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Proteolytic processing of the cellular prion protein (PrP\textsuperscript{C}) in canine cancer cells and cancer stem cells in response to DNA damage

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Declaration

I declare that the work presented in this thesis was performed by myself, unless otherwise specified.

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Abstract

Cancer is a group of diseases associated with abnormal cell growth, space occupation and metastasis. Tumours consist of a heterogeneous mix of cells including a small population of cells, called cancer stem cells (CSCs) that are able to self-renew and differentiate into all cell types of the tumour, and are proposed to drive tumourigenesis. Traditional chemotherapy kills most of differentiated cancer cells, but CSCs are inherently resistant and are able to reinitiate tumour growth. Accumulating evidence indicates that targeting both cancer cells and CSCs is required to elicit a cure. Therefore, it is a necessity to understand the biology of all the cells within a tumour. Cellular prion protein (PrP\textsuperscript{C}) is an extracellular membrane glycoprotein that is mainly known for its association with transmissible spongiform encephalopathies. Several types of human cancer express high levels of PrP\textsuperscript{C} including: breast, gastric, glioblastoma and colorectal. Notably, PrP\textsuperscript{C} has roles in self-renewal, anti-apoptosis and differentiation. However, the role and proteolytic processing of PrP\textsuperscript{C} in canine cancer cells and CSCs remain unclear.

In this project, we aimed to investigate the expression levels and relative amounts of different isoforms of PrP\textsuperscript{C} in a panel of different canine cancer cell lines; to study the proteolytic processing of PrP\textsuperscript{C} in response to doxorubicin, a commonly used chemotherapy; and to develop and optimise an assay to determine the relative amount of secreted PrP\textsuperscript{C} in cell culture medium.

We have found that PrP\textsuperscript{C} is detected in several canine cancer cell lines by western blot and that different cancer cell lines have different \textit{PRNP} expression patterns. In response to DNA damage by doxorubicin, both a canine mammary inflammatory carcinoma (Lily) and a canine glioma (J3T) cell line, showed increases at the protein and the mRNA level of PrP\textsuperscript{C}. PrP\textsuperscript{C} is subject to proteolytic processes, including: α-cleavage, β-cleavage and shedding, which produce C1/N1, C2/N2 and full-length isoforms respectively. We have demonstrated that the ratio of full-length PrP\textsuperscript{C} to C1 increases after DNA damage treatment. Conversely, the ratio of full-length PrP\textsuperscript{C} to C1 decreased in CSCs. We also identified that different isoforms are degraded independently: the half-life of C1 becomes longer under the stress of chemotherapy, whereas, the half-life of full-length becomes shorter. Together these data indicate
that the different isoforms may have independent biological significance in cancer cells. PrP\(^C\) is also cleaved after the GPI anchor and secreted into the cell culture medium. To investigate the secreted isoforms of PrP\(^C\) we developed and optimised an immunoprecipitation protocol.

In conclusion, PrP\(^C\) and its isoforms are detectable in different canine cancer cells and is expressed at different levels. The role of PrP\(^C\) in cancer cells and CSCs may differ when stressed under chemotherapy, and we present evidence that C1 may act distinctively from full-length PrP\(^C\) in response to chemotherapy.

Future studies are needed to investigate the role of different isoforms of PrP\(^C\) in canine cancer cells and CSCs in specific pathways relating to cell survival, apoptosis and metastatic potential, and to evaluate PrP\(^C\) and its isoforms as a therapeutic target in oncotherapy.
Cancer is a disease of abnormally growing cells, which can occupy the surrounding tissues and organs, eventually spreading to other parts of the body. Tumours consist of a mix of different types of cells including a small population of cells, called cancer stem cells (CSCs) that are able to drive tumour growth. A cure for cancer remains elusive. Traditional chemotherapy or radiation therapy kill most of the cancer cells, but CSCs are resistant to these therapies, and are responsible for relapse after chemotherapy and radiation therapy. CSCs can be compared to the root of a plant, and may even give rise to a more aggressive tumour. Targeting CSCs is a prospective way to cure cancer. It is a necessity to discover a therapeutic target to kill all cancer cells including CSCs.

A protein named cellular prion protein (PrPC) is found in brain of most mammals. The biological function of PrPC remains largely unknown. Recently the attention of researchers was raised by the higher amount of PrPC found in human cancer, where it was not expected to appear. It has been proven that PrPC also has a role in renewal and survival of CSCs. These findings suggest that PrPC could be an important factor that enables cancer cells to grow. With advances in veterinary medicine there are increasing numbers of pet dogs suffering from cancer. Many types of cancer in dogs are very similar to humans, which affect health, condition and survival. We believe that tumours, which arise naturally in companion animals, could be a good model for the human disease. Here we have shown that PrPC can be detected in several types of canine cancer cell lines, at different levels. PrPC exists on the cell surface of normal cells, and the full-length form of PrPC can be chopped into smaller fragments called isoforms.

In this project, we are aimed at investigating the changes of these different isoforms of PrPC in response to chemotherapy and to investigate the details of PrPC released from the cell surface after treating with chemotherapy drugs.

We selected two cell lines, a glioma and an inflammatory mammary carcinoma to investigate the amount of various protein isoforms of PrPC that appear in these cells after treatment with a widely used chemotherapeutic drug. We found that each isoform has a different response to the drug. We also observed that it takes different lengths of time to degrade different PrPC isoforms. We speculate that this biological
response may influence the CSCs that are important for the tumour survival. To investigate the details of these processes we developed a new method to detect PrP\textsuperscript{C} that has been released from the cell surface.

In conclusion, we demonstrated that chemotherapy alters the amount of PrP\textsuperscript{C} isoforms in canine cancer cells. Furthermore, we have shown that each isoform is processed by the cell differently in response to treatment with a chemotherapy drug, and this alters the relative ratio of the amount of each isoform in the cell. Further research is required to determine if this biologically relevant and if each isoform has a different role in response to the anti-tumour drug.

Further investigation is also required to determine if the activity of PrP\textsuperscript{C} in canine cancers correlates to tumour drug-resistance and therefore tumour survival. Targeting PrP\textsuperscript{C} or any functional isoforms of PrP\textsuperscript{C} in canine cancer cells or CSCs could potentially inhibit tumour growth and be of therapeutic benefit to the patient.
# Table of content

Declaration ................................................................................................................. i  
Acknowledgement ....................................................................................................... ii  
Abstract ......................................................................................................................... iii  
Lay summary ................................................................................................................... v  
1 Chapter 1 Introduction ................................................................................................. 3  
  1.1 Cancer .................................................................................................................... 3  
  1.1.1 Hallmarks of cancer .......................................................................................... 3  
  1.1.2 Cancer stem cells ............................................................................................. 4  
  1.1.3 Canine cancer model: a comparative model to study human cancer. ................. 5  
  1.2 The cellular prion protein (PrP^C) in human cancer cells ....................................... 6  
  1.2.1 Breast cancer ................................................................................................... 7  
  1.2.2 Colorectal cancer .......................................................................................... 8  
  1.2.3 Gastric cancer ................................................................................................. 9  
  1.3 Depletion of PrP^C reveals its roles in cancer stem cells ........................................ 9  
  1.4 PrP^C .................................................................................................................. 10  
  1.4.1 Structure of PrP^C ............................................................................................ 10  
  1.4.2 Endocytosis of PrP^C ..................................................................................... 11  
  1.4.3 Proteolytic processing of PrP^C ....................................................................... 12  
  1.4.4 Interaction with PrP^C .................................................................................... 14  
  1.5 The role of PrP^C in normal cells ......................................................................... 15  
  1.5.1 Zebrafish embryos ......................................................................................... 16  
  1.5.2 Nervous system .............................................................................................. 16  
  1.5.3 Stem cells ....................................................................................................... 17  
  1.5.4 Immune system .............................................................................................. 18  
  1.5.5 Other tissues .................................................................................................. 18  
  1.5.6 Diverse roles of PrP^C: signal transduction and cell adhesion ......................... 19  
  1.6 Prion Diseases ..................................................................................................... 20  
  1.7 Previous findings of PrP^C in canine cancer cells ................................................. 20  
  1.8 Aims .................................................................................................................... 21  
Reference ...................................................................................................................... 21  
2 Chapter 2 Materials and Methods.............................................................................. 27  
  2.1 Animal cells and tissues ....................................................................................... 27  
  2.2 Cell culture .......................................................................................................... 27  
  2.2.1 Adherent cells ............................................................................................... 27  
  2.2.2 Sphere cells ................................................................................................... 28  
  2.2.3 Harvesting cells ............................................................................................. 28  
  2.3 Drug treatment .................................................................................................... 29  
  2.4 Half-life experiment ............................................................................................ 29  
  2.5 Protein assays ...................................................................................................... 29  
  2.5.1 Cell lysis ....................................................................................................... 29  
  2.5.2 Bradford assay ............................................................................................. 29  
  2.5.3 PNGase Digestion ......................................................................................... 30  
  2.5.4 Protein precipitation ...................................................................................... 30  
  2.5.5 Western blot analysis .................................................................................... 30
1 Chapter 1 Introduction

1.1 Cancer

1.1.1 Hallmarks of cancer

Cancer is a group of diseases characterised by abnormal cell growth, and metastasis. Fundamentally, cancer cells are derived from normal cells that progressively accumulate mutations leading to tumourigenesis. This process may take many years. Over the decades, the complexity of cancer has been depicted at physical, molecular, cellular and genetic level. However, due to the diversity of different types of cancer a rationalized and logical approach is required to understand the fundamentals of this disease. Six ‘hallmarks of cancer’ have been defined by Hanahan and Weinberg, including: sustaining proliferative signaling, enabling replicative immortality, evading growth suppressor, resisting cell death, activating invasion and metastasis and inducing angiogenesis (Hanahan and Weinberg 2011) (Fig. 1.1).

Fig. 1.1 Six hallmarks of cancer. (A). Tumour is capable of hijacking proliferative signals from normal tissues and controlling the release of these signals by producing growth factor, stimulating normal cell to supply cancer cells with growth factor and activating ligand-mediated receptors. (B). Telomeres is an enzyme shorten the proliferation of non-immortalized cells and also exists mostly in immortalized cells include human cancer cells. Telomere shortening limits proliferation of normal cells. Cancer cells are capable of activating telomerase there by inhibit telomere shortening, which leads to unlimited replication. (C). There are two key tumour suppressors of proliferation—encode to RB and TP53. Cancer cells avoid the function of the RB pathway, which serves as an inhibitor of cell proliferation. TP53 hinder intracellular progression signal when growth elements are below the
normal level. (D). Tumour cells develop two main strategies to bypass the programming of cell death. In most case, they cause the loss of TP53 tumour suppressor to reduce the apoptotic effect. On the other hand, tumour cells up-regulate the antiapoptotic proteins (Bcl-2 and Bcl-x<sub>L</sub>) and down-regulate the pro-apoptotic proteins (Bax and Bak). (E). Tumour malignancy is developed by pathological invasion and metastasis, the cancer cells attach to the other cells and the extracellular matrix (ECM). Absence of E-cadherin, a crucial cell adhesion molecule, is essential in this progress. The carcinoma cells potentially down-regulate the expression of E-cadherin thereby stimulate invasion and metastasis. (F). Formation of new vessels (angiogenesis) is essential for the growth of tumour to supply nutrients and oxygen. Angiogenesis widely presents in human and animal models from early stage, which induced by the vascular endothelial growth factor-A (VEGF-A), extracellular matrix-degrading protease (e.g. MMP-9) and fibroblast growth factor (FGF), and inhibited by thrombospondin-1 (TSP-1). In addition, malignant cancer cells spread throughout the body via blood stream (Hanahan and Weinberg 2011).

1.1.2 Cancer stem cells

There are two models for cancer origin and metastasis: the clonal evolution model and the hierarchical model. The clonal evolution model states that any cell in the body has the potential for malignant transformation, and all cells in the resulting tumour have a similar regenerative potential following treatment. By contrast, the hierarchical model proposes that tumours are composed of a heterogeneous mix of cells that are specialised by the process of differentiation, and are sustained by a small subpopulation of cells called cancer stem cells (CSCs), that are able to drive tumourigenesis. CSCs share similar characteristics with normal stem cells, including self-renewal, differentiation and homeostatic control (Dalerba, Cho et al. 2007). CSCs are inherently resistant to traditional chemotherapy and ionising radiation and are responsible for tumour relapse after oncotherapy. Oncotherapy, such as chemo- or radiation therapy, often kills most non-tumourigenic cancer cell progeny except CSCs.

