high PrP$^C$ expression and poor patient prognosis has previously been examined in pancreatic ductal carcinoma and briefly, in breast cancer. The authors showed that in pancreatic ductal carcinoma (PDAC) high PrP$^C$ expression has correlated with poor prognosis, with the risk of death four times higher in patients with PrP$^C$(+) tumours (median survival of 5 months) compared to patients with PrP$^C$(-) tumours (median survival of 20 months) [261]. Furthermore, an analysis of breast cancer microarray databases showed that high PRNP expression levels are associated with poor prognosis and metastatic events [256].

Our analysis demonstrated a significant correlation between high PRNP expression levels and poor patient prognosis, furthermore, we observed a significantly higher expression of PRNP in basal breast carcinoma cells in comparison to luminal. This
higher expression in basal-subtype compared to luminal-subtype breast carcinoma cells was an expected result as PRNP was been previously shown to be higher in breast carcinomas with the basal subtype in comparison to the luminal subtype [256]. With higher PRNP expression already shown to correlate with poor patient prognosis and higher PRNP expression in basal subtype carcinomas, we can therefore infer that basal subtype may correlate with poor patient prognosis. While we see a significant correlation between high PRNP expression levels and poor survival in all breast cancer, grades and subtypes, we do not see a correlation with basal subtype, all grade breast carcinomas alone. However, within basal subtype, if we look specifically at high grade, metastatic (lymph-node-positive) breast carcinomas, we see a significant correlation between high PRNP expression and poor patient prognosis, indicating no correlation with low grade, non-metastatic breast carcinomas. Intriguingly, previous studies have shown high PrP\textsuperscript{C} expression levels increase the metastatic potential of other cancers such as colorectal [206, 262] and gastric cancer cells [13]. For example, high expression of PRNP has previously been shown to correlate with increased risk of colorectal cancer metastasis [262] promoting colorectal cancer metastasis through the epithelial-mesenchymal transition [206]. Furthermore, PrP\textsuperscript{C} significantly increased metastatic potential of several gastric cancer cell lines [13]. If PrP\textsuperscript{C} is involved in the development of breast cancer metastasis, this may explain why we observed a significant correlation between high PRNP levels and poor patient survival in only high grade, metastatic tumours.

With high PRNP expression levels correlating with poor survival in breast cancer patients, particularly in those with metastatic breast cancer, we assessed if the expression of PrP\textsuperscript{C} at both the mRNA and protein levels increased with increasing metastatic potential. We observed a significant increase in PRNP mRNA expression levels in those metastatic cells, however we see a concomitant decrease in total protein
levels in metastatic cells. Interestingly, PrP\textsuperscript{C} over-expression has been demonstrated in other metastatic cancers relative to non-metastatic cancers. For example, immunohistochemical staining of gastric cancer showed higher PrP\textsuperscript{C} expression in metastatic gastric cancers in comparison to non-metastatic [13] of the same cancer type. The loss of PrP\textsuperscript{C} protein in the highly metastatic cells examined in this study may be caused by a number of factors, such as loss of immunoreactivity due to altered folding, degradation, up-regulation of ectodomain shedding from the cell surface, secretion of intact PrP\textsuperscript{C} into the medium, or packaging of PrP\textsuperscript{C} into exosomal vesicles and release into the medium. Shedding and/or export of PrP via exosomes are utilized methods of transferring misfolded PrP (PrP\textsuperscript{Sc}) to uninfected cells in prion diseases [240, 241].

To begin to address which of these account for the loss of PrP\textsuperscript{C} in the highly metastatic breast cancer cells, we performed experiments to determine differences (if any) in the PrP\textsuperscript{C} concentration of extracellular medium of non-metastatic and highly metastatic breast cancer cells, and to determine if PrP\textsuperscript{C} within the highly metastatic cells exhibit features of altered folding. Our data supports the up-regulation of export (for example via exosomes) or ectodomain shedding of PrP\textsuperscript{C} into the extracellular medium, rather than changes in folding and loss of immunoreactivity.

We observed that the highly metastatic breast cancer cells have lower PrP\textsuperscript{C} on the cell surface, and higher PrP\textsuperscript{C} levels in the extracellular medium in comparison to the non-metastatic cells. This suggests that PrP\textsuperscript{C} is being exported or shed more efficiently from the highly metastatic breast cancer cells, in comparison to the non-metastatic cells. Ectodomain shedding of PrP\textsuperscript{C} is a normal shedding in cultured cells performed by the sheddase ADAM10 [110-112], however, export of intact PrP\textsuperscript{C} via exosomes has also been previously demonstrated [116, 118-120]. Our data suggests that both of these functions may be occurring as we do not see a drop in molecular weight suggesting presence of intact PrP\textsuperscript{C} in the extracellular medium but not ruling out shed PrP\textsuperscript{C} which
would have a lower molecular weight. This observed export or shedding of PrP<sub>C</sub> may be a method of inducing further metastasis, conferring on other cells the cancerous or metastatic phenotype. This possible function is further examined in Chapter 6.

When metastatic and non-metastatic breast cancer cells are treated with BFA, total PrP<sub>C</sub> levels are increased in both cell lines, but the increase is more significant in the metastatic cell line. Previous studies have shown that total PrP<sub>C</sub> levels increase when breast cancer cells are treated with BFA [256], however an increase in cell surface protein levels only has not previously been shown. As BFA inhibits export of proteins from the endoplasmic reticulum [263] as well as blocking exosome secretion [257] we expected to see an increase in total PrP<sub>C</sub> protein expression but a decrease in plasma membrane surface PrP<sub>C</sub>. Unexpectedly, we observed an increase (or unchanged) in plasma membrane surface PrP<sub>C</sub> expression. This indicates that it is still being allowed to be transported to the plasma membrane by alternate pathways. Intriguingly, BFA has been shown not to affect all endocytosis [263]. PrP has been shown to cycle between the endocytic compartment and cell surface in culture neuroblastoma cells [106]. If BFA were not affecting this pathway, this may account for the increased or unchanged PrP<sub>C</sub> levels of parental and HM LNM5 BFA-treated cell.

We further observed a decrease in PrP<sub>C</sub> protein levels in the media of parental cells following BFA treatment, but an increase in BFA-treated HM LNM5 cell. However the increase observed in the media of HM LNM5 cells may be due a high level of cell lysis when treated with BFA, permitting all proteins within cells to be released into the media (indicated by the detection of β-actin). As a decrease in PrP<sub>C</sub> protein levels in BFA-treated parental cells was observed (with minimal or no β-actin detected), we can conclude BFA is affecting export or shedding of PrP<sub>C</sub> into the media. Intriguingly, BFA has been shown to affect ADAM10 function [264, 265] but not exosomal pathway [266]
in the export of proteins other than PrP\textsuperscript{C}. While not conclusive, this may explain the incomplete inhibition of PrP\textsuperscript{C} export into the extracellular media.

