THE ROLE OF BIRC6, A MEMBER OF THE INHIBITOR OF APOPTOSIS PROTEIN (IAP) FAMILY, IN THE SURVIVAL OF HUMAN PROSTATE CANCER CELLS

by

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ABSTRACT

Prostate cancer is the most commonly diagnosed cancer and third leading cause of cancer deaths in Canadian men. Prostate cancers typically begin as androgen-dependent tumours susceptible to growth arrest/apoptosis induced by ablation of androgens. Although initially effective, androgen ablation frequently leads to the development of castration-resistant (androgen-independent) prostate cancer, which is generally also resistant to other available treatments. Development of castration-resistant prostate cancer is characteristically associated with marked increases in resistance to apoptosis. BIRC6 is a member of the Inhibitors of Apoptosis Protein (IAP) family which protects a variety of cancer cell lines from apoptosis. In the present study, we have investigated whether BIRC6 plays a role in prostate cancer and could potentially be useful as a novel therapeutic target. Analysis of a variety of human prostate cancer cell lines and clinical specimens for BIRC6 protein expression, using Western blot and immunohistochemical analyses, respectively, showed that BIRC6 protein is markedly expressed by the prostate cancer cell lines and by clinical cancer specimens, as distinct from benign prostate cells/tissue. In addition, analysis of the clinical specimens showed that elevated BIRC6 protein expression was found to be particularly associated with cancers of Gleason score 6-8 and with the development of castration-resistant disease. Specific, siRNA-induced reduction of BIRC6 expression in LNCaP cells led to a marked reduction in cell proliferation, associated with an increase in apoptosis markers and a decrease in

autophagosome markers, indicating that BIRC6 plays a major protective role in the proliferation of LNCaP cells by inhibiting apoptosis and perhaps by enhancing autophagy. Taken together, the data suggest an important role for BIRC6 in prostate cancer growth and progression, particularly, in the development of treatment resistance. In conclusion, this study indicates - for the first time - that the *BIRC6* gene and its product are potentially valuable targets for therapy of human prostate cancers. BIRC6-targeting drugs may be especially useful for sensitization of cancer cells in combination therapy.

PREFACE

Human prostate cancer tissue specimens were obtained from patients, with their informed consent, following a protocol approved by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board (REB NUMBER: H04-60131). Immunohistochemical scoring of human prostate cancer tissue sections was conducted at the Prostate Centre at the Vancouver General Hospital by Dr. Ladan Fazli.

TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

Atg: Autophagy-Related Gene (i.e., Atg5, Atg12, Atg8)
ASO: Anti-Sense Oligonucleotides
BIR: Baculoviral IAP Repeat
BIRC: Baculoviral IAP Repeat-Containing Protein
BIRC6: Baculoviral IAP Repeat-Containing Protein 6
BSA: Bovine Serum Albumin
c-IAP: Cellular Inhibitor of Apoptosis Protein
CRPC: Castration-Resistant Prostate Cancer
DAB: 3', 3'-Diaminobenzidine
DIABLO: Direct IAP Binding Protein with Low Isoelectric Point, pI
DISC: Death Inducing Signalling Complex
DNA: Deoxyribonucleic Acid
DR4: Death Receptor 4
DR5: Death Receptor 5
FAB: French-American-British AML Subtype Classification
FADD: Fas-Associated Death Domain
FasL: Fas Ligand
GnRH: Gonadotropin-Releasing Hormone
h: Hour
H&E: Hematoxylin and Eosin
HIAP: Human Inhibitor of Apoptosis Protein
Hsp27: Heat shock protein 27
HTRA2/OMI: HtrA Serine Peptidase 2
IAP: Inhibitors of Apoptosis Protein
MAP1LC3/LC3: Microtubule-Associated Protein 1 Light Chain 3
min: Minutes
ML-IAP: Melanoma Inhibitor of Apoptosis Protein
mTOR: Mammalian Target of Rapamycin
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

KIAP: Kidney Inhibitor of Apoptosis Protein

LC3B-I: Subunit of MAP1LC3

LC3B-II: Phosphatidylethanolamine-Conjugated form, of LC3B-I

NAIP: Neuronal Apoptosis Inhibitory Protein

P: *P*-Value

PARP: Poly ADP-Ribose Polymerase

PBS: Phosphate Buffered Saline

PI3K: Class III Phosphatidylinositol 3-Kinase

PIN: Prostatic Intraepithelial Neoplasia

PSA: Prostate-Specific Antigen

PVDF: Polyvinylidene Fluoride

RPMI-1640: Roswell Park Memorial Institute-1640

S.D.: Standard Deviation

SDS: Sodium Dodecyl Sulfate

siRNA: Small Interfering Ribonucleic Acid

SMAC: Second Mitochondria-Derived Activator of Caspase

TBS: Tris Buffered Saline

TMA: Tissue Microarrays

TNFα: Tumour Necrosis Factor α

TNFR1: Tumour Necrosis Factor Receptor 1

TRAIL: Tumour Necrosis Factor-Related Apoptosis Inducing Ligand

UBC: Ubiquitin-Conjugating

VAD-FMK: Carbobenzoxy-Valyl-Alanyl-Aspartyl-[O-Methyl]-Fluoromethylketone

XIAP: X-Linked Inhibitor of Apoptosis Protein

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CHAPTER 1: INTRODUCTION

1.1 Prostate cancer

Prostate cancer is the most commonly diagnosed cancer and third leading cause of cancer deaths in Canadian men. It was estimated that, in 2009, 25,500 Canadian men would be diagnosed with prostate cancer and that 4,400 would die of the disease (Canadian Cancer Society 2009). If the malignancy is localized to the prostate, surgery and radiation therapy can be curative, however, 30-50% of patients will experience local recurrence or metastases (Pound, Partin et al. 1999; D'Amico, Schultz et al. 2000). Men with metastatic prostate cancer are faced with a less favourable prognosis, having