Tumour recurrence is correlated with an increase of circulating tumour cells (CTCs) in mesenchymal tumours compared with that of epithelial origin, which is significantly related to epithelial and mesenchymal transition (EMT) (Chang 2016). EMT is a process that epithelial cells lose their cell-cell junctions and apico–basolateral polarity, result in generating migratory and invasive mesenchymal stem cells, gaining scaffolding or anchoring functions and having multifunctional roles, which has an essential role in cancer metastasis, EMT is
involved in acquisition of stemness by reducing the cell-cell adhesion ability of epithelial cells and enhancing mesenchymal characteristics of cells. Induction of EMT in vitro leads to an increase in the percentage of CSCs (Mani, Guo et al. 2008). Many conventional therapies are aimed at eliminating non-cancer cells but preserve proliferative and differentiating qualities of the tumour by failing to kill the CSC population.

Inhibiting stemness is a potential way to reduce the exposure of CSCs by inhibiting or eradicating their ability for tumorigenesis (Fig. 1.2).

**Fig. 1.2 Conventional therapy vs. therapy of targeting cancer stem cells.** Conventional therapies are aimed at targeting cancer cells and adjacent cells by DNA damage or inhibiting proliferation. In this progression, presence of a portion of cancer stem cells have potential role in tumour relapse. However, eliminating stemness by targeting CSCs is a potential treatment for inhibiting tumour growth and curing cancer.

### 1.1.3 Canine cancer model: a comparative model to study human cancer.

Companion animals share many characteristics with humans, which is of benefits for studying cancer *in vitro* and *in vivo*. The canine genome has been sequenced and it has been shown that the canine genome sequence exhibits closer
similarities to the human genome than the rodent genome (Pinho, Carvalho et al. 2012). A great deal of our basic knowledge of cancer comes from rodent models of human cancers, unfortunately pre-clinical drug trials utilizing these models often fail to translate into the clinic as these tumours are genetically and pathologically different from human cancer. In contrast, spontaneous cancers that naturally arise in dogs are very similar to human cancers, including histological appearance, tumour genetics, molecular targets, biological behaviour and response to conventional therapies (Paoloni and Khanna 2008). Recently, canine mammary carcinoma has been utilized as a model for human breast cancer to seek out novel therapeutic targets (Queiroga, Raposo et al. 2011). The epidemiological studies of these two species have found that the spontaneous canine breast cancer shares a comparable age group with human. Both benign and malignant breast tumours in canines are morphologically and histologically similar to human breast cancer (Strandberg and Goodman 1974). In addition, a significant overlap in ten thousand deregulated orthologous genes between canine and human mammary tumour samples was observed (Uva, Aurisicchio et al. 2009). Comparative analysis between two species has revealed the homology of many cancer-related pathways from human breast cancer and canine mammary carcinoma, including: the phosphatidylinositol 3-kinase (PI3K)/AKT pathway, KRAS, phosphatase and tensin homolog (PTEN), Wnt-β-catenin, and mitogen-activated protein kinase (MAPK) cascade (Pinho, Carvalho et al. 2012). Research in the field of cancer genome identification, and the study of epidemiology and pathogenesis has led to greater understanding of tumour biology and progression as well as the development and evaluation of novel oncotherapies.

1.2 The cellular prion protein (PrP<sup>C</sup>) in human cancer cells

The field of cancer research has been recently focused on the development of cancer, and drug resistance. The development of cancer includes three main steps: initiation, promotion and progress (Farber 1984). Initiation of cancer has been analysed at many aspects: the metabolism of original cells for initialising; the analysis of genetic changes in original cells; the molecules involved in cancer cell
initiation; and the essential pathways in initial cells proliferation. Promotion of cancer cells is related to cell proliferation that requires multiple promoting stimuli and is influenced by environment. In the progression process, invasion and metastasis are two main steps for tumourigenesis. The development of cancer is a stepwise process that involves many molecular pathways including those for cell proliferation, avoidance of cell death, tumour invasion and metastasis. Sub-populations of cancer cells are also drug resistance. To investigate the drug resistance within a tumour, complicated mechanisms are considered, including: alterations in genetics, epigenetics and involved cellular molecules. Many studies have shown that drug resistance is due to involvement of essential molecules that including antibodies, cytokines and growth factors (Teicher 2007). Discovering an essential molecule related to cancer development and drug resistance would offer a potential prognostic marker and therapeutic target. Unexpectedly, the cellular prion protein (PrPC) is found overexpressed in several types of human cancer including breast, colorectal and gastric cancer (Diarra-Mehrpour, Arrabal et al. 2004) (Antonacopoulou, Grivas et al. 2008) (Liang, Pan et al. 2007). PrPC is a glycoprotein, encoded by PRNP gene that is found in nervous system. PrPC is an essential molecule in the conversion process to pathogenic isoform—the scrapie prion protein (PrPSc) in transmissible spongiform encephalopathies (TSEs also known as prion diseases). Several studies have shown putative roles of PrPC in cancers, including: signal transduction, differentiation, anti-apoptosis, proliferation and tumourigenesis (Yang, Zhang et al. 2014). These reports suggest that studying PrPC can potentially provide novel insights into mechanisms of carcinogenesis and drug resistance.

1.2.1 Breast cancer

Breast cancer is a type of cancer with a high rate of incidence and recurrence, which has always been taken as a model for metastatic cancer research. PrPC is found in breast cancer and has been widely investigated for its roles in cell-death resistance, chemotherapy resistance, invasion and metastasis.

The endoplasmic reticulum (ER) is the cellular site for synthesis, modification, and trafficking of secretory and cell-surface proteins. ER stressed-induced
apoptosis has been suggested in the development of human breast cancer (Dery, Jodoin et al. 2013). Elevated PRNP gene expression has been detected in human breast cancer and may protect cancer cells from ER stress-mediated apoptosis. (Dery, Jodoin et al. 2013). By triggering the releasing cytochrome c from mitochondria and nuclear condensation, overexpressed PrP<sup>C</sup> contributed to resistance to tumour necrosis factor (TNF)-α-induced cell-death in a human breast cancer cell line (MCF-7) (Diarra-Mehrpour, Arrabal et al. 2004). On the other hand, PrP<sup>C</sup> prevents Bax-mediated apoptosis by inhibiting Bax-proapoptotic conformation in human neurons and MCF-7 cells, but PrP<sup>C</sup> is not involved in caspase-mediated cell death (Roucou, Giannopoulos et al. 2005).

In breast cancer, P-glycoprotein (P-gp) is a crucial mediator for drug resistance and P-gp expression has been shown to increase after exposure to anti-tumour drugs (REF). Multidrug resistance (MDR) is a significant factor of chemotherapeutic failure in breast cancer. The interaction between PrP<sup>C</sup> and P-gp promotes paclitaxel-induced invasion and plays a role in anti-apoptosis in MDR breast cancer cells (Li, Cao et al. 2009). Furthermore, both CD44 and PrP<sup>C</sup> have been proven to be overexpressed in MDR breast cancer cells. CD44 is a cellular protein exerts many functions in tumour progression especially cell proliferation, cell adhesion, self-renewal, invasion and metastasis (Basakran 2015). The depletion of PrP<sup>C</sup> or CD44 inhibits invasion, metastasis, proliferation and drug-resistance in MDR breast cancer cells. It has been confirmed that PrP<sup>C</sup> physically and functionally interacts with CD44, enhancing cell invasion by regulating EGFR, CD147, MMP2, and MMP9 in MDR cells (Cheng, Tao et al. 2014). In conclusion, PrP<sup>C</sup> is involved in anti-apoptotic activity, invasiveness and multidrug resistance in breast cancer cells.

**1.2.2 Colorectal cancer**

The expression of PRNP is higher in colorectal cancer (CRC) than normal colon tissue (Antonacopoulou, Grivas et al. 2008). PrP<sup>C</sup> has been shown to modulate the expression of special AT-rich sequence-binding protein-1 (SATB1) via the Fyn-SP1 pathway (a cell growth related pathway). SATB1 was downregulated significantly in PrP<sup>C</sup>-depleted cells (Wang, Qian et al. 2012). PrP<sup>C</sup> binding to heat
shock organizing protein (HOP) drives metastasis and invasion of colorectal cancer cells and is associated with induction of EMT (de Lacerda, Costa-Silva et al. 2016). Moreover, PrP antibody treatment induced apoptosis by a measured reduction of Bcl-2 expression in a nude mouse bearing human colon cancer cell line (HCT 116) xenografts, (McEwan, Windsor et al. 2009). In conclusion, PrP<sup>C</sup> expression correlates to metastasis, invasion and anti-apoptosis in CRC.

### 1.2.3 Gastric cancer

PrP<sup>C</sup> is highly expressed in gastric cancer cells and as an important molecule in cancer development and drug resistance. PrP<sup>C</sup> reduces apoptosis in gastric cancer by inhibiting reactive oxygen species (ROS) and up-regulating Bcl-2 (an anti-apoptosis marker) (Mehrpour and Codogno 2010). Moreover, overexpressed PrP<sup>C</sup> promotes proliferation with PI3K/Akt pathway, and may also induce transactivation of Cyclin D1 and facilitate the G1/S transition in the cancer cell cycle in several human gastric cancer cell lines. (Liang, Pan et al. 2007). PrP<sup>C</sup> facilitates invasion and metastasis (in vivo) by MEK/ERK pathway activation and MMP11 trans-activation in which the PrP N-terminus was proposed to be of pivotal importance (Pan, Zhao et al. 2006). PrP<sup>C</sup> is also associated with multidrug resistance. PrP<sup>C</sup> could down-regulate adriamycin-induced apoptosis and may alter the expression of Bcl-2 and Bax in the gastric carcinoma cell line SGC7901 (Du, Pan et al. 2005). These observations show that diverse functions of PrP<sup>C</sup> are of great significance in tumourgenesis as well as drug-resistance.

### 1.3 Depletion of PrP<sup>C</sup> reveals its roles in cancer stem cells

Transplantation and linage tracing assays have demonstrated heterogeneous populations of cancer cells and CSCs in multiple cancers. CSCs can generate cancer cells, drive tumourigenesis and mediate metastasis. The downregulation of PrP<sup>C</sup> suggests that it is a potential mediator of CSC biology and may be involved in self-renewal, anti-apoptosis, differentiation and drug-resistance.

CD44 is a membrane receptor protein associated with CSC properties, including modulating cell adhesion, motility and metastasis. In orthotropic transplantation models of CRC CSCs, PrP<sup>C</sup><sup>+</sup> was shown to be associated with CD44 in colorectal
CSCs and it promoted epithelial-mesenchymal transition (EMT) of CD44^PrP^C+ cells via the ERK2 (MAPK1) pathway, thereby, enhancing metastasis (Du, Rao et al. 2013). Monoclonal antibodies against PrP^C significantly inhibited the invasion and metastasis of CRC (Du, Rao et al. 2013). In conclusion, the PrP^C-CD44 interaction or PrP^C regulates tumourigenesis of CSCs and may be a potential target for metastatic CRC oncotherapy.

In glioblastoma, an association between the expression of PrP^C and proliferation of CSCs has been found; it was shown that PrP^C down-regulation in glioblastoma cells significantly inhibited expression of the self-renewal markers NANOG and Sox2 thereby reducing sphere-forming activity, a CSC characteristic (Corsaro, Bajetto et al. 2016). Additionally, PrP^C down-regulation reduced the expression of GFAP causing loss of differentiation activity in GEM CSCs (Corsaro, Bajetto et al. 2016). Taken together, PrP^C displays a significant role in proliferation and differentiation of GEM CSCs.

The evidence presented indicates that PrP^C may be involved in multiple aspect of cancer and CSC biology. Further research is required to substantiate these findings and determine the validity of PrP^C and its associated ligands as potential therapeutic targets.

1.4 PrP^C

1.4.1 Structure of PrP^C

PrP^C is encoded by the PRNP gene and is a glycoprotein of 208 amino acids with an apparent molecular weight of approximately 35Kd. The domain structure of human PrP^c is shown in figure 1.3. and contains 42% α-helix and 3% β-sheet (Huang 2015). The C-terminal globular domain of PrP^C is modified with two N-glycan chains and contains a disulfide bridge (Altmeppen, Puig et al. 2012). PrP^C is attached to the cell plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. The N-terminal domain of PrP^C is unstructured, containing a glycine-rich, octapeptide repeat region which binds copper ions. Cu^{2+} binding may be an indispensable function promoting proliferation and G1/S transition of human gastric cancer cell (Liang, Pan et al. 2007). (Fig. 1.3)
Fig. 1.3 Structure of human PrP<sup>C</sup>. PrP<sup>C</sup>, consists of a signal peptide (1-22), five octapeptide repeats (51-91), a highly-conserved hydrophobic domain (106-126), three α-helices (residues 143-152, 171-191 and 199-221), two antiparallel β-strands (residues 127-129 and 166-168), a disulfide bond (between residues 179 & 214), two N-linked glycosylation sites (residue 180 & 196) and a signal sequence for a glycosylphosphatidylinositol (GPI) anchor (231-253) (Altmeppen, Puig et al. 2012).