The physical form of PrP in cancer has only been determined in one study of gastric cancer cells, where they found that PrP can be digested with proteinase K, unlike the misfolded infectious form PrP\textsuperscript{Sc}. While unable to determine the exact folding of PrP in non-metastatic and metastatic breast cancer cells, we could examine if PrP in these cells have typical characteristics of PrP\textsuperscript{Sc} such as proteinase K digestion sensitivity and aggregation. This would further provide an address the loss of PrP\textsuperscript{C} protein in highly metastatic cells was further due to altered folding and therefore loss of immunoreactivity. Our data showed PrP\textsuperscript{C} in non-metastatic and metastatic breast cancer cells can also be fully digested by concentrations of proteinase K that are sufficient to digest PrP\textsuperscript{Sc}. Our data also showed that while there is an increase in amyloid protein aggregates in the highly metastatic cells, it does not appear to be due to an increase in PrP\textsuperscript{C} aggregation. While this does not verify the physical form of PrP\textsuperscript{C}, it indicates that PrP in the highly metastatic cells is not misfolded in the same way as PrP\textsuperscript{Sc}. Overall, our results indicate that PrP\textsuperscript{C} has a role in the development of metastatic breast cancer. We demonstrate that when export of PrP\textsuperscript{C} is inhibited, highly metastatic cells have higher or equivalent PrP\textsuperscript{C} levels as the non-metastatic cells, showing that export may be responsible for the low PrP\textsuperscript{C} protein levels in highly metastatic breast cancer cells. This is further consistent with a role for PrP\textsuperscript{C} in conferring a metastatic phenotype on none-metastatic cells.

5.6 CONCLUSION

PrP\textsuperscript{C} has been previously implicated in the metastasis of cancers such as gastric and colon cancer and additionally its higher-level expression correlates with poor prognosis in pancreatic cancer. Here, we determined that high \textit{PRNP} expression levels correlate
Chapter 5: The role of expression and export of PrP\textsubscript{C} in breast cancer metastasis

with poor prognosis in breast cancer, particularly when cancers have metastasised. We further established \textit{PRNP} is significantly over-expressed at the mRNA level in metastatic breast cancer cells, however PrP\textsubscript{C} protein levels are significantly decreased. We demonstrated that the loss of PrP\textsubscript{C} protein may be due to the up-regulation of PrP\textsubscript{C} export or ectodomain shedding from the cell surface, into the extracellular media. The up-regulation of PrP\textsubscript{C} shedding may have a potential function in conferring on other cells the metastatic phenotype, and therefore the shedding or export process is a potential marker for diagnosis or target for therapeutic intervention. Further experimentation is required to determine the exact pathway(s) PrP\textsubscript{C} is exported in metastatic breast cancer cells and if complete inhibition of PrP\textsubscript{C} export can reduce metastasis of breast cancer.
Chapter 6: Consequences of *PRNP* knock-down on breast cancer metastatic potential, doxorubicin sensitivity and regulators of protein folding
6.1 ABSTRACT

Previously, we have established the high expression of PrP\textsuperscript{C} in metastatic breast cancer cells, in comparison to non-metastatic cells at the mRNA level, but lower expression of total PrP\textsuperscript{C} protein. We have determined that increased rate of PrP\textsuperscript{C} export or ectodomain shedding from the plasma membrane contributes to the lower PrP\textsuperscript{C} steady state protein expression in highly metastatic breast cancer cells. To determine if the release of PrP\textsuperscript{C} into the media has a role in increasing the metastatic potential of non-metastatic cells, we treated non-metastatic breast cancer cells with the conditioned media of highly metastatic breast cancer cells. We showed that the presence of PrP\textsuperscript{C} in the media contributed to increased migration and proliferation of non-metastatic cells. However, \textit{in vivo} we found that knocking down PrP\textsuperscript{C} with siRNA in highly metastatic cells prior to injection into mice, did not decrease the rate of metastasis, however, this may have been due to the unstable knock-down of PrP\textsuperscript{C}. Additionally, increased PrP\textsuperscript{C} expression has been linked to the acquisition of drug resistance in breast and gastric cancers. To further confirm this, and extend this analysis to treatment of metastatic breast cancer, we determined that knock-down of PrP\textsuperscript{C} expression sensitised both non-metastatic and metastatic breast cancer cells to treatment with doxorubicin. Overall, we further show the importance of PrP\textsuperscript{C} and its potential as a target for the development of more effective therapeutic interventions.

6.2 INTRODUCTION

Following Chapter 5, where the differences in expression level of PrP\textsuperscript{C} in metastatic in comparison to non-metastatic breast cancer cells was determined, this chapter encompasses the work to begin to determine the effect of PrP\textsuperscript{C} knock-down in breast cancer cells and the effect on the metastatic phenotype and drug sensitivity, further confirming the potential role of PrP\textsuperscript{C} in breast cancer progression and acquisition of
drug resistance. Firstly, we examine the effect of PrP<sup>C</sup> depletion on the expression level of genes involved in protein folding in non-metastatic breast cancer cells. This stage also ascertained the effectiveness of the chosen PRNP-specific siRNA on high-level PrP<sup>C</sup> protein expressing cells breast cancer cells and influence on cell growth. The determined protocol could then be used to knock-down PRNP in the metastatic breast cancer cells in the following experiments. Secondly, we determine the effect of PrP<sup>C</sup> depletion on the metastatic potential of highly metastatic breast cancer cells in comparison to non-metastatic cells. Thirdly, we assessed if PrP<sup>C</sup> can confer metastatic phenotype on the non-metastatic breast cancer cells. Finally, we determine the effect of PrP<sup>C</sup> depletion on the effectiveness of chemotherapeutic treatment of breast cancer cells with doxorubicin.