PrP<sup>C</sup> is post-translationally modified at three sites before cleavage: a disulfide bond, two N-linked glycosylation sites and a GPI anchor. Naturally occurring un-, mono- and di-glycosylated PrP<sup>C</sup> forms can be isolated from cells or tissues and visualized by western blotting. GPI-anchored proteins are widely found in eukaryotic cell membranes, they follow particular regulation for sorting and trafficking, in the secretory and endocytic pathways (Fujita and Kinoshita 2012). Emerging evidence has shown that PrP<sup>C</sup> possesses many similarities as the other GPI-anchored proteins.

1.4.2 Endocytosis of PrP<sup>C</sup>

Endocytosis and intracellular trafficking of PrP<sup>C</sup> are important in the generation of prion diseases, and also may affect PrP<sup>C</sup> function (Prado, Alves-Silva et al. 2004). Like other membrane proteins, PrP<sup>C</sup> is synthesized in the rough endoplasmic reticulum (ER). After passing through the Golgi network it attaches to the extracellular membrane (Harris 1999). It has been proposed that PrP<sup>C</sup> detaches from the rafts and moves into non-raft regions after binding with Cu<sup>2+</sup> in the octapeptide repeat region. Following this, the N-terminal region binds to a transmembrane adaptor, inducing endocytosis of PrP<sup>C</sup> through clathrin-coated pits (Taylor, Watt et al. 2005). Clathrin-coated pits have shown to be important for PrP<sup>C</sup> internalization (Sunyach, Jen et al. 2003). Classical endocytic organelles also participate in PrP<sup>C</sup> trafficking (Prado, Alves-Silva et al. 2004). PrP<sup>C</sup> is subsequently internalized into endosomes. Internalized PrP<sup>C</sup> is then subjected to proteolytic degradation or re-cycled. There are interactions between
PrP\textsuperscript{C} and secreted exosomes, which can be released from the membrane into the extracellular environment (Harris 1999). These exosomes may be used by cells to secrete molecules into targeted multivesicular bodies (MVBs) which are a subset of endosomes (Shyu, Kao et al. 2000). Low-density lipoprotein receptor-related protein 1 (LRP1), which is a cell-surface endocytic receptor with many ligand binding sites, is proven to be involved in these processes. LRP1 was shown to be required to control biosynthetic and endocytic trafficking of PrP\textsuperscript{C} via clathrin-coated pits in neuron cells (Taylor and Hooper 2007).

1.4.3 Proteolytic processing of PrP\textsuperscript{C}

PrP\textsuperscript{C} is subject to proteolytic processing, which has been suggested to be of importance for the multiple functions of PrP\textsuperscript{C}. There are three main cleavage events: \(\alpha\)-cleavage, \(\beta\)-cleavage and ectodomain shedding (Fig. 1.4&1.5). \(\alpha\)-cleavage, around codon 111, destroys the hydrophobic region (residue 105-120), giving rise to the N1 fragment of 11kDa and the C1 fragment of 15kDa. \(\beta\)-cleavage in the octapeptide repeat region results in the N2 (9kDa) and the C2 (17kDa) fragment. Shedding releases almost full-length PrP\textsuperscript{C} leaving a few amino acids with GPI-anchor on the membrane.

Fig. 1.4 Western blot analysis from cat brain homogenate. Two N-linked glycosylated sites were removed by treating with PNGase F. Cat brain homogenate was analysed by western blot, and immunoblotting with primary antibody BC6 and secondary antibody IRDye® goat anti-mouse IgG (H+L). Proteolytic products, full-length PrP, C1 and C2, are simultaneously present in cat brain.
Fig. 1.5 Proteolytic processing of PrP\textsuperscript{C}. α-cleavage destroys the hydrophobic domain, giving rise to N1 fragment and C1 fragment. β-cleavage separates the N2 and C2 fragments at the octapeptide repeat region. PrP\textsuperscript{C} shedding releases almost full-length PrP with a C-terminal GPI-anchor on the membrane remaining (Altmeppen, Puig et al. 2012).

### 1.4.3.1 α-cleavage

The main cleavage event in PrP\textsuperscript{C} is α-cleavage. C1 is produced in substantial amounts in extracellular medium, approximately 5-50% of total PrP, depending on the cell type (Altmeppen, Puig et al. 2012). Some proteases have been shown to be involved in α-cleavage, including the A-disintegrin and metalloproteinase 10 (ADAM 10) and ADAM 17. Inhibiting ADAM 10 significantly reduced α-cleavage upon stimulation of ADAM 17 (Vincent, Paitel et al. 2001). The α-cleavage site lies in a region which is suggested to be the neurotoxic domain, an essential structure for the conversion of PrP\textsuperscript{C} to its pathogenic counterpart scrapie prion protein (PrP\textsuperscript{Sc}). Thus, α-cleavage destroys the neurotoxic domain and thereby protecting PrP\textsuperscript{C} from propagation in prion diseases (Altmeppen, Prox et al. 2013). Surprisingly, α-cleavage is associate with myelinitrophic function in chronic demyelinating polyneuropathy (CDP). The C1 fragment was found abundantly in PrP\textsuperscript{C} mutant mice compared to PrP-knockout mice. Cleavage of axonal PrP\textsuperscript{C} exhibited a signal transduction function involving surrounding schwann cells (Bremer, Baumann et al. 2010). Moreover, N1 is an important trigger for neuroprotection and a regulator in anti-apoptosis by modulating the p53 pathway in an ischemia model of rat retina (Guillot-Sestier, Sunyach et al. 2009).

### 1.4.3.2 β-cleavage

β-cleavage is a less prominent cleavage under physiological conditions. However, the C2 fragment was found to be the main truncated product of PrP in
prion infected diseases revealing a pathogenic relevance (Dron, Moudjou et al. 2010). It has also been reported that PrP\textsuperscript{C} protects cells from oxidative stress. A dramatic increase of the C2 fragment was detected under stress of reactive oxygen species. This is particularly important as oxidative stress contributes to the pathogenesis of prion diseases (Watt, Taylor et al. 2005).

### 1.4.3.3 Ectodomain shedding of PrP\textsuperscript{C}

GPI-anchored proteins can actively shed from the membrane of one cell by phospholipases and attach to other cells by insertion of their lipid anchors into the cell membrane. Studies have shown that two types of GPI-specific phospholipases, GPI-phospholipase C (GPI-PLC) and GPI-phospholipase D (GPI-PLD) are involved in the cleavage of GPI-anchors (Low 2000). Like the other GPI-anchored proteins, the release of PrP\textsuperscript{C} is mediated by phospholipases and metalloprotease. However, PrP\textsuperscript{C} is less sensitive to phospholipase cleavage compared to another GPI-anchored protein-- decay-accelerating factor (DAF) on the same cell type, and has a much shorter half-life on the cell surface (Li, Liu et al. 2003). The reason that PrPC is less sensitive than DAP is not clear. The C-terminal peptide of the protein functions at GPI attachment as a signal. The presence of the anchor itself appears to confer some important functional and behavioral attributes on proteins to which it is attached. The hypothesis that the GPI anchor may result in the release of the protein from the cellular membrane as a signal by specific endogenous enzymes has been postulated after the existence of GPI-anchors is now widely accepted (Lauc and Heffer-Lauc 2006).

### 1.4.4 Interaction with PrP\textsuperscript{C}

#### 1.4.4.1 Protein ligands

It is widely assumed that proteolytic modification and glycosylation of PrP\textsuperscript{C} will affect the interaction between PrP\textsuperscript{C} and protein molecules. As PrP\textsuperscript{C} is a GPI-anchored protein predominantly existing on the extracellular membrane, it exerts its functions by interacting with other transmembrane molecules, thereby enabling signal transduction. It has been shown that PrP\textsuperscript{C} interacts with macromolecules at the cell membrane in the secretory pathway, including membrane proteins (37-kDa laminin receptor, laminin and caveolin-1),
cytoplasmic proteins (stress-inducible protein 1, Bcl-1 and Fyn kinase, etc.), synaptic vesicles (Mehrpour and Codogno 2010), and the nuclear protein CBP70 (Rybner, Finel-Szermanski et al. 2002). Each PrP<sup>C</sup> interaction is likely to have different functional properties, such as, internalisation mediation, neuritogenesis, neuroprotection and signal transduction. The 37-kDa laminin receptor, which has been shown to be overexpressed in several cancer cell lines, is a transmembrane protein binding to PrP<sup>C</sup> that mediates internalisation through clathrin coated pits (Hundt, Peyrin et al. 2001, Zuber, Mitteregger et al. 2008). Stress inducible protein 1 (STI1, also known as HOP) is a specific PrP<sup>C</sup> ligand that induces neuritogenesis and neuroprotection in neurons (Zanata, Lopes et al. 2002, Lopes, Hajj et al. 2005). Tyrosine kinase fyn is enriched in brain synaptosomes where its interaction with PrP<sup>C</sup> has been implicated in signal transduction (Mouillet-Richard, Laurendeau et al. 1999). Investigations of both PrP<sup>C</sup> and its ligands provide more profound reading in the mechanisms of PrP<sup>C</sup> or interactions functioning.

**1.4.4.2 Cu<sup>2+</sup>**

The N-terminal domain of PrP<sup>C</sup> contains a multiple octapeptide repeat region that is flexible and able to bind copper ions (Cu<sup>2+</sup>). There is increasing evidence to support a functional role for PrP<sup>C</sup> in copper metabolism and several groups have investigated the physiological meaning of this association (Zomosa-Signoret, Arnaud et al. 2008). After binding with copper, PrP<sup>C</sup> acts as a superoxide dismutase (SOD), thereby protects cells against oxidative stress. Thus, PrP<sup>C</sup> interacts with copper exerts a neuroprotective role in accordance with the copper binding amounts (Lo, Shyu et al. 2007). Overall, PrP<sup>C</sup>-copper interaction exhibits roles of antioxidant and neuroprotection.

**1.5 The role of PrP<sup>C</sup> in normal cells**

As discussed before, PrP<sup>C</sup> has been detected in many cell and tissues. The highest levels of PrP<sup>C</sup> expression are found in the CNS, which includes the hippocampus, hypothalamus and olfactory bulb, which all have rapid cell-renewal. To localise
PrPC, PRNP mRNA expression has been detected in the CNS and peripheral organs, including the heart, lung, and intestine in mouse embryos (Halliez, Passet et al. 2014).

1.5.1 Zebrafish embryos

To identify the role of PrPC in the embryo or initial cell development, zebrafish (Danio rerio) has been widely used as a vertebrate animal model where a large amount of PrPC is found. A list of PrP encoding genes: prp1, prp2 and prp3 were assigned in the zebrafish (Nourizadeh-Lillabadi, Seilo Torgersen et al. 2010). The second paralogue (prp2) was thought to share the most similarities with the mammalian Prnp gene in terms of structure and function. Depletion of prp2 revealed that PrP is involved in biological processes including: anti-apoptosis, neurogenesis and embryonic development (Nourizadeh-Lillabadi, Seilo Torgersen et al. 2010). Down-regulation of PrP affected E-cadherin based cell adhesion and cell movement, disrupting embryonic development in the zebrafish embryo. Moreover, PrPC itself mediated homophilic cell-cell adhesion and induced intracellular signalling via Src-related kinases in zebrafish embryos (Malaga-Trillo and Sempou 2009). In conclusion, PrPC exerts essential functions in the zebrafish embryo that could reflect its biological modulations in vertebrates.

1.5.2 Nervous system

PrPC is ubiquitously expressed in the developing and mature mammalian CNS. However, the role of PrPC in the CNS remains to be elucidated. Recent studies have shown that PrPC induces rapid differentiation and polarization in synapse development and enhances neuritogenesis in incubation of cultured hippocampal neurons with recombinant PrPC (Deleglise, Lassus et al. 2013). In neuritogenesis, PrPC endocytosis is a necessary step to modulate STI1-dependent ERK1/2 signaling (Caetano, Lopes et al. 2008). PrPC acts as an indispensable molecule in neuritogenesis in particular by its contribution to cell adhesion on a matrix rich in laminin (Mehrpour and Codogno 2010).

Bcl-2-associated protein X (Bax) is the main pro-apoptotic protein in neuronal cells. PrPC protects primary human neurons from Bax-induced cell death by preventing the Bax conformational change. Thus in the nervous system, PrPC has been proposed to
have a neuroprotective role as a Bax inhibitor (Roucou, Giannopoulos et al. 2005). PRNP<sup>+</sup> neuronal cells are more susceptible to Bax-induced apoptosis under serum-free conditions (Kuwahara, Takeuchi et al. 1999). PrP<sub>C</sub> is also up-regulated after focal cerebral ischemia (Weise, Crome et al. 2004), and PrP<sub>C</sub> overexpression decreases neuronal loss after ischemic insult. (Shyu, Lin et al. 2005). Moreover, PrP<sub>C</sub> expression is neuron-specific in differentiated neural cells and it increases multipotent neural precursor differentiation in vitro and proliferation in neurogenic regions in vivo (Steele, Warfel et al. 2005). In conclusion, PrP<sub>C</sub> plays an important role in neurogenesis in terms of neuron-renewal and neuroprotection.

1.5.3 Stem cells

PrP<sub>C</sub> may also be involved in stem cell biology. There is accumulating evidence showing that PrP<sub>C</sub> regulates human bone marrow stem cell differentiation. CD34 is a stem cell marker. PrP<sub>C</sub> is found presented very early in human hematopoiesis as it is detected in CD34<sup>+</sup> bone marrow stem cell population by flow cytometry (Dodelet and Cashman 1998). Further, in human premyeloid cell line (HL-60), PrP<sub>C</sub> drives differentiation into macrophage-like cells upon phorbol ester treatment. Instead, HL-60 cells into granulocyte differentiation is reduced by PrP<sub>C</sub> down-regulation (Dodelet and Cashman 1998). In murine bone marrow, approximately 50% of small lymphocytes and progenitor cells express PrP<sub>C</sub>, most of them are CD43<sup>+</sup> progenitor cells (Liu, Li et al. 2001). Recent studies have also shown that PrP<sub>C</sub> is expressed on the surface of long-term repopulating hematopoietic stem cells in adult bone marrow. Thus, PrP<sub>C</sub>-knockout mice are confined to self-renew in hematopoietic stem cells with a dramatically decrease of repopulation after tertiary transplantation (Zhang, Steele et al. 2006). In human embryonic stem (ES) cells, PrP<sub>C</sub> has anti- or pro-proliferation effects, depending on growth conditions for self-renewal or differentiation (Lee and Baskakov 2013). These convincible investigations suggest that PrP<sub>C</sub> exists in stem cells-rich region and exerts its function for stem cell differentiation, self-renewal and proliferation.
1.5.4 Immune system

PrP<sup>C</sup> is expressed abundantly in the immune system. PrP<sup>C</sup> has been detected on human T and B lymphocytes, natural kill (NK) cells, platelets, monocytes, dendritic cells and follicular dendritic cells (Isaacs, Jackson et al. 2006). CD4 and CD8 are T cell surface proteins binding their respective class II and class I major histocompatibility complex (MHC) induce T cell recognition and activation after activating by antigen presenting cell (APC). High expression level of PrP<sup>C</sup> was found in peripheral blood lymphocytes, both CD8<sup>+</sup> and CD4<sup>+</sup> T cells. Furthermore, age-related PrP<sup>C</sup> expression was determined under a comparison of ranged ages, which showed that the elder population expressed significant higher than children (Durig, Giese et al. 2000, Politopoulou, Seebach et al. 2000). Moreover, application of antibody against PrP<sup>C</sup> modulates T cell activation, leads to reorganizations of lipid raft components and enhance processing of signaling proteins. PrP<sup>C</sup> is also upregulated during NK cell differentiation (Zomosa-Signoret, Arnaud et al. 2008). These findings suggest that PrP<sup>C</sup> functionally important in differentiated lymphocytes that play a role in the development of immune system.

1.5.5 Other tissues

Although PrP<sup>C</sup> is found mostly in nervous, immune and regenerative systems, studies have shown that PrP<sup>C</sup> can also be detected in dental tissues, mammary glands and muscles. However, neither PrP<sup>C</sup> nor PrP mRNA have been found in the liver (Horiuchi, Ichikawa et al. 1995).

It is widely known that PrP<sup>C</sup> is capable of binding copper and manganese. Analysis of developing murine teeth from PRNP<sup>−/−</sup> animals showed reduced copper concentration but no differences in the manganese content. This finding suggests that the copper binding capability of PrP<sup>C</sup> may influence the activation of copper binding enzymes in interacting with collagen, which is an essential component of pre-dentin and desmodontal fibers during tooth formation (Schneider, Korkmaz et al. 2007, Zhang, Kim et al. 2011)

PrP<sup>C</sup> has been found in milk and mammary glands of domestic ruminants. It has been shown that all fragments of PrP<sup>C</sup> can be detected in ovine and caprine milk by immunoprecipitation (Franscini, El Gedaily et al. 2006, Maddison, Whitelam
et al. 2007). PrP\textsuperscript{C} is highly distributed at basolateral membranes of mammary gland epithelial cells. Thus, apocrine secretory vesicles are stained with a strong signal. These findings suggest that transmission of PrP\textsuperscript{C} is mainly affected by apocrine secretion in milk fat globules (Didier, Gebert et al. 2008). PrP\textsuperscript{C} is also detected abundantly in skeletal muscles. The expression of PrP\textsuperscript{C} changes in the processes of myogenesis within different muscle fibres. PrP\textsuperscript{C} is involved in regenerating skeletal muscles in adult mice. Analysis of PRNP\textsuperscript{−/−} mice showed slower recovery of locally damaged muscle fibers compared to wild type mice (Stella, Massimino et al. 2010).

1.5.6 Diverse roles of PrP\textsuperscript{C}: signal transduction and cell adhesion

The physiological function of PrP\textsuperscript{C} in cell-cell contacts has been widely investigated recently. PrP\textsuperscript{C} is involved in signal transduction pathways as shown by antibody-mediated cross-linking. PrP\textsuperscript{C} interaction with caveolin-1 to contribute to signal transduction via the tyrosine kinase Fyn pathway (Mouillet-Richard, Ermonval et al. 2000). Tyrosine kinase Fyn pathway is a pro-oncogene that associated with cell signaling in cancer development. It is also reported that PrP\textsuperscript{C} is a functional agent in communications of differentiated epithelial cells while interacting with c-Src, which is also a pro-oncogene which promote cancer progression by enhancing signal transduction (Morel, Fouquet et al. 2008). Moreover, PrP\textsuperscript{C} is related to the activation of Src-related kinases, thereby stimulates homophilic cell-cell adhesion and induces intracellular signaling (Malaga-Trillo and Sempou 2009). Ca\textsuperscript{2+}-independent cell signaling and adhesion are partially mediated by PrP\textsuperscript{C} in the transition of E-cadherin to the plasma membrane in zebrafish, mouse and drosophila cells (Malaga-Trillo and Sempou 2009). PrP\textsuperscript{C} is overexpressed in neuroblastoma cells enhancing cation-independent aggregation. In contrast, incubation of PrP-antibody leads to a contrary effect (Mange, Milhavet et al. 2002). To conclude, PrP\textsuperscript{C} is thought to be as a molecule involved in cell adhesion and signal transduction. These findings may advance our understanding of the roles of PrP\textsuperscript{C} in normal cell and cancer cells.
1.6 Prion Diseases

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a group of neurodegenerative diseases caused by transformation of PrP\textsuperscript{C} to pathogenic isoform PrP\textsuperscript{Sc}, which can be generated endogenously or introduced into the body from the environment. PrP\textsuperscript{Sc} polymers act as infectious agents by propagating the conversion of normal PrP\textsuperscript{C} to abnormal prions. The covalent structure of the two PrP isoforms is identical. However, they can be distinguished by their distinctive physical characteristics. PrP\textsuperscript{C} is soluble in detergents and readily digested in proteases. In contrast, PrP\textsuperscript{Sc} is a protease-resistant protein, which is only soluble in detergent (Peretz, Scott et al. 2001). This conversion results in neuronal degeneration and loss by an unknown mechanism (Cobb and Surewicz 2009).

Scrapie, the first prion disease is identified, has been known in sheep since 1732 throughout the world (Collinge 1999). At first, the transmission of scrapie was thought to be conveyed by a virus. Dr Stanley B. Prusiner coined the word ‘prion,’ derived from the term ‘proteinaceous infectious particle’, in 1982 to describe the scrapie agent as being different from any known virus (Prusiner 1982). The human phenotypes of TSEs have a wide range, including Creutzfeldt-Jakob disease (CJD), kuru, Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Animal TSEs include scrapie of sheep and goat, bovine spongiform encephalopathy and chronic wasting disease of elk and mule deer (Nair and Johnson 2011).

1.7 Previous findings of PrP\textsuperscript{C} in canine cancer cells

PrP\textsuperscript{C} expression in canine cancer cells has not been evaluated. Previous work in our lab (by a summer student Sophia Moore) has shown that PrP\textsuperscript{C} is strongly expressed in several established canine cancer cell lines, with unique glycosylation patterns of PrP\textsuperscript{C} evident in the different cell lines. Of particular note, PrP\textsuperscript{C} isolated from the canine inflammatory mammary carcinoma cell line, Lily, expressed mainly the truncated form of PrP\textsuperscript{C}, C1, and very little of the full-length form; this has not been documented before in either human or canine cells, and offers a unique opportunity to study the function of this isoform.
1.8 Aims

PrP^C is highly conserved in mammals and is associated with cell survival, protection against apoptosis and is required for long-term replicative potential of haemopoietic stem cells. In human cancer cells PrP^C has been shown to be involved in numerous cellular processes including: cell proliferation, antiapoptosis, invasion, metastasis and drug-resistance. However, PrP exists as multiple isoforms due to heterogenous glycosylation and proteolytic cleavage, and the relative expression levels and functions of each isoform is yet to be investigated. In this study, we aimed to investigate the gene and protein expression pattern of PrP^C in different canine cancer cells and corresponding CSCs, and to investigate changes in the relative levels of PrP^C isoforms after treatment with chemotherapeutic agents. To investigate shedding of full-length PrP^C into the extracellular milieu, we developed an immunoprecipitation assay to isolate PrP^C from the tissue culture media.

Reference


Chapter 2 Materials and Methods

2.1 Animal cells and tissues

All canine cancer cells have been stored at -198°C. The cat brain homogenate was taken from our laboratory tissue archive at -80°C. The sources of canine cancer cell lines are listed in table 2.1.

<table>
<thead>
<tr>
<th>Canine cancer cell line</th>
<th>Location</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lily</td>
<td>Mammary gland</td>
<td>Dr De Maria Raffaella, University of Turin, Italy</td>
<td></td>
</tr>
<tr>
<td>J3T</td>
<td>Glioma</td>
<td>Dr Micheal Behrens, Translational Genomics Research Institute (TGen), Phoenix AZ, USA</td>
<td></td>
</tr>
<tr>
<td>CSKOS (previously called KOS-003)</td>
<td>Osteosarcoma</td>
<td>Chand Khanna, National Institutes of Health, 9000 Rockville Pike, Bethesda, Maryland USA</td>
<td>(Pang, Gatenby et al. 2014)</td>
</tr>
<tr>
<td>K9TCC</td>
<td>Bladder</td>
<td>Dr Deborah Knapp Purdue University West Lafayette, Indiana USA</td>
<td>(Pang, Gatenby et al. 2014)</td>
</tr>
<tr>
<td>REM134</td>
<td>Mammary gland</td>
<td>Prof Rod Else, University of Edinburgh, UK</td>
<td>(Pang, Cervantes-Arias et al. 2011)</td>
</tr>
</tbody>
</table>

Table 2.1 The sources of canine cancer cell lines

2.2 Cell culture

2.2.1 Adherent cells

All cell culture media were purchased from Life Technology (Gibco, Life Technology, UK). The canine glioma cell line J3T and the canine osteosarcoma cell
line CSKOS were grown in T75 flasks (Thermo Fisher Scientific, Denmark) with Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum (Life Technologies, UK) and 1% penicillin/streptomycin (pen/strep) (Life Technologies, UK). The canine mammary inflammatory carcinoma cell line, Lily was grown with Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum (Invitrogen, Paisley, UK) and 1% penicillin/streptomycin (Life Technologies, UK), insulin (20 µg/ml) (Sigma Biochemicals, Dorset, UK) and human epidermal growth factor (EGF) (10 ng/ml) (Peprotech, NJ, USA). These cells were generally grown in 5% CO₂/humidified air at 37°C. When cells reached 80% confluency, adherent cells were enzymatically dissociated by 0.25% trypsin-EDTA (Gibco, Life Technology, UK), seeded at a number of 0.8 x 10^6 with 12 ml of fresh medium in T75 flasks.

2.2.2 Sphere cells

For sphere cell culture, J3T, K9TCC and REM134 cells were seeded as single cells in ultralow attachment 6-well plates (Corning, CA, USA) at low cell density (1.5 x 104 cells/mL). Cells were grown in serum-free medium containing DMEM/F12 supplemented with progesterone (20 nM), putrescine (100 µM), sodium selenite (30 nM), transferring (25 µg/mL), insulin (20 µg/mL) (Sigma Biochemicals, Dorset, UK), human recombinant bFGF (10 ng/mL) (Peprotech, NJ, USA) and human EGF (10 ng/mL) (Peprotech, NJ, USA). Plates were incubated in 5% CO₂/humidified air at 37°C. Growth factors (10 ng/mL bFGF and EGF, final concentration) were added to each well.