While the exact mechanism that underlies the conformational change of PrP<sup>C</sup> to PrP<sup>Sc</sup> remains elusive, it is known that protein folding may be altered by a number of chaperones, such as members of the heat shock protein family. Chaperones such as these have been shown to play a role in preventing misfolding while others promote conversion to a misfolded form [267]. For example, some forms of PrP<sup>C</sup> that are retained in the ER due to incomplete processing, are bound by the ER-luminal chaperone protein BiP. BiP can bind the defective PrP<sup>C</sup> for extended periods, i.e. until such time as the defective PrP<sup>C</sup> can be retro translated to the cytoplasm and degraded by the proteasomal pathway. This quality control process helps to maintain correct folding of PrP<sup>C</sup> [268]. In this study, we examine the expression level of a wide array of proteins involved in protein folding in breast cancer cells with and without high-level PrP<sup>C</sup> expression. This will begin to determine if depletion of PrP<sup>C</sup> influences their expression, which would indicate that they may physically interact with PrP<sup>C</sup> and perhaps have a role in maintaining the correct folding of PrP<sup>C</sup>.
Studies on colon cancer cells have shown that over-expression of PrP<sup>C</sup> enhances cell migration and invasion [269]. Additionally, PRNP knock-down in gastric cancer cells has been shown to suppress metastatic characteristics such as invasiveness and adhesion [13]. To determine the role of PrP<sup>C</sup> in breast cancer metastasis, we used siRNA to deplete PrP<sup>C</sup> in both non-metastatic and metastatic breast cancer cells and assessed the impact on proliferation and migration in vitro demonstrating an alteration in proliferation and migration of both non-metastatic and highly metastatic breast cancer cells. We further assessed the effect of PrP<sup>C</sup> depletion in vivo, assessing the number of metastases formed in mice following injection with PrP<sup>C</sup>-depleted highly metastatic cells, in comparison to control highly metastatic cells. While we did not see a significant alteration in rate of metastasis, this may have been due to the incomplete and unstable knock-down of PRNP. Furthermore, we continued to explore the role of shedding or export of PrP<sup>C</sup> by examining if preconditioned media from highly metastatic cells (with or without high-level PrP<sup>C</sup> expression) can confer metastatic potential to non-metastatic cells. We show how PrPC can confer a metastatic phenotype onto non-metastatic cells through export into extracellular medium.

Over-expression of PrP<sup>C</sup> has been shown to correlate with poor response to chemotherapy treatment in breast cancer patients [235]. Additionally, silencing of PrP<sup>C</sup> in breast cancer cells has been previously shown to sensitise breast cancer cells to chemotherapeutic treatment [195, 270]. However, the effect of PrP<sup>C</sup> on drug sensitivity of metastatic breast cancer cells has yet to be determined. Here, we assessed the effect of PrP<sup>C</sup> depletion on the proliferation and migration in metastatic and non-metastatic breast cancer cells following treatment with doxorubicin. We show that PrP<sup>C</sup> expression affects sensitivity to the drug doxorubicin.

This chapter highlights the importance of PrP<sup>C</sup> in the progression of breast cancer and acquisition of drug resistance. It demonstrates the requirement of further
experimentation to determine how PrP\textsuperscript{C} can be targeted to improve chemotherapeutic intervention in breast cancers, particularly in metastatic breast cancers.

6.3 METHODS

6.3.1 Cell culture
Cancer cell lines used throughout this study include: MDA-MB-231 Parental (Par) and MDA-MB-231 HM LNM5. See Chapter 3, section 3.1.1 for protocols.

6.3.2 PRNP silencing
MDA-MB-231 Par/HM LNM5 cells were seeded at 1.5x10\textsuperscript{5} cells per 35 mm well, incubated over night and then transfected with PRNP-siRNAs or universal negative control (Scr) siRNAs. See Chapter 3, section 3.1.7 for transfection protocol.

6.3.3 RT-qPCR Array
A Heat Shock Proteins and Chaperones RT\textsuperscript{2} Profiler PCR Array (Qiagen) was used for assessing changes in expression of genes that encode protein folding proteins. The MDA-MB-231 Par cell line was transfected with PRNP-specific or universal negative control siRNA (as above). Data was analysed using the online data analysis tool for the Qiagen Heat Shock Proteins and Chaperones RT\textsuperscript{2} Profiler PCR Array. RNA samples were prepared by following the manufacturers’ protocol using an RNAeasy minikit (Qiagen). cDNA was prepared using a QuantiTect Reverse Transcription Kit (Qiagen) following the manufactures’ protocol. The prepared array underwent real-time quantitative PCR, performed on a Roche Lightcycler® 96.

Gene included in array:

**HSP90 Family Members**: HSP90AA1, HSP90AB1 (HSPCB), HSP90B1 (TRA1).
**HSP70 Family Members:** HSPA14, HSPA1A (HSP70-1A), HSPA1B, HSPA1L, HSPA2, HSPA4 (hsp70), HSPA4L (Osp94), HSPA5 (GRP78), HSPA6 (HSP70B), HSPA8, HSPA9.

**HSP60 Family Members:** HSPD1.

**HSP40 Family Members:** DNAJA1, DNAJA2, DNAJA3, DNAJA4, DNAJC21, DNAJB1, DNAJB11, DNAJB12, DNAJB13, DNAJB14, DNAJB2, DNAJB5, DNAJB6, DNAJB7, DNAJB8, DNAJB9, DNAJC1, DNAJC10, DNAJC11, DNAJC12, DNAJC13, DNAJC14, DNAJC15, DNAJC16, DNAJC17, DNAJC18, DNAJC3, DNAJC4, DNAJC5, DNAJC5B, DNAJC5G, DNAJC6, DNAJC7, DNAJC8, DNAJC9, SERPINH1 (HSP47).

**Small Heat Shock Proteins:** HSPB1 (HSP27), HSPB2, HSPB3, HSPB6, HSPB7, HSPB8, HSPE1.

**Other Protein Folding Regulators:** ADCK3 (CABC1), ATF6, BAG1, BAG2, BAG3, BAG4, BAG5, CCS, CCT2, CCT3, CCT4, CCT5, CCT6A, CCT6B, CCT7, CRYAA, CRYAB, HSFI (TCF5), HSF2, HSF4, HSPH1 (HSP105), PFDN1, PFDN2, SIL1, TCP1, TOR1A.