2.2.3 Harvesting cells

To harvest adherent cells, the cell monolayer in each flask was washed with 5 mL cold PBS, scraped with 1 mL cold PBS on ice, and collected. To harvest spheres, cells were harvested with 2 mL PBS per well and collected. Cells were pelleted by centrifugation at 3000rpm for 3 minutes at 4°C. Supernatant was removed. Cell pellets were kept at 4°C.
2.3 Drug treatment

Cells were grown until approximately 80% confluent and then treated with 5 μM doxorubicin (MHRA, Germany). Cells were harvested cells at 0, 2, 4 and 8 hours after treatment. After harvesting, cells were lysed following the procedure in section 2.5.1.

2.4 Half-life experiment

Cells were grown until approximately 80% confluent and then pre-treated with 5 μM doxorubicin for 3 hours, the vehicle control group received equal volume of Dimethyl sulfoxide (DMSO). Both groups of cell flasks were incubated in 5% CO2/humidified air at 37°C. Cells were then treated with 30μM cycloheximide and harvest at 0, 1, 2 and 4 hours after treatment.

2.5 Protein assays

2.5.1 Cell lysis

Pelleted cells were manually homogenized in 100μL of cell lysis buffer (7 M urea, 0.1 M DTT, 1 M DTT, 0.05% Triton X-100, 10% Triton, 25 mM NaCl, 20 mM HEPES-KOH, pH 7.6) supplemented with protease inhibitors (10 μM PMSF and 10 μM NEM final concentration), and incubated on ice for 30 minutes. After incubation the cell lysates were centrifuged at 13000 x g for 10 minutes at 4°C, the supernatants were removed and stored at -70°C.

2.5.2 Bradford assay

A Bradford assay were performed to determine total protein concentration of cell lysates. To make a standard curve, 10 mg/ml bovine serum albumin (BSA) stock solution (>99% pure grade, Sigma) was serially diluted to 4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL and 0.125 mg/mL. 1 μL of diluted BSA solution or cell lysate was added to 200μL 1x Bradford reagent (Bio-Rad, US), resulting in a colour change. The absorbance of protein-dye complex was measured by Victor 3
machine at wavelength of 595 nm (PerkinElmer, USA). Each sample was assayed in triplicate.

2.5.3 PNGase Digestion

Peptide: N-Glycosidase F, also known as PNGase F, is commonly used to hydrolyse N-glycan chains from glycopeptides. PrP\textsuperscript{C} was deglycosylated by a PNGase digestion kit (New England BioLabs, UK). Cell lysate (100 μL) was denatured by 10 μL of denaturing buffer (10x) at 100°C for 10 minutes. 10 μL of GlycoBuffer 2 and 10 μL of NP40 were added to further denatured the cell lysate at room temperature. Reactions were split 1:2 for PNGase (positive) and PNGase (negative). 2 μL of PNGase F was added into PNGase (positive) reactions and incubated at 37°C for 2 hours with tilting and shaking. PNGase (negative) reactions were then incubated with 2 μL of PMSF (100 mM) for 2 hours at 4°C.

2.5.4 Protein precipitation

Four volumes of ice-cold acetone were added into the PNGase digested protein lysate and PNGase negative control and incubated overnight on ice. The sample was centrifuged at 13,000 x g, at 4 °C for 10 minutes, the supernatant was removed and the pellet was air-dried. For gel electrophoresis, the pellet was suspended in 10 μl LDS sample buffer (4x) (106 mMTrisHCl, 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM Phenol Red, pH 8.5) (Invitrogen).

2.5.5 Western blot analysis

Proteins were precipitated in acetone as described in section 2.5.3, dried and solubilized in LDS sample buffer (4x) as above and sample reducing agent (10x) (500mM dithiothreitol (DTT)) (Invitrogen, USA). Protein samples were heated at 95°C for 10 minutes then loaded (50 μg/lane) and electrophoretically separated on a 12% NuPAGE Bis-Tris gel (Bis-Tris-HCl buffer (pH 6.4), Acrylamide, Bis-acrylamide, APS, Ultrapure water, pH, 7.0) (Invitrogen) in an Xcell SureLock® tank (Invitrogen) with Novex® running buffer (20x) (1x: 50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3) (Invitrogen) at 150 V for 1 hour. Proteins were
transferred onto polyvinylidene fluoride (PVDF) membranes (Roche) in the Xcell SureLock tank with Novex® transfer buffer (20x) (1x: 25 mM Bicine, 25 mM Bis-Tris (free base), 1 mM EDTA, pH 7.2) at 25 V for 1 hour. Each membrane was washed with PBS 3 times for 5 minutes each time.

2.5.5.1 Imaging with LI-COR®

Molecular weight markers (Chameleon Duo Marker, LI-COR) ranging from 8 kDa to 260 kDa were loaded for size reference before gel electrophoresing. Each membrane was blocked with blocking solution (Odyssey, LI-COR) for 1 hour at room temperature and incubated with primary antibody mouse anti-PrP antibodies (BC6, dilution factor 1:5,000) (Sandra McCutcheon, Roslin Institute, Edinburgh, UK) and loading control α-tubulin (dilution factor 1:5,000) (Abcam) diluted in blocking solution. Protein was revealed with the secondary antibody IRDye® goat anti-mouse IgG (H+L) (dilution factor 1:6,000) (Abcam), detected by LI-COR® Odyssey imaging system (UK) and quantified using Image Studio® software.

2.5.5.2 Imaging with chemiluminescence

Molecular weight markers (Amersham ECL Rainbow Marker, GE Healthcare) from 12 kDa to 225 kDa were used for size reference before gel electrophoresing. Each membrane was blocked with blocking solution (5% (w/v) milk/ 0.1% (v/v) PBS-Tween) for 1 hour at room temperature and blotted with BC6 (dilution factor 1:5,000) (Roslin Institute, Edinburgh, UK) and α-tubulin (dilution factor 1:5,000) (Abcam, Canada) diluted in blocking solution. Immunoreactivity was shown with IgG coupled to horseradish peroxidase (HRP) (dilution factor 1: 5,000) (Abcam). Secondary antibodies were diluted in blocking solution and incubated with the membrane for 90 minutes. The membrane was washed 6 times for 5 minutes with 0.1% (v/v) PBS-Tween after secondary antibody incubation. The membrane was developed by enhanced chemiluminescence (ECL, Amersham, GE healthcare, UK) and visualized by autoradiography (Amersham, GE healthcare, UK).
2.6 Real-time quantitative PCR (RT-qPCR)

Cells were grown until 80% confluent and harvested as previously described (section 2.2.3). Total RNA was isolated using RNeasy® mini kit (QIAGEN) from 80% confluent grown cells according to the manufacturer's protocol. Total RNA was digested with DNase I stock solution (Invitrogen) according to the manufacturer's protocol. RNA concentration was measured by Nano Drop (Thermo Fisher Scientific), and RNA integrity was checked by gel electrophoresis. Reverse transcription was accomplished with 500 μg of RNA using Omniscript® reverse transcription reagents (QIAGEN). Real-time qPCR (RT-qPCR) was conducted with cDNA and both the sense and antisense oligonucleotides in a volume of 10 μl of Platinum® SYBR Green qPCR SuperMix-UDG (Invitrogen, USA) and monitored and assessed in a Stratagene® Mx3000P (Thermo Fisher Scientific) system. Values were normalized to MRP-S25 or to GAPDH expression for cells. The primers were shown as below:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRNP</td>
<td>5'-ACCACCACCACCAAGGGGGA-3'</td>
<td>5'-TTGTGCCTGACTCGTCAAC-3'</td>
</tr>
<tr>
<td>MRP-S25</td>
<td>5'-TCTTGGGAAGAACAAGGAA-3'</td>
<td>5'-AGTGGGCTGGGTGAAAGAAG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GGGAAGATGTGGGGTGAC-3'</td>
<td>5'-GGAGCCATGCGTGAG-3'</td>
</tr>
</tbody>
</table>

Table 2.2 Primer sequence used for RT-qPCR.

The PCR conditions were 1 cycle of 2 minutes at 50°C and 2 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, followed by 1 cycle of 1 minute at 95°C, 30 seconds at 60°C and 30 seconds at 95°C. Fluorescence of reacting samples were measured by StrataGene® Mx3000P and normalised by calculating Δc(t) using Microsoft® Office Excel.
2.7 Immunoprecipitation

2.7.1 Cell culture media preparation for immunoprecipitation

Approximately 12 ml of cell culture media were collected from one flask. Protease inhibitors (10 µM PMSF, 10 µM NEM, final concentration) were required to prevent protease digestion of PrP\textsuperscript{C}. Four volumes of chilled methanol were added to precipitate total protein and incubated at -20°C for 1 hour or overnight. Subsequently, proteins were pelleted by centrifugation at 15,000 x g for 10 minutes at 4°C, and were resuspended with RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% (v/v) Triton, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate, supplemented with 10 mM PMSF and 10 mM NEM). Prepared samples were used for binding with either pre-immobilized protein G antibodies or free antibodies. The primary antibody is bound to either nude magnetic beads or protein A/ G magnetic bead. Nude beads were used to bind anti-PrP antibody directly (Fig. 2.1). Therefore we used IgG, protein G magnetic beads for indirect binding (Fig. 2.2).

2.7.2 Immunoprecipitation using magnetic beads (IP)

Dynabeads® antibody coupling kit (ThermoFisher Scientific) was used for preparing magnetic bead-antibodies (BC6) complexes according to the manufacturer’s protocol. Prepared cell lysates (section 2.5.1) or cell culture media were incubated with bead-BC6 complexes overnight at 4°C with rotation. A magnetic field was applied to pull beads to the side of the tube and the supernatant removed carefully. After washing with 3 x 500 µL of washing buffer (0.1 M sodium phosphate, pH 8.0), PrP was eluted from beads with 0.1 M citric acid to prevent contamination from antibody crosslinking. The coupled magnetic beads are reusable. We adjusted the pH of both the bead suspension and eluted PrP supernatant to pH 8.0 with sodium hydroxide (pH 10.0) immediately after elution. PrP was precipitated by adding 4 volumes of chilled methanol and incubating at -20°C for approximately 1 hour. Precipitated PrP was centrifuged at 13,000 x g for 10 minutes. The supernatant was removed the pellet was resuspended with 10 µL of cell lysis buffer and denatured by NuPAGE® sample loading buffer (4x) (Invitrogen) supplemented with reducing agent (Invitrogen) and distilled water at 70 °C for 10 minutes. We followed
procedures of western blot (section 2.5.4). The blots were visualised by chemiluminescence (section 2.5.4.2).

![Image of immunoprecipitation process]

**Fig. 2.1** Immunoprecipitation with nude beads (IP). Antibodies were coupled with magnetic beads. Antigens were captured by antibodies. Captured antibodies were detached from protein suspension by applying magnetic field. Non-specific protein may be washed by washing buffer. Targeted antigens were eluted from beads with citric acid.

### 2.7.3 Immunoprecipitation using protein G magnetic beads (co-IP)

500 µL cell lysate or 500 µL cell culture medium was incubated with 5 µg anti-PrP goat polyclonal antibody (Santa Cruz) at 4°C for 1 hour with tilting and rotation. 25 µL Protein G beads suspension (0.02% sodium azide, 0.1% BSA, 0.5% Tween® 20, 1x PBS, pH 7.4) (New England BioLabs) was added to samples at 4°C for 1 hour of incubation. Protein-beads complexes were separated from samples by applying magnetic field. Supernatant was removed. The complexes were washed with 500 µL washing buffer (0.1 M sodium phosphate, pH 8.0) for 3 times. Protein-bead complexes were denatured by adding NuPAGE® sample loading buffer (4x) (Invitrogen) supplemented with reducing agent (Invitrogen) and distilled water at 70 °C for 10 minutes. We then applied magnetic field to samples then loaded
supernatant on NuPAGE® gel and proformed western blot. (section 2.5.4). The blots were visualised by chemiluminescence system (section 2.5.4.2).

**Fig. 2.2** Immunoprecipitation with protein G magnetic beads (co-IP). Antibodies were incubated with antigens. Antibodies were then coupled with protein G magnetic beads. Captured antigen-antibody-protein G-bead complexes were separated from sample suspension by applying magnetic field. Non-specific protein may be washed by washing buffer. Targeted antigens were loaded to NuPAGE® gel.

### 2.8 Statistical analysis

All values represent a condition’s mean ± SD. Statistical analysis was performed with SPSS (version 22; IBM, Inc., Armonk, New York) software using Student’s t-test was used to evaluate for significance between 2 groups.

### Reference

3 Chapter 3 Results

3.1 PrP<sup>C</sup> found in cancer cells

To investigate the role of PrP<sup>C</sup> in canine cancer cells, we first detected the PRNP expression in a panel of established canine cancer cell lines (KTOSA and CSKOS osteosarcoma, J3T glioma, REM134 mammary carcinoma and K9TCC bladder carcinoma), grown in culture as adherent cells or as spheres in suspension (CSCs). The ability of cells to form spheroids, under defined conditions, in vitro is a CSC characteristic. All selected cancer cells and CSCs exhibited PrP<sup>C</sup> expression, to differing degrees, when analysed by RT-qPCR. As shown in Fig. 3.1, J3T cells expressed the most PrP<sup>C</sup> in adherent cells than other cell lines tested. REM 134 and K9TCC showed less PrP<sup>C</sup> expression than CSKOS and KTOSA cells. In sphere cells (CSCs), K9TCC cells expressed more PrP<sup>C</sup> as compared to J3T and REM134 CSCs. J3T sphere cells expressed much less PrP<sup>C</sup> compared to adherent cells. However, REM134 and K9TCC adherents expressed similar amounts of PrP<sup>C</sup> as compared to corresponding CSC populations. In general, adherent cancer cells express more PrP<sup>C</sup> than CSCs (the expression level ranking shows as below in table 3.1). Taken together, these results indicate that different canine cancer cell lines and corresponding CSCs express different levels of PrP<sup>C</sup>. 
Fig. 3.1 PrP<sup>C</sup> mRNA expression of canine cancer cells and CSCs relative to GAPDH or MRP-S25 expression. The Bar chart shows different mRNA expression levels of PrP<sup>C</sup> on tested cell lines analysed by RT-qPCR (real-time quantitative PCR). All PRNP expression levels are calculated by ΔΔt and by comparison with the PRNP expression of KTOSA. Data are expressed as means ± SD. n=3 biological replicates.

<table>
<thead>
<tr>
<th>PRNP mRNA expression level ranking</th>
<th>Cell line</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>J3T</td>
<td>Nervous system</td>
</tr>
<tr>
<td>2</td>
<td>CSKOS</td>
<td>Bone</td>
</tr>
<tr>
<td>3</td>
<td>KTOSA</td>
<td>Bone</td>
</tr>
<tr>
<td>4</td>
<td>K9TCC- sphere</td>
<td>Bladder</td>
</tr>
<tr>
<td>5</td>
<td>J3T- sphere</td>
<td>Nervous system</td>
</tr>
<tr>
<td>6</td>
<td>REM134</td>
<td>Mammary gland</td>
</tr>
<tr>
<td>7</td>
<td>K9TCC</td>
<td>Bladder</td>
</tr>
<tr>
<td>8</td>
<td>REM134- sphere</td>
<td>Mammary gland</td>
</tr>
</tbody>
</table>
Table 3.1 PRNP mRNA expression level ranking in the panel of canine cancer cell lines used and the location from which they were originally derived.

3.2 Chemotherapy treatment effects PRNP gene expression

Previous research in the lab has shown that an inflammatory mammary carcinoma cell line called Lily, expresses more C1 than full-length PrP^C protein (data not shown). To determine the expression pattern of PrP^C in Lily cells in response to chemotherapy, we treated Lily cells with 5µM doxorubicin and harvested at indicated time points (Fig. 3.2A). Western blot analysis was utilised to look at doxorubicin-induced changes in PrP^C protein levels. Unfortunately, α-tubulin was inconsistent as a loading control, despite using a Bradford assay to measure protein concentration and ensure that an equal amount of protein was loaded across samples (a representative example is shown in Table 3.2).

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0 (-DOX)</th>
<th>2 (+DOX)</th>
<th>4 (+DOX)</th>
<th>8 (+DOX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lily cell lysate concentration (mg/ml)</td>
<td>14.115</td>
<td>11.882</td>
<td>13.917</td>
<td>9.693</td>
</tr>
</tbody>
</table>

Table 3.2 Bradford assay for Lily cell lysates upon time-course experiment.

Our results indicate that PrP^C protein was elevated in response to doxorubicin, a widely-used chemotherapy drug, in Lily cells and the maximum effect was achieved at 4 hours after treatment (Fig. 3.2A). In addition to looking at changes in protein expression, we looked at changes of PRNP gene expression in response to chemotherapy by qPCR (Fig. 3.2B). Results showed that PRNP expression was similarly increased after 4 hours and then decreased within 4 to 8 hours post-treatment. To conclude, both PrP^C protein and PRNP gene expressions are evaluated in response to chemotherapy and peaking at 4 hours post-treatment.
Fig. 3.2 Doxorubicin induces PrP\textsuperscript{C} protein and PRNP gene expression in Lily cells. Lily cells were treated with 5\mu M doxorubicin, and harvested at 0, 2, 4, 8 hours. A. Western blot analysis of PrP\textsuperscript{C} protein expression of Lily cancer cells. PrP\textsuperscript{C} was detected by mouse anti-PrP (BC6) and IRDye\textregistered goat anti-mouse IgG (H+L), visualized by LIcor\textregistered machine. B. qPCR analysis of PRNP gene expression in Lily cells. mRNA was measured by StrataGene\textregistered Mx3000P. Data are expressed as means ± SD. n=6 biological replicates. Representative images were selected from western blot experiments that have been repeated four times.
To determine if different canine cancer cells are sharing the same pattern of PrP$^C$ expression under the stress of chemotherapy, we treated J3T cells with 5µM doxorubicin and harvested at indicated time-points (Fig. 3.3A). We found that PrP$^C$ expression increased continuously in J3T cells after treating with doxorubicin from 0 to 8 hours (Fig. 3.3A). To verify the PRNP gene expression of J3T cells at mRNA level, we utilised qRT-PCR to show that PRNP gene expression increases over time after treatment with doxorubicin (Fig. 3.3B). Collectively, J3T cells express increasingly PrP$^C$ protein and PRNP gene expression after treatment with doxorubicin.
Fig. 3.3 Doxorubicin induces PrP\textsuperscript{C} protein and PRNP gene expression in J3T cells. J3T cells were harvested at 0, 2, 4 and 8 hours after treating with 5µM doxorubicin. A. Western blot analysis for J3T cell lysates after treatment. Representative images were selected from western blot experiments that have been repeated six times. B. PRNP gene expression was measured in J3T cells. Data are expressed as means ± SD. n=6 biological replicates. t-test was performed. P values that are significantly different are as shown as indicated. *p<0.05; **p<0.005 vs. control.

Previous data showed that CKCOS cells had a high basal of PRNP gene expression compared to other cell lines (Fig. 3.1). CSKOS cells were treated with 5µM doxorubicin and harvested at increasing time-points, and we determined associated
changes in PRNP gene expression. Interestingly, the changes of PRNP gene expression significantly decreased after doxorubicin treatment (Fig. 3.4). These results are in contrast to those obtained for Lily and J3T cells (Fig. 3.2&3.3).

**Fig. 3.4** Doxorubicin induces a decrease in PRNP gene expression in CSKOS cells. CSKOS cells were treated with 5µM doxorubicin and harvested at 0, 2, 4 and 8 hours. mRNA was extracted from cells. qPCR was conducted and measured by StrataGene® Mx3000P. Data are expressed as means ± SD. n=4 biological replicates. t-test was performed. P values that are significantly different are as shown as indicated. **p<0.005 vs. control.

### 3.3 Proteolytic processing of PrP<sup>C</sup> in cancer cells and cancer stem cells

To investigate proteolytic processing of PrP<sup>C</sup>, we used Peptide: N-Glycosidase (PNGase) F to remove N-linked glycans. After treatment with 5µM doxorubicin over the indicated time course, Lily cell lysates were treated with PNGase F. Upon treatment with PNGase F, PrP<sup>C</sup> protein can be visualised by western blotting as two isoforms: full-length and C1 (Fig. 3.5A). Line chart showed the ratio of full-length and C1 in total normalized to total protein (Fig. 3.5B). In response to doxorubicin the amount of C1 decreased while the amount of full-length increased over time as a
proportion of total protein. These data indicate that basally Lily expresses more C1 than full-length, and that this ratio switches in response to cytotoxic stress.

Fig. 3.5 Proteolytic processing of PrP\(^\text{C}\) in Lily cells in response to doxorubicin. After treated with 5\(\mu\)M doxorubicin, Lily cells were harvested at 0, 2, 4 and 8 hours. Cell lysates were treated with PNGase F to remove N-linked glycans. A. De-glycosylated PrP\(^\text{C}\) isoforms (full-length and C1) were detected by mouse primary antibody (BC6) and secondary antibody (IRDye\(^\text{®}\) goat anti-mouse IgG (H+L)), and visualized by Li-cor\(^\text{®}\) Blot Scanner. B. Bands were quantified by Image Studio\(^\text{®}\) software, and the ratio of isoforms calculated (FL: full-length). Representative images were selected from western blot experiments, which have been repeated four times.

To determine the proteolytic events in J3T cancer cells and corresponding CSCs upon treatment of 5\(\mu\)M doxorubicin, cell lysates were treated with PNGase F. We detected changes of two isoforms (full-length and C1) in J3T cancer cells after
treated with doxorubicin (Fig. 3.6A). The amount of full-length PrP\(^C\) decreased compared to the amount of C1, which increased over time after doxorubicin treatment (Fig. 3.6B). These results contrasted with those of Lily cells (Fig. 3.5B).

![Proteolytic processing of PrP\(^C\) in J3T cells after doxorubicin treatment](image)

**A**

<table>
<thead>
<tr>
<th>Time (hr)</th>
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<th>2</th>
<th>4</th>
<th>8</th>
</tr>
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<tbody>
<tr>
<td><strong>Adherent</strong></td>
<td>Full-length</td>
<td>C1</td>
<td>Full-length</td>
<td>C1</td>
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<tr>
<td><strong>α-tubulin</strong></td>
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**B**

![Line chart showing the ratio of full-length and C1 to total PrP\(^C\)](image)

**Fig. 3.6** Proteolytic processing of PrP\(^C\) in J3T cells after doxorubicin treatment. J3T cells were treated with 5\(μ\)M doxorubicin, and harvested at 0, 2, 4 and 8 hours. A. The PrP\(^C\) expression was evaluated by western blot. After immunoblotting with BC6 and secondary antibody, full-length PrP\(^C\) and C1 were detected by LIcor® Blot Scanner. Representative images were selected from western blot experiments replicated four times. B. The amount of each isoforms was normalized by Image Studio® software. Line chart shows the ratio of full-length and C1 to total PrP\(^C\) (FL: full-length). α-tubulin was performed as a loading control.

We then looked at the PrP\(^C\) protein expression levels in J3T CSCs (Fig. 3.7A) in response to doxorubicin. J3T CSCs were treated with 5\(μ\)M doxorubicin and harvested over the indicated time course. In contrast to J3T adherent cells we show that there is a higher basal level of C1 in CSCs than full-length PrP\(^C\), and that this
changes over time after treatment; C1 levels decrease and full-length levels increase (Fig. 3.7B). Taken together, these results show that PrP$^C$ is proteolytically processed in response to doxorubicin treatment, and that there are fundamental differences in the processing of PrP$^C$ between non-CSCs and CSCs.

![Proteolytic processing of PrP$^C$ in J3T CSCs after treatment with doxorubicin. J3T CSCs were treated with 5µM doxorubicin and harvested at 0, 2, 4 and 8 hours. A. The PrP$^C$ expression and proteolytic processing was evaluated by western blot both full-length PrP$^C$ and C1 were detected. B. Line chart shows the ratio of each isoform to total PrP$^C$ normalized by Image Studio® (FL: full-length). α-tubulin was performed as a loading control.](image)

**3.4 Half-life of PrP$^C$ proteolytic products**

Half-life of a protein is the time it takes for that the protein to degrade to half of its initial amount. To describe the decay of PrP$^C$ in cancer cells after DNA damage has been triggered by doxorubicin, we determined the half-life of PrP$^C$ by treating cells
with cycloheximide, a protein synthesis inhibitor. Cells were pre-treated with 5µM doxorubicin for 3 hours, then treated with 30µM cycloheximide and harvested at 0, 1, 2, and 4 hours. Dimethyl sulfoxide (DMSO) was used as vehicle control. Upon PNGase F digestion the relative amounts of the proteolytic products of PrP<sup>C</sup> were identified by western blotting. We compared the half-lives of two detected isoforms. For Lily cells, the half-life of C1 (3.6 hr) increased by doxorubicin treatment compared to negative control (2.5 hr). Interestingly, the half-life of full-length PrP<sup>C</sup> was shorter than control group when under the stress of chemotherapy (Fig. 3.8).