**House-Keeping Genes:** ACTB, B2M, GAPDH, HPRT1, RPLP0, HGDC

### 6.3.4 Cell proliferation assays

Cell lines examined in proliferation assays include MDA-MB-231-Par/HM LNM5. Proliferation assays were performed under the conditions listed in Table 6.1. Following transfection with siRNA, 2x10⁴ cells were seeded into each well of a 24 well plate and incubated overnight. The appropriate conditions or treatments (Table 6.1) were applied and the cell cultures were placed into an InCucyte™ (Essence Bioscience, Michigan, USA) for up to 192 hours. InCucyte™ is a live-cell imaging system and was programmed to image the center of each well every hour for assess
cell proliferation. The acquired data was analyzed using the provided software to calculate confluence each cell line. Acquired data was inputted into GraphPad 5.2.1 and the effects of the treatments (Table 6.1) were compared. The data shown represents the means of experimental triplicates from two independent experiments.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Transfected with</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>Par</td>
<td>PRNP siRNA</td>
<td>No treatment control</td>
</tr>
<tr>
<td></td>
<td>PRNP siRNA</td>
<td>Doxorubicin (1 µM)</td>
</tr>
<tr>
<td></td>
<td>PRNP siRNA</td>
<td>HM LNM-conditioned media</td>
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<tr>
<td></td>
<td>PRNP siRNA</td>
<td>PRNP knock down–HM LNM5-conditioned media</td>
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<tr>
<td></td>
<td>Negative control siRNA</td>
<td>No treatment control</td>
</tr>
<tr>
<td></td>
<td>Negative control siRNA</td>
<td>Doxorubicin (1 µM)</td>
</tr>
<tr>
<td></td>
<td>Negative control siRNA</td>
<td>HM LNM5-conditioned media</td>
</tr>
<tr>
<td></td>
<td>Negative control siRNA</td>
<td>PRNP knock down- HM LNM5 conditioned media</td>
</tr>
<tr>
<td>HM LNM5</td>
<td>PRNP siRNA</td>
<td>No treatment control</td>
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<td></td>
<td>PRNP siRNA</td>
<td>Doxorubicin (1 µM)</td>
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<tr>
<td></td>
<td>Negative control siRNA</td>
<td>Doxorubicin (1 µM)</td>
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</table>

For proliferation and migration experiments using condition media from HM LNM5 cells, steps were taken to ensure siRNA was not affecting observed results. 12 hours following transfection with either PRNP siRNA or negative control (SCR) siRNA, HM LNM5 cells were washed twice with PBS before adding growth culture medium to ensure no traces of siRNA remained. Once cells reached confluence, conditioned media was removed from HM LNM5 cells and centrifuged at 1,000 rpm for 5 mins, before being applied to parental cells.

6.3.5 Assays of cell migration

Following transfection of MDA-MB-231 Par/HM LNM5 cells with siRNA (as described above), $3 \times 10^5$ cells were seeded overnight in each well of a 96 well plate until 100% confluent (48 hours). Confluent cells were wounded with WoundMaker™ (Essence Bioscience, Michigan, USA) that contains 96 spaced metal
spokes to wound cell cultures and ensuring uniform wound formation. The plates were then incubated for 96 hours in the InCucyte™, programmed to image cells at the centre of each well every 3 minutes. The data were analysed using the provided software to identify differences in migration under specific treatments (Table 6.1). The data shown is a representation of experimental triplicates from two independent experiments.

6.3.7 Mouse in vivo

All mouse in vivo work was performed at QIMR Berghofer. 10 Balb/c nude were used, ordered from Animal Resource Centre (ARC) under project p674 and animal ethics A07607M, and cared for by QIMR Berghofer animal husbandry specialists. Mice were sacrificed after 42 days by QIMR specialists. Cells were prepared by the author, and injected by QIMR animal handling specialists. Images were taken by QIMR animal handling specialists. MDA-MB-231 HM LNM5 cell line was transfected with PRNP-specific or universal negative control siRNA as described above, however, following transfection the cells were cultured for 24 hours instead of 48 hours. Cells were then collected by trypsinisation and then pelleted by centrifugation for 5 mins at 1,000 rpm at room temperature. Trypsin was removed and cells washed with 10 ml PBS. Cells were again pelleted by centrifugation at 1,000 rpm and then PBS was removed. 25x10^6 cells were resuspended in 1.25 ml PBS. 1.25 ml Matrigel was then mixed with PBS/cell mixture on ice. 0.5 ml of cell/PBS/Matrigel mixture (5x10^6 cells) was injected subcutaneously into each mouse (5x injections of PRNP-siRNA-transfected and 5x injections of negative control siRNA-transfected) by experienced animal handlers. After seven days, each developed tumour in each mouse was injected with an equal concentration of PRNP siRNA or universal negative control siRNA/Lipofectamine complex (final
Chapter 6: Consequences of PRNP knock-down

concentration 10 nM) and allowed to grow seven days. MDA-MB-231 HM LNM5 cells express luciferase. The mice were therefore assessed for metastases via luciferase fluorescence imaging and comparing PRNP-and negative-siRNA control transfected HM LNM5 cells using the Perkin Elmer IVIS bioluminescence spectral imager.

6.3.7 Kaplan-Meier Plotter

The Kaplan-Meier plotter online analysis tool was used to assess the effect of PRNP high-level expression on the survival probability of breast cancer patients [254] using over 4000 samples derived from breast cancer patients [255]. The following cohorts were analysed and subsequent plots retrieved from [254]:

Relapse-free survival:
1) All grades, no chemotherapy
2) All grades, chemotherapy

6.4. RESULTS

6.4.1 Effect of PRNP knock down on non-metastatic cells

To determine the influence of PRNP expression on the expression of genes encoding proteins involved in the regulation of protein folding, PrP was knocked down with PRNP-specific siRNA. This stage identified the level of PRNP-specific siRNA required to successfully deplete PrPC. PRNP was knocked down using siRNA in MDA-MB-231 parental breast cancer cells and RT qPCR and microarray used to assess the effect on expression of genes encoding proteins involved in the regulation of protein folding.
6.4.1.1 Knock-down of PRNP

ON-TARGET *PRNP* siRNA or universal negative control (Scr) siRNA were transfected into MDA-MD-231 parental (Par) cells. This successfully depleted PrP<sup>C</sup> levels by 95% as shown by western blot analysis (Figure 6.1A/B). The influence of *PRNP* knock-down on the growth rate was assessed. Growth rate of Par cells was significantly reduced when *PRNP* was knocked down. Increased doubling time from 28.3 hours increasing to 30.4 hours (Figure 6.1C) was observed.
Figure 6.1 *PRNP* knock-down slowed the growth rate of parental breast cancer cells. A) Western blot verification of *PRNP* knock-down B) β-actin-normalised protein band density relative to that of the untransfected control * Indicates statistically significant difference to untransfected control (P<0.05) and ** indicates statistically significant difference to SCR siRNA-transfected control (P<0.05) C) Growth rate of SCR siRNA-transfected control and *PRNP* knock-down *PRNP*-siRNA transfected parental cells. Solid lines represent observed growth rate. Dashed lines represent calculated non-linear growth equations. SCR siRNA-transfected growth equation: Y=171729*exp(0.001641*X). *PRNP* siRNA-transfected cell growth equation Y=131735*exp(0.002695*X) where X=time. * Indicates statistically significant difference to Scr-siRNA transfected control P<0.05. Error bars indicate standard error of the mean of triplicates from two independent experiments.

6.4.1.2 Knock-down of *PRNP* affects expression of genes encoding proteins involved in regulation of protein folding

Parental MDA-MB-231 cells were transfected with ON-TARGET *PRNP* siRNA or universal negative control scrambled (SCR) siRNA, then RNA was prepared from
Chapter 6: Consequences of PRNP knock-down

the cells 72 hours post transfection. The RNA was subjected to RT-qPCR, analysing an array of 96 genes encoding proteins involved in the regulation of protein folding (See section 6.3.3) for gene list. Numerous genes displayed up- or down-regulation upon PRNP knock-down (Figure 6.2). Using the provided online data analysis tool (Qiagen), the most stable house-keeping gene (of 6 house-keeping genes) was shown to be RPLP0 and subsequently this house-keeping gene used for all ∆∆Ct calculations.

**Figure 6.2** PRNP knock-down in MDA-MB-231 parental effects the expression of numerous genes encoding proteins involved in protein misfolding A) Verification of PRNP knock-down assessed by western blot B) Magnitude of fold-change in expression of each gene. Numbers represent fold change.

The three genes with the most up-regulated expression the genes encoding two HSP40 family members (DNAJB5, DNAJc16) and HSP60 family member (HSPD1), while the three genes with the most down-regulated expression were the genes encoding protein folding regulator CRYAB, HSP40 family member DNAJA4 and small heat-shock protein HSPB7 (Figure 6.3B). CRYAB is the gene that encodes
Crystallin alpha-B, which has been shown previously to be involved in breast cancer [271] and breast cancer metastasis [272]. This gene was therefore chosen for further analysis.

Figure 6.3 PRNP knock-down in MDA-MB-231 parental cells effects the expression genes encoding proteins involved in the regulation of protein misfolding

A) Fold-change in expression of the three genes with the most up-regulated expression relative to the control group, normalized with house-keeping gene RPLP0. B) Fold change in expression of the three genes with most down-regulated expression relative to the control group, normalized with house-keeping gene RPLP0. Graphs represent a single experiment.
6.4.1.3 CRYAB expression is down-regulated in PRNP knock-down breast cancer cells

Expression of crystallin alpha-B at the mRNA and protein levels was further analysed in MDA-MB-231 parental cells, transfected with PRNP siRNA or negative control (SCR) siRNA. CRYAB expression level was shown to be significantly reduced (by 0.8-0.9-fold) when PRNP was knocked down (Figure 6.4A), thus confirming the down-regulation observed in the HSP array (Figure 6.3B). Down-regulation of crystallin alpha-B was further confirmed by an observed reduction in protein levels by 50% when PRNP was knocked down (Figure 6.4B/C).

Figure 6.4 PRNP knock down down-regulated crystalline alpha-B expression. A) RT-qPCR analysis of CRYAB gene expression level following PRNP knock-down. Error bars indicate standard error of the mean of data from triplicate experiments. *Indicates statistically significant difference (P<0.005). B) Western blot analysis of crystalline alpha-B protein expression levels following PRNP knock-down. C) β-actin adjusted crystallin alpha-B protein band density relative to SCR-siRNA transfected control. Western blot analysis of crystalline alpha-B was conducted only once due to difficulty of staining.
Expression level of \textit{CRYAB} mRNA was further assessed in the MDA-MB-231 metastatic series. It was determined that \textit{CRYAB} expression levels were significantly reduced in metastatic breast cancer cells, (0.6-0.9 fold decrease in expression) (Figure 6.5).

![Figure 6.5 CRYAB expression levels are significantly lower in metastatic breast cancer cells in comparison to parental breast cancer cells. Error bars indicate the standard error of the mean of data from triplicate experiments.* Indicates statistically significant differences from parental cell line (P<0.05).]

6.4.2 Effect of \textit{PRNP} knock down on metastatic capabilities

6.4.2.1 \textit{PRNP} knock down slows proliferation and migration in metastatic and non-metastatic breast cancer cells

The effect on the metastatic potential of HM LNM5 cells of \textit{PRNP} knock down with siRNA was analyzed through the ability of cells to migrate and proliferate. \textit{PRNP} knock down in both the parental and HM LNM5 cells slowed both cell migration and cell proliferation, however, proliferation was more strongly reduced in the HM LNM5 cells (Figure 6.6).
### Figure 6.6 PRNP silencing diminishes the metastatic capabilities of non-metastatic and highly metastatic breast cancer cells

A) Scratch assay of negative control SCR-siRNA-transfected and PRNP siRNA-transfected MDA-MB231-parental and HM LNM5 cells as analysed by InCucyte™ over 24 hours. Images represent duplicate experiments.

B) Cell proliferation of SCR siRNA-transfected and PRNP siRNA-transfected MDA-MB-231 parental and HM LNM5 cells, every hour over 192 hours as analysed by InCucyte™ and graphically represented using GraphPad 5.2.1.

### 6.4.2.2 PrP<sup>C</sup> contributes to the transfer of a metastatic phenotype from highly metastatic cells to non-metastatic

Previously, was demonstrated export of PrP<sup>C</sup> is up-regulated in HM LNM5 cells. To begin to establish if the export of PrP<sup>C</sup> affects the metastatic potential of previously non-metastatic cells that came into contact with extracellular PrP<sup>C</sup>, we treated parental breast cancer cells with conditioned media from HM LNM5 cells that had been transfected with either PRNP siRNA or negative control SCR siRNA. We observed that when parental (Par) cells are treated with conditioned media from HM LNM5 cells that retain PrP<sup>C</sup> expression (i.e. negative control SCR-siRNA transfected), cell migration and proliferation is significantly increased in comparison...
to those Par cells treated with conditioned media from HM LNM5 cells that had been transfected with PRNP siRNA (Figure 6.7).

**Figure 6.7 Treatment of Parental cell line with conditioned media from HM LNM5 cells that have been transfected with SCR siRNA show increased metastatic potential.** A) i) Representation of wound healing over 24 hours of triplicate experiments and ii) graphical representation of mean confluency of centre of each wound in each well parental cells treated with conditioned media from HM LNM5 cells transfected with negative control SCR siRNA or PRNP siRNA * indicated statistically significant difference from control (p<0.05) B) proliferation assay over 96 hours in parental cells treated with conditioned media from HM LNM5 cells transfected with negative control SCR siRNA or PRNP siRNA * indicated statistically significant difference from control (p<0.05).
6.4.2.3 Effect of PRNP knock-down on metastasis in vivo

The effect of PRNP-knock down on breast cancer metastasis in vivo was assessed by observing the rate of metastasis in nude mice injected with HM LNM5 cells that had been transfected with negative control SCR siRNA or PRNP siRNA. The rate of metastasis of PRNP knock-down HMLNM5 cells in mice was not reduced relative to SCR siRNA transfected HM LNM5 cells, with metastases in both groups 42 days post-injection (Figure 6.8).