<table>
<thead>
<tr>
<th></th>
<th>Lily</th>
<th>J3T</th>
</tr>
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<tbody>
<tr>
<td>DOX (5µM)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CHX (30µM)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Time</td>
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<td>6</td>
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</table>

**Fig. 3.8** Half-life experiment of Lily cells. Western blot image for half-life experiment in Lily cells treated with DMSO (control) or doxorubicin (5µM) for 3 hours, and cells were then harvested at 0, 1, 2, 4 hours after treating with cycloheximide (30µM). Cell pellets were digested with PNGase F to deglycosylate N-linked glycans and immunoblotted with anti-PrP (BC6) and anti-mouse secondary antibody. α-tubulin was performed as a loading control.

Similarly, for J3T cells we observed that the half-life of C1 was increased after doxorubicin treatment, and that the half-life of full-length was shorter after doxorubicin treatment compared to control cells (Fig. 3.9). These results indicate that the two isoforms of PrP<sup>C</sup> may have functional roles in the cellular response to DNA damage.
**3.5 The secreted isoforms of PrP<sup>C</sup> are detected in cell culture media by immunoprecipitation.**

There are three main proteolytic cleavages of PrP<sup>C</sup>: α-cleavage, β-cleavage and shedding. α-cleavage cleavages from the hydrophobic region, giving rise to C1 (15kDa) and N1 (11kDa) fragments. β-cleavage destroys the octapeptides repeat region, producing C2 (17kDa) and N2 (9kDa) fragments. We hypothesised that PrP<sup>C</sup> is constitutively found in the media of cultured canine cancer cells which is subjected by cleavage of GPI-peptides and proteolytic procedures. PrP<sup>C</sup> is cleaved at the GPI-anchor, shedding nearly full-length PrP<sup>C</sup> from extracellular membrane into the extracellular milieu. To investigate the secreted isoforms of PrP<sup>C</sup> in cell culture media with regard to proteolytic processing, we evaluated several methods of immunoprecipitation. Cell culture media was collected from 80% confluent cell culture flasks. All of the proteins in the cell culture media were precipitated by methanol and resuspended in cell lysis buffer. Incubating with pre-coated anti-PrP antibodies, PrP<sup>C</sup> secreted isoforms in cell culture media were precipitated and detected by western blot.

**3.5.1 Validation of immunoprecipitation by magnetic beads**

We first used magnetic beads coupled to anti-PrP antibody. The antibody-beads complexes were then added into protein suspension. The PrP-antibody-beads complexes were pulled out from protein suspension by applying a magnetic field. PrP<sup>C</sup> was verified by western blotting. As a positive control cell lysates were used to compare to cell culture media. We were able to successfully detect both full-length PrP<sup>C</sup> and C1 isolated from cell lysates and cell culture media, of both J3T and Lily cells (Fig. 3.10). Non-PNGase F-treated cells were shown (Fig. 3.10A). For Lily cells, C1 is detected more than full-length in both cell culture media and cell lysate (Fig. 3.10A; right). In J3T cells, both full-length and C1 were detected in the cell media.
lysate. However, only C1 was detected in the cell culture medium (Fig. 3.10A; left). Furthermore, the detection of both full-length PrP\textsuperscript{C} and C1 was validated by PNGase F digestion (Fig. 3.10B). A faint band of C1-deglycosylated and a strong band of C1-unglycosylated were shown for J3T cell culture media (Fig. 3.10B; left). Also, only C1-unglycosylated was present for both Lily cell culture media and cell lysate (Fig. 3.10B). In summary, we have optimised an immunoprecipitation technique to detect PrP\textsuperscript{C} in cell culture media.

![Image](image1.png)

**Fig. 3.10** Immunoprecipitation validation of Lily and J3T cells. Cell lysates and cell culture media were collected and antibody-coupled magnetic beads added to immunoprecipitate PrP\textsuperscript{C}. PrP\textsuperscript{C} was bound to BC6 and pulled out by solution with low pH. Precipitated PrP\textsuperscript{C} was immunoblotted with anti-PrP (BC6) and anti-mouse secondary antibody (goat anti-mouse IgG HRP). Western blot was visualized by chemiluminescence. A. Western blot image of immunoprecipitated PrP\textsuperscript{C} without deglycosylation. (exposure time: overnight, approximately 17 hours). B. Immunoprecipitated PrP\textsuperscript{C} treated with PNGase F (exposure time: overnight, approximately 17 hours).

To demonstrate the results shown before, we repeated the same experiment. PrP\textsuperscript{C} precipitated from cell culture media was not detected in non-PNGase F-treated cells. Upon PNGase F treatment, faint bands of deglycosylated C1, fully glycosylated/mono-glycosylated and deglycosylated full-length were exhibited on western blot image (Fig. 3.11). To conclude, this repeated experiment proves that the method detects separated cleavage products of full-length PrP in cell culture media, which was not previously shown (Fig. 3.10).
Fig. 3.11 Immunoprecipitation validation of Lily and J3T cells. PrP\textsuperscript{C} was bound to BC6 and pulled out by a solution with low pH. Precipitated PrP\textsuperscript{C} was immunoblotted with anti-PrP (BC6) and anti-mouse secondary antibody (goat anti-mouse IgG (HRP)). The blot was visualised by chemiluminescence. A. Western blot analysis for PrP\textsuperscript{C} eluted from magnetic beads in Lily and J3T cell lysates and cell culture media. B. After eluted from beads, PrP\textsuperscript{C} was treated with PNGase F, which showed on western blot image. Exposure time: 5 minutes.

3.5.2 Validation of immunoprecipitation by protein G magnetic beads

To optimise the immunoprecipitation, we generated a new protocol using protein G magnetic beads to bind anti-PrP antibodies. Compared to magnetic beads, protein G magnetic beads are pre-coated with protein G and specially bind to IgG antibodies. To enrich binding sites of beads, we chose polyclonal rabbit anti-PrP antibody. Western blot analysis shows a strong band for J3T cell culture medium precipitation, which indicates that protein G beads coupled with polyclonal antibodies had a high binding efficiency with PrP\textsuperscript{C} (Fig. 3.12A). The western blot was repeated using J3T cell culture medium and cell lysate, and we were able to detect PrP\textsuperscript{C} from precipitations of both cell culture medium and cell lysate (Fig. 3.12B).

Representative data show that protein G magnetic beads immunoprecipitation is potentially more efficient than magnetic beads. Collectively, immunoprecipitation with protein G magnetic beads is valid for precipitating PrP\textsuperscript{C} in J3T cell culture.
medium.

**Fig. 3.12** PrP<sup>C</sup> immunoprecipitation using protein G magnetic bead. PrP<sup>C</sup> was precipitated from J3T cell culture medium by polyclonal rabbit anti-PrP antibodies. The blot was detected by BC6 and secondary antibody goat anti-mouse IgG (HRP) and visualized by immunoluminescence. A. Western blot image showed precipitated PrP<sup>C</sup> from J3T cell culture medium. Exposure time: 1 minute. B. Western blot analysis presents immunoprecipitation of PrP<sup>C</sup> in J3T cell culture medium and cell pellet. Exposure time: 30 seconds. Representative images were selected from western blot experiments, which have been repeated for four times. Cat brain homogenate was used as a positive control.

In conclusion, we have optimised two methods of PrP<sup>C</sup> immunoprecipitation that are valid in cancer cell culture media.
4 Chapter 4 Discussion

Canine cancers share many characteristics with human cancers. The rodent cancer model has been investigated widely, but is genetically and pathologically different from human cancer and has many limitations (Rowell, McCarthy et al. 2011). Most importantly, tumors arise spontaneously in humans, but must be induced in most mouse models due to vast genetic and environmental differences (Rowell, McCarthy et al. 2011). In contrast, spontaneous cancers do arise in dogs featuring many similarities with human cancers, including histological appearance, tumour genetics, molecular targets, biological behaviour and response to conventional therapies (Paoloni and Khanna 2008). There is some evidence indicating that using dogs in translational medicine can hugely accelerate drug development in human cancer (Rowell, McCarthy et al. 2011). Previous research has demonstrated that PrP\textsuperscript{C} is an essential component in the processes of transmissible spongiform encephalopathies (TSEs) as a normal counterpart of pathogenic scrapie prion protein (PrP\textsuperscript{Sc}) (Harris 1999). Surprisingly, the normal cellular cellular prion (PrP\textsuperscript{C}) exists widely in different types of cells and tissues and has been studied in human cancers, including breast, colorectal and gastric cancer, and has been shown to have a role in proliferation, anti-apoptosis, tumourigenesis and drug-resistance (Yang, Zhang et al. 2014). PrP\textsuperscript{C} is modified by two N-linked glycans and subjected to proteolytic processing (Altmeppen, Puig et al. 2012). In canine cancer the role of PrP\textsuperscript{C} and its isoforms has not been investigated. Our studies have analysed PrP\textsuperscript{C} expression in different cancer cell lines, and we have found that PrP\textsuperscript{C} truncated forms follow different proteolytic mechanisms in two canine cancer cell lines when treated with conventional oncotherapy.

4.1 Diverse PrP\textsuperscript{C} expression levels between different cancer types

Our studies provide evidence that many established canine cancer cell lines express PrP\textsuperscript{C}, including: canine glioma (J3T), osteosarcoma (CSKOS and KTOSA), inflammatory mammary carcinoma (REM134 and Lily) and bladder cancer (K9TCC) cell line (Fig. 3.1). Different cancer cell lines have varying expression levels of PrP\textsuperscript{C}. In this study, only a limited number of cell lines have been tested and
future experiments are needed to determine if PrP\textsuperscript{C} expression and cancer type are associated. Future research could then be extended to determine if PrP\textsuperscript{C} can be a potential drug target or used as a prognostic marker. The canine glioma cell line (J3T) expresses the most of PrP\textsuperscript{C} among selected cancer cells. The canine osteosarcoma cell lines (CSKOS and KTOSA) rank second and third. The canine inflammatory mammary carcinoma (REM134) and the canine bladder cancer (K9TCC)’s PRNP gene expression were the lowest (Table.3.1). PrP\textsuperscript{C} is predominantly found in the nervous system and has been proven to elicit neuroprotection and neurogenesis (Roucou, Giannopoulos et al. 2005, Steele, Warfel et al. 2005). J3T is a canine glioma cell line, which is derived from nervous system. It is therefore expected that PRNP gene expression is higher in J3T cells compared to the other cell lines such as K9TCC, a bladder cell line. To our best knowledge, the existence of PrP\textsuperscript{C} in bladder cancer has not been investigated yet. These results may suggest that PrP\textsuperscript{C} characteristics are different in different types of cancer cells and may support cells to self-renew and differentiate which has also been suggested for embryonic stem cells (Lee and Baskakov 2013).

In terms of different expression levels of PrP\textsuperscript{C} in canine cancer cells and cancer stem cells, our results show that there is no correlation between them. Cancer stem cells are a small subset of cancer cells, with distinct properties such as, tumourigenesis and drug resistance. Recent evidence shows that PrP\textsuperscript{C} is involved in cell renewal, cell proliferation and antiapoptosis in human cancer cells (Yang, Zhang et al. 2014) and cancer stem cells (CSCs) (Du 2013; Corsaro, 2016). However, our findings reveal that PRNP gene expression level of CSCs is much lower than heterogeneous cancer cells.

4.2 Chemotherapy induces changes in PrP\textsuperscript{C} protein expression and PRNP gene expression

Chemotherapy is treatment with drugs that interfere with the machinery of cancer cells and causing irreversible damage to cancer cells (Herr and Debatin 2001). Doxorubicin (trade name termed Adriamycin) is a commonly used chemotherapy drug for a wide range of cancers, including many kinds of carcinoma, sarcoma and
blood cancers (Hande 1998). Doxorubicin is a highly effective DNA intercalating agent that triggers permanent DNA damage to cancer cells in the treatment of breast cancer and soft tissue sarcomas (Hande 1998). Operatively, common chemotherapy often kills most non-tumourigenic cancer cell progeny except CSCs, which are able to reinitiate tumour growth (Han, Shi et al. 2013). Chemotherapy treatments show that PRNP gene and PrP<sub>C</sub> protein expression in Lily and J3T cells was significantly increased by doxorubicin treatment (Fig. 3.2&3.3), which links to previous studies that have shown that PrP<sub>C</sub> plays a role in anti-apoptosis in human cancer cells (breast cancer, colorectal cancer and gastric cancer) contributing to tumourigenesis and drug resistance (Yang, Zhang et al. 2014). Lily is an inflammatory mammary carcinoma cell line for which we were unable to identify a suitable loading control; β-actin and GAPDH levels were variable. We tried to compensate for this by quantifying total protein concentration by Bradford assay (Table. 3.2), yet still found inconsistencies in loading. As this occurred repeatedly under treatment of chemotherapy drug, we propose that the cytoskeleton of Lily cells may be disrupted by doxorubicin treatment. In line with this, would be the observation that Lily cells grow slower with increasing cell passage suggesting that an unstable cytoskeleton may cause slower cell growth. Interestingly, we found that PRNP gene expression in CSKOS was downregulated following chemotherapy (Fig.3.4). This result indicates that PrP<sub>C</sub> may have different roles in different cancer types.