**Figure 6.8** In vivo monitoring of injected HM LNM5 cells transfected with PRNP siRNA or SCR siRNA does not show decrease in metastases when PRNP is knocked down. A) Verification of PRNP knock down by western blot using 3F4 antibody B) Tumour spread in mice over 42 days post-injection with MDA-MB-231 HM LNM5 cells transfected with SCR siRNA or PRNP siRNA. Cells were injected into mammary tissue, and then imaged using luciferase bioluminescence. Coloured patches represent tumours.
6.4.3 High level PRNP expression correlates with poor patient survival post-chemotherapy

Assessment of PRNP expression levels in patients with lymph-node-positive tumours showed a correlation between high PRNP expression and poor survival in post-chemotherapy patients, while no correlation between high level PRNP expression and survival was observed in non-chemotherapy-treated patients (Figure 6.9).

Figure 6.9 High level PRNP expression correlated with poor patient survival in post-chemotherapy treated breast cancer patients as assessed using Kaplan-Meier plotter. A) High PRNP expression correlates with poor survival in all grades, basal subtype, chemotherapy-treated patients with lymph node-positive tumours, statistical significance p=0.043. B) High PRNP expression levels do not correlate with poor survival in non-chemotherapy-treated patients with lymph node positive tumours p=0.36. Ticks indicate death of patients.
6.4.4 PRNP knock down sensitizes non-metastatic and metastatic breast cancer cells to doxorubicin treatment

The influence of PrP\(^{C}\) expression on the response of chemotherapy treatment in metastatic and non-metastatic breast cancer cells was assessed by proliferation rate. Parental and HM LNM5 cells were transfected with PRNP siRNA and subsequently treated with doxorubicin. Proliferation of each sample of cells was then assessed over 144 hours. We observed that PRNP knock-down significantly slowed proliferation of Parental and HM LNM5 cells without doxorubicin treatment (Figure 6.10A). PRNP knock-down also sensitised Parental and HM LNM5 cells to doxorubicin treatment (Figure 6.10A/B). PRNP knock-down cells showed a further reduction in proliferation rate during doxorubicin treatment, with Parental and HM LNM5 cells both reaching only ~25% confluence (Figure 6.10B) during doxorubicin treatment when PRNP was knocked down, compared to parental and HM LNM5 SCR siRNA-transfected cells which reached confluence of 50 and 60% respectively (Figure 6.10B).
Figure 6.10 PRNP knock-down in Parental and HMLNM5 cells sensitizes cells to doxorubicin treatment. A) Comparison of proliferation rates of Parental and HM LN5M cells transfected with PRNP siRNA or SCR siRNA with and without 1 µM doxorubicin treatment. B) Proliferation rates of Parental and HMLNM5 cells transfected with PRNP siRNA or Scr siRNA transfected in the presence of 1 µM doxorubicin treatment. Points represent the mean percent confluence of experimental triplicates. Error bars indicate the standard error of the mean of triplicates. * Indicates statistically significant differences.
6.5 DISCUSSION

In the previous chapter, we found differences in PrP<sup>C</sup> expression at the gene and protein level in the breast cancer metastatic series MDA-MB-231 as well as a correlation between poor patient survival and high PRNP expression, indicating a potential role of PrP<sup>C</sup> in breast cancer metastasis. In this chapter we began to investigate the effect of PRNP knock down on metastatic and non-metastatic breast cancer cells when. We show that PRNP knock-down has a variety of consequences for both metastatic and non-metastatic breast cancer cells and effects cell proliferation, cell migration and sensitivity to chemotherapeutic drugs as well as the expression of various genes encoding proteins involved in protein folding.

Knock-down of PRNP has been (previously) performed on cancer cells, however assessment of the effect of PRNP knock-down on the expression of a wide array of genes encoding proteins involved in protein folding, as performed here, has not previously been performed. While depletion of PrP<sup>C</sup> affected the expression of a number of genes, one gene (CRYAB) that was down-regulated was of particular interest. CRYAB encode αB crystallin, a major structural protein of the ocular lens and a member of the small heat shock protein family. αB crystallin is a molecular chaperone that prevents the aggregation of denatured proteins after stress exposure [273]. CRYAB has been implicated in a number of cancers, including colorectal [274], squamous cell carcinoma of the oral cavity [275], breast cancer [271] and breast cancer metastasis [272], however a link between PRNP/PrP<sup>C</sup> and CRYAB/αB crystallin in cancer has not previously been reported. Analysis of gene expression data has shown PRNP and CRYAB are co-expressed (i.e. have similar expression level across conditions in a gene study) [276, 277] and co-localise (i.e. have a similar tissue-specific distribution) [277, 278]. We show that when PrP<sup>C</sup> is depleted, CRYAB expression is also down-regulated, indicating that αB crystallin may interact with PrP<sup>C</sup>. Furthermore, we show a much
higher expression of \textit{CRYAB} in non-metastatic breast cancer cells, than in metastatic breast cancer cells. Interestingly, previous research has shown \textit{CRYAB} is up-regulated in basal-like breast cancer cells [279] and furthermore, high expression in primary breast carcinomas correlated with poor survival in patients [272], consistent with our findings of higher expression in parental cells and basal-like breast cancer cells. However, the connection between PrP\(^C\) and \(\alpha\)B crystallin remains unclear and further analysis is required to elucidate the mechanism responsible for the down-regulation of \textit{CRYAB} when \textit{PRNP} is knocked-down.

\textit{PRNP} knock-down further affected the metastatic potential of breast cancer cells, particularly on the most highly metastatic cells. This finding is consistent with findings from previous studies of colorectal and gastric cancer cells in which the expression of PrP\(^C\) was shown to mediate invasive and metastatic capacities [13, 206]. Additionally, we observed that an increase metastatic potential can be conferred onto non-metastatic cancer cells by incubation with conditioned media from highly metastatic cancer cells, particularly those expressing PrP\(^C\). Such a transfer of metastatic potential from metastatic cells to non-metastatic cells by PrP\(^C\) has not previously been demonstrated.

However, while a knock-down of \textit{PRNP} resulted in a decreased rate in proliferation and migration in cell culture, we did not observe any slowing of primary tumour growth or metastasis \textit{in vivo}. This may be attributable to the instability of \textit{PRNP} siRNA knock-down. While \textit{PRNP} may have initially been knocked-down in the HM LN5 cells prior to injection, the knock-down would not have been stable over the entire period (with siRNA knock-down typically lasting between 5-7 days as indicated by the supplier). It was not examined here if tumours that formed \textit{in vivo} had regained the expression of \textit{PRNP} as it was assumed they would over this time-frame due to the limited half-life of the \textit{PRNP}-specific siRNA as stated by the manufacturers (section Chapter 3, Section 3.1.7). While a further injection of \textit{PRNP} siRNA directly into the tumour (once
established at day 7) may have lengthened the PRNP knock-down, metastasis did not occur until later stages (after day 28), where the PRNP siRNA would have no longer have been effective/present. To establish a more successful knock-down of PrP in future experiments, tumours should be injected with PRNP-specific siRNA every 3-5 days as recommended by the supplier. Furthermore a time-course experiment should be conducted to determine the exact length of time the PRNP expression remains knocked-down within cells. In previous studies, mice injected with colorectal cancer cells in which PRNP had been knocked-down using shRNA, showed less metastases and prolonged survival [206]. In future, a stable PRNP knock-down or the use of PRNP knock-out mice would further indicate if PrP<sup>C</sup> directly affects metastasis of breast cancer.

The correlation between high PRNP expression and poor patient survival in post-chemotherapy-treated patients indicates that PrP<sup>C</sup> may influence the sensitivity of some breast carcinomas to treatment. We showed that knock-down of PRNP increased the sensitivity of both non-metastatic and metastatic breast cancer cells to doxorubicin treatment, with cells exhibiting a slower proliferation rate following transfection with PRNP siRNA when placed subsequently under doxorubicin treatment. This result is consistent with the results of previous studies of breast cancer cell lines undergoing treatment with alternate chemotherapeutic agents. For example, Meslin et. al. [195] showed that down-regulation of PRNP in high-PRNP-expressing adriamycin-resistant MCF7 cells sensitised the cells to TRAIL-mediated death by facilitating the activation of Bax and down-regulation of Bcl-2 expression. Additionally, it has been shown the Estrogen Receptor (ER)-negative/ PrP<sup>C</sup>-negative tumours display high sensitivity to chemotherapy [260]. In other cancer types, such as gastric cancer, PrP<sup>C</sup> over-expression has been shown to suppress adriamycin-induced apoptosis [12] and this suppression has further been shown to be prevented by the inhibition of the PI3K/Akt pathway,
suggesting that high \( \text{PrP}^C \) expression levels may induce drug resistance via PI3/Akt activation [17].

In contrast to the results presented in this study and others discussed previously, Yu et al. [270] in a study on MDA-MB-435 breast cancer cells showed that silencing of \( PRNP \) had the opposite effect on doxorubicin sensitivity, resulting in the cells become more resistant to treatment. While both cells lines are triple negative (i.e. do not have estrogen, progesterone or human epidermal growth factor receptors), they do have differences which may account for this inconsistency in the effect of \( PRNP \) knock-down on doxorubicin sensitivity. For example, MDA-MB-231 originated from an adenocarcinoma, while MDA-MB-435 originated from an infiltrating ductal adenocarcinoma. Additionally, there has been speculation that the current clones of MDA-MB-435 may be contaminated with a melanoma cell line [280, 281]. However, this lack of consistency in results may indicate that \( \text{PrP}^C \) influence different processes in different cancers. If this is the case it may result in a different effect of \( PRNP \) knock down on drug sensitivity in different cancers. This shows the importance of identifying the pathways affected by \( \text{PrP}^C \) over-expression and the mechanism(s) by which \( \text{PrP}^C \) mediates drug resistance.

### 6.6 CONCLUSION

Overall, we have shown the potential of \( \text{PrP}^C \), not only as a target for future treatment in breast cancer, but also as a potential prognostic marker for tumour progression. Furthermore, we have begun to show how \( \text{PrP}^C \) may contribute to the transfer of a metastatic phenotype from highly metastatic cells to previously non-metastatic cells. To completely understand the role of \( \text{PrP}^C \) in the acquisition of a drug-resistant and metastatic phenotype, additional research is required. Experimentation would be required to determine not only the mechanisms by which \( \text{PrP}^C \) mediates its effects, but
also in which cancer types PrP<sup>C</sup> can influence sensitivity to chemotherapeutic agents, particularly in which cancer types that are drug-resistant.
Chapter 7: Summary and conclusions
Cancer incidence in Australia continues to increase with each passing year, more than doubling since 1982 [174]. While treatments have become more effective over the past decade, many cancers are showing resistance to current treatments. Furthermore, metastasis of cancer is a continuing, potentially fatal, complication of cancer. This highlights the importance of determining the cause(s) of cancer development, cancer progression and chemotherapeutic resistance of cancer as well as finding potential targets for the development of new treatments or increasing the effectiveness of established treatments. While the exact role of PrP$^C$ remains elusive, we do know it has a role in numerous cell functions, including to provide protection from programmed cell death [156, 158]. It is therefore understandable that previous research has already demonstrated that PrP$^C$ may be involved in a number of cancers, including breast, colon, gastric, and pancreatic cancers. The work presented in this thesis has demonstrated how PrP$^C$ expression (at the gene and protein levels), influences the development, progression and drug resistance of different cancer types. Overall, this study has seven main findings.

The first finding is that PrP$^C$ expression level varies, not only between different cancer types, but also within a cancer type, with the PRNP mRNA expression level in cancer cell lines not always correlating with the PrP$^C$ protein level. We found that one cause of these differences is a difference in the stability of the PrP$^C$ protein between the cell lines. For example, HT29 cells had lower PRNP mRNA expression levels than the other colon cancer cell lines, however a higher PrP$^C$ protein level and we showed that this was due to the greater stability of the PrP$^C$ protein in the HT29 cells. While PrP$^C$ may have potential use as a diagnostic marker for certain cancers (such as melanoma, prostate or colon cancers), this finding demonstrates the importance of not only examining the PRNP mRNA expression level in cancers, but the PrP$^C$ protein level as well. Assessment of the PRNP mRNA and PrP$^C$ protein expression levels in patient tissue
samples (comparing normal and cancerous tissue samples) would have been highly advantageous to conduct within this study, and should be considered in future research. Use of only cell lines is a limitation of this study as we were unable to match cell lines derived from non-cancerous tissues with those derived from cancers of the same tissue.

Secondly, we demonstrated that PrP\textsuperscript{C} influences cisplatin-sensitivity in colon cancer cell lines with high PrP\textsuperscript{C} expression. Furthermore, we found that PrP\textsuperscript{C} interferes with FOXO3a activation, thereby conferring cisplatin-resistance in colon cancer cells. We showed that cisplatin-sensitivity can be restored to a highly cisplatin-resistant cell line by depleting PrP\textsuperscript{C} levels, thereby restoring the ability of FOXO3a to be activated and translocate into the nucleus where it can transactivate expression of genes that mediate cisplatin-induced apoptosis. In the future, this mechanism should be examined in additional cancers that are treated using cisplatin or other chemotherapeutic drugs that induce apoptosis via this pathway. Additionally, it should be determined which genes have their expression transactivated by activated FOXO3a following nuclear translocation and whose expression results in the induction of apoptosis in cancer cells. It should further be determined which (if any) proteins cause the high-level expression of PrP\textsuperscript{C} in cisplatin-resistant colon cancer cells, including determining whether inactivation of FOXO3a itself is involved.