4.3 Lily and J3T cells exhibit different α-cleavage

PrP<sub>C</sub> is a GPI-anchored protein with two N-linked glycans. In order to assess the proteolytic processes of PrP<sub>C</sub>, both glycans were removed by PNGase treatment. PrP<sub>C</sub> is proteolytically processed at three cleavage sites: α-cleavage, β-cleavage and shedding. The α-cleavage gives rise to C1 and N1 fragments, the β-cleavage separates C2 and N2 fragments and the shedding produces almost full-length PrP<sub>C</sub>. In our studies, two main cleavages (α-cleavage and shedding) were observed by using BC6 PrP-antibody, which is a monoclonal PrP-antibody only detecting C-terminal PrP. We have previously shown that the Lily cell line is unique because it mainly expresses the C1 fragment while in the other canine cancer cell lines tested
both full-length and C1 are detected (data not shown). In the present study, Lily produced increasing amounts of full-length PrP\textsuperscript{C} in response to doxorubicin-induced cytotoxic stress (Fig. 3.5). Our data shows that the ratio of the C1 isoform compared to the full length PrP\textsuperscript{C} form in J3T cells increased due to the stress of chemotherapy (Fig. 3.6). Conversely, in J3T CSCs the ratio of full-length PrP\textsuperscript{C} increased compared to the ratio of C1 (Fig. 3.7).

To determine the life-cycle of PrP\textsuperscript{C}, we treated cells with doxorubicin and determined the half-life of PrP\textsuperscript{C} using a protein synthesis inhibitor, cycloheximide. We showed that the half-life of C1 in J3T cells was prolonged in response to doxorubicin. Combined with the results of drug treatment time course, we hypothesized that the $\alpha$-cleavage may potentiate the drug-resistance in J3T cells. In J3T CSCs, the level of isoforms was opposite to normal cancer cells. As the supporting evidence is not sufficient, the role of PrP\textsuperscript{C} in J3T cancer cells remains putative. In Lily cells, the amount of C1 protein reduced while full-length increased. In response to doxorubicin Lily cells have a longer half-life of C1 and a shorter half-life of full-length PrP\textsuperscript{C} compared to the control cells. In this scenario, the relationships between full-length PrP\textsuperscript{C} and C1 fragment and if there is a proteolytic conversion mechanism between full-length PrP and C1 fragment are yet unknown.

Of note, previous data has shown that C2 fragment is not proteolytically converted to C1 fragment (Sunyach, Cisse et al. 2007). Hence, it is tempting to investigate whether the full-length and C2 catabolite to C1 as a secondary proteolytic processing.

Previous research indicates that the protease that mediates the $\alpha$-cleavage of PrP\textsuperscript{C} is either the lysosomal serine protease type or the A-disintegrin-and-metalloproteinase (ADAM) family group (Altmeppen, Prox et al. 2013). The ADAMs belong to a zinc protease superfamily which has been proven to be involved in various biological processes, including: signal transduction, protein-protein interactions and protein processing (Seals and Courtneidge 2003). To date, ADAM 10 and ADAM 17 have been implicated in the $\alpha$-cleavage of PrP\textsuperscript{C} (Altmeppen, Prox et al. 2013). Accordingly, it has been suggested that ADAM 10 and ADAM 17 play an important role in etoposide (a widely used anti-tumour drug)-induced cell-death (Lizama, Ludwig et al. 2011). Doxorubicin may also activate ADAM 10 and ADAM 17,
thereby triggering α-cleavage producing N1/C1 fragment. The α-cleavage may therefore have a functional purpose in response to chemotherapy. Since there is no investigation involving in the relationship between canine cancer and PrP\(_C\) proteolytic processing, this hypothesis will be interesting to research further. To verify this assumption, we can compare the protein expression levels of ADAMs and PrP\(_C\) isoforms upon chemotherapy treatment in the future.

Previous evidence has shown that the N1 fragment inhibits staurosporine-induced caspase-3 activation via the p53 pathway, thereby displays neuroprotection functions in retinal ganglion cells. p53 is tumour suppressor protein that mediates cell cycle progression. In contrast, other evidence shows that the C1 fragment significantly induces the caspase-3 activation, relying on the presence of p53, in two human embryonic kidney cell lines (Sunyach, Cisse et al. 2007). Accordingly, the J3T glioma cell line is derived from the nervous system. Hence, it is possible that C1 of J3T cells has a neuroprotective role under the stress of chemotherapy.

These results are potentially associated with previous findings, which found that the PrP\(_C\) N-terminus (octapeptide repeat region) is involved in copper binding and has a role in resisting oxidative stress (Brown, Boon-Seng et al. 1999). Oxidative stress is an important trigger for apoptosis (Kannan and Jain 2000). Thus, PrP\(_C\) antibodies reduced cell-death by inhibiting the expression of Bcl-2, which proved that PrP\(_C\) is associated with Bcl-2-induced anti-apoptosis in colorectal cancer cells (McEwan, Windsor et al. 2009). Hence, it is hypothesized that the PrP\(_C\) N-terminus binds to copper to inhibit oxidative stress, thereby reducing apoptotic activity. As previous studies shown that BC6 PrP-antibody has a high affinity to PrP\(_C\), which is a C-terminal monoclonal antibody, we then decided to detect PrP by BC6. Nevertheless, an appealing topic is risen to emphasize the distinct biological functions between alternative isoforms. Future experiments to demonstrate if there are different responses for alternative products of PrP\(_C\) correlated with p53 pathway or oxidative stress by down-regulating or up-regulating experiments of PrP\(_C\) or its isoforms, are needed.

Compared to α-cleavage, full-length PrP\(_C\) shedding has been investigated much less. From what has been already discovered, PrP\(_C\) shedding activation is subjected to
phospholipases and involved in cell signaling (Altmeppen, Puig et al. 2012). We cannot determine if full-length PrPC exerts functions, which are similar to the N1/C1 fragment due to the time limitation of this project. However, it will be interesting to delineate the function of each isoform of PrPC with regard to different hallmarks of cancer. Collectively, our results are the first demonstration that the expression of PrPC could be determined in canine cancer cells and the proteolytic processing of PrPC could be altered by chemotherapy drug treatment in two canine cell lines.

**4.4 Validation of immunoprecipitation**

In our studies, we first discovered that PrPC expression was upregulated in Lily and J3T cell lines in response to chemotherapy treatment. Furthermore, proteolytic processing of PrPC in Lily and J3T cells has been shown by deglycosylation with PNGase F treatment. However, the pattern of cleavage remains unclear, particularly whether the proteolytical generation of C1 fragment and shedding from the plasma-membrane of full-length PrPC are linked. To investigate these processes, we employed immunoprecipitation to isolate PrPC isoforms from the in cell culture media. This should provide data to demonstrate the possible relationship between the observed isoform ratios and shedding into the media.

Compared to agarose beads, magnetic beads have a higher yield as using magnetic to pull off protein complexes rather than centrifuging. Based on that, the protein of interest has the highest purity-specificity after elution from magnetic beads. Immunoprecipitation with magnetic beads can be performed using a direct (IP) or indirect method (co-IP). Table of comparison between nude beads and protein G magnetic beads is shown as below (Table 4.1). Nude beads have ultra-low non-specific binding, but they are adapted to all kinds of antibody. Protein G magnetic beads are specific to IgG with low non-specific binding.

<table>
<thead>
<tr>
<th></th>
<th>Magnetic beads (nude beads)</th>
<th>Protein G magnetic beads (pre-immobilized beads)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Binding properties</strong></td>
<td>Covalent antibody binding</td>
<td>Non-covalent antibody binding</td>
</tr>
<tr>
<td><strong>Antibody co-eluted off the beads</strong></td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 4.1 Comparison between magnetic beads and protein G magnetic beads. (This table is partly adapted from ThermoFisher Scientific® Antibody-binding product selection guide)

<table>
<thead>
<tr>
<th>Type of ligand</th>
<th>All antibodies</th>
<th>IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-specific binding</td>
<td>Ultra-low</td>
<td>Low</td>
</tr>
</tbody>
</table>

Firstly, PrP<sup>C</sup> and its isoforms were bound by BC6-convalent-bound magnetic beads and detected by immunoprecipitation in both Lily and J3T cell culture media and cell lysates (control group) exposed to lumifilm overnight. In J3T cell culture medium, a large amount of a 26kDa protein was found in western blot, which could represent either glycosylated C1 or unglycosylated full-length PrP<sup>C</sup> (Fig. 3.10A & 3.10B). There are two possibilities for explanation: 1) fully glycosylated C1 has remained largely in both J3T and Lily cell culture media and PNGase treatment did not fully digest the N-linked glycans of the C1 fragment. As shown in (Fig. 3.10B), fully glycosylated C1 was mostly detected from Lily cell culture media; 2) unglycosylated full-length PrP<sup>C</sup> and some minor amount of di-glycosylated C1 existed in both J3T and Lily cell culture media (Fig. 3.10A). After PNGase digestion, a faint band of deglycosylated C1 appeared below the unglycosylated full-length of J3T cell culture medium (Fig. 3.10B). The majority of full-length PrP<sup>C</sup> found in Lily culture medium is unglycosylated. To validate of this immunoprecipitation technique, we repeated the experiment. Unfortunately, no PrP<sup>C</sup> was detected in both Lily and J3T cell culture media without PNGase F digestion (Fig. 3.11A). The blot exhibited faint bands of di-deglycosylated or mono-deglycosylated full-length PrP<sup>C</sup> and deglycosylated full-length or un-deglycosylated C1 are detectable after PNGase digestion in J3T cell culture medium (Fig. 3.11B). These results are shown in the blot with a short exposure time (5 minute). To improve on this result, the blot was exposed overnight, but it was overexposed. However, this work reveals that full-length PrP<sup>C</sup> is detectable in J3T cell culture medium. The band around 30kDa of PNGase F treated J3T cell culture medium remains to be elucidated.

Due to inconsistency of results that we acquired from nude magnetic beads precipitation, we decided to use protein G magnetic beads precipitation (co-IP) utilising polyclonal antibodies against more epitopes of PrP<sup>C</sup>, with the aim of improving binding Our results showed that the signals from precipitation of PrP-
protein G-beads were stronger than the signals from immunoprecipitation by BC6-convalent-beads. This co-IP technique was more effective than using protein G beads or polyclonal PrP<sup>C</sup> antibody. Further studies are needed to investigate the proteolytic processing of PrP<sup>C</sup> in extracellular environment after treating with chemotherapy drug utilising this validated co-IP technique. Collectively, our studies firstly showed the proteolytic processing of PrP<sup>C</sup> of canine cancer cells in cell culture media. Also, we validated two techniques for detecting extracellular PrP<sup>C</sup> and its products that are secreted by canine cancer cells. These finding provide a perspective for future studies in investigating the function of secreted PrP<sup>C</sup> isoforms after proteolytic processing.

In this study, we validated two immunoprecipitation techniques but did not optimise the exposure time for immunoprecipitation. Further experiments are needed to demonstrate the extracellular PrP<sup>C</sup> isoforms existence, regulation of PrP<sup>C</sup> isoforms under drug treatment and additional proteolytic processes. To further optimise this experiment, we must appreciate the inherent limitations of immunoprecipitating PrP<sup>C</sup> from cell culture media. Firstly, we may lose protein during each step: protein precipitation, elution and magnetic beads precipitation. Secondly, the PrP<sup>C</sup> is readily degraded by proteases (Swietnicki, Petersen et al. 1998) during the period of harvesting culture media and protein precipitation, even if we supplement with protease inhibitors. However, if we are only interested at the changing trend of PrP<sup>C</sup> products, immunoprecipitation is an attractive option. Future experiments to elucidate the function of PrP<sup>C</sup> could also involve knockout/overexpression of each of these isoforms.

**Reference**

Conclusion

PrP\textsuperscript{C} has been investigated in human cancers, whereas PrP\textsuperscript{C} expression has not been evaluated in canine cancers. We have provided evidence that \textit{PRNP} gene expression is detected in cancer cell lines and varies under chemotherapy treatment. We have investigated in details the proteolytic processing of PrP\textsuperscript{C} protein in two different cell lines. Two immunoprecipitation techniques have been validated to detect PrP\textsuperscript{C} isoforms secreted from cultured cancer cells. These findings give evidence of the existence of PrP\textsuperscript{C} and its isoforms in canine cancer cell culture media and detection of extracellular PrP\textsuperscript{C} truncated forms. Collectively, PrP\textsuperscript{C} may have diagnostic or therapeutic potential in canine cancer.