The third finding of this study is that high PRNP mRNA expression correlates with poor prognosis in patients with breast carcinomas, particularly metastatic breast carcinomas of the basal subtype. These results indicate that PrP\textsuperscript{C} may be a useful prognostic marker or a potential marker for metastasis of breast carcinomas of the basal subtype. This result was interesting as we previously showed that in colorectal cancer, the cell line with lower PRNP mRNA level showed cisplatin resistance while higher PRNP mRNA expression showed cisplatin-sensitivity indicating lower PRNP mRNA expressing cancers correlate with poor prognosis. However, our finding is consistent with those of
others who showed high PRNP mRNA expression correlated with drug resistance [199], metastasis [13, 206] and poor prognosis [256, 261] in pancreatic, breast, colon and/or gastric cancers. This finding highlights the importance of looking at both the PRNP mRNA expression levels and PrP^C protein expression levels in all cancer types when determining its role in cancer development or progression.

In light of the third finding, the fourth finding of this study is a difference in PrP^C expression at the protein and mRNA levels between a breast derived cell line, and non-metastatic and metastatic breast cancer cells. It was demonstrated that the breast-derived cell line had the lowest expression of both PRNP mRNA and PrP^C protein. Furthermore, we demonstrated that while PRNP mRNA expression level increases with an increase in metastatic potential in a clonal set of breast cancer cell lines (MDA-MB-231), the PrP^C protein level decreases, particularly in the highly metastatic HM LN55 cell line. We found that the reduced PrP^C protein level may be due to an increased rate of PrP^C export into the extracellular medium. We further showed that blockade of protein secretion by treatment with BFA restored high intracellular PrP^C protein levels and reduced secreted, released or shed PrP^C. This mechanism may have the potential to distribute metastatic or drug resistance phenotypes to other cells and therefore warrants additional examination to determine the effect that increased PrP^C export has on non-cancerous cells, non-metastatic cells (as we have done here) or drug-sensitive cells. Additionally as with our first finding, these results showed the importance of looking at both the PRNP mRNA expression levels as well as the PrP^C protein expression levels and revealed that factors other than stability of the PrP^C protein may contribute to the differences observed between PRNP mRNA expression levels and PrP^C protein expression levels. For example, shedding, secretion of intact PrP^C, or release of PrP^C may occur in cancer cells other than metastatic breast cancer cells. While we assessed the stability of the PrP^C protein in colon cancer cells to determine the molecular
mechanisms that underlie the protein expression differences, we did not assess potential differences in shedding, secretion or release rate, which may additionally contribute to the observed difference in PRNP mRNA expression level and PrPC protein expression level.

The fifth finding is that PrPC in breast cancer cells may not be misfolded in the same way as PrPSc in either metastatic or non-metastatic cells. We demonstrated that PrPC present in either cell line was not resistant to digestion by low levels of proteinase K and did not increase in aggregation propensity with increasing metastatic potential of the cells it was expressed in. However, while PrPC may not be misfolded in the same way as PrPSc, these results do not rule out any misfolding of PrPC in these breast cancer cell lines. Additionally, we were limited by our inability to compare to a positive control (such as the use of PrPSc-infected N2a mouse neuroblastoma cell line) due to Australian and Griffith University regulations. Further research is required to determine the folded structure of the PrPC protein in cancer cells and to establish whether any differences exist in PrPC folding between the non-metastatic cancerous and metastatic cancerous breast–derived cell lines.

The following finding is that PrPC influences the metastatic potential of breast cancer cells, enhancing both proliferation and migration of cells. While we did not show a decrease in metastatic potential of breast cancer tumours in vivo following siRNA-mediated knock-down of PrPC, we did demonstrate that PrPC is essential for conditioned media of metastatic cells to confer a metastatic phenotype on previously non-metastatic cells. The use of PRNP-targeted siRNA is a limitation of this study as it provided only an incomplete (~90%) and unstable PRNP knock-down with PRNP expression being restored after 5-7 days following transfection. Additional research is required to determine if stably transfected PRNP-knock down cancer cells can still develop
metastases in vivo and if non-metastatic cells conditioned with growth media from metastatic cells acquire a metastatic phenotype that persists *in vivo.*

Lastly, we demonstrated the influence of PrP<sup>C</sup> on the of drug sensitivity, showing that when PrP<sup>C</sup> is depleted using siRNA, breast cancer cells (both metastatic and non-metastatic) become more sensitive to treatment with doxorubicin. This result was consistent with our second finding of restored sensitivity to cisplatin in the colorectal cancer cell line HT29. Further research is required to determine if PrP<sup>C</sup> expression level correlates with resistance to other chemotherapeutic drugs and the mechanisms by which PrP<sup>C</sup> expression results in drug resistance, e.g. whether it involves the FOXO3a pathway demonstrated here or other pathways shown to be affected by PrP<sup>C</sup> in previous studies such as the PI3K/Akt pathway, Bax/Bcl pathways, MEK/ERK pathway or TRAIL-mediated apoptosis.

In summary, this study has demonstrated the potential involvement of PrP<sup>C</sup> in cancer development and breast cancer metastasis and chemotherapeutic resistance in both colon and breast carcinomas. Furthermore, we have demonstrated the requirement for further research in the future, to not only establish the mechanisms that underlie PrP<sup>C</sup> involvement in cancer development and metastasis, but also to assess the potential of PrP<sup>C</sup> as a therapeutic target for inhibition to sensitize drug-resistant tumours to therapy. We additionally showed that PrP<sup>C</sup> may be a potential prognostic marker for breast carcinomas and a diagnostic marker for breast cancer metastasis.
Chapter 8: References


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Supplementary figure 4.1 PRNP mRNA expression of control samples. Brain pool cDNA sample was subjected to quantitative polymerase chain reaction to demonstrate high level of PrP expression, in comparison to non-cancerous control 293T. * Indicates statistically significant difference. Error bars indicate standard error of the mean of three independent experiments.

Supplementary figure 4.2 PrP<sup>C</sup> expression in siRNA transfected colon cancer cells. Prior to treatment to determine effect on cell’s sensitivity to cisplatin, an aliquot of PRNP and Scr siRNA transfected cells were subjected to whole cell lysis and subsequent SDS-PAGE and western blot analysis to confirm depletion of PrP<sup>C</sup> expression.

Supplementary figure 5.1 PrP<sup>C</sup> expression in mouse metastatic breast cancer series. Representational image of western blot analysis of mouse breast cancer metastatic series with mouse brain lysate as positive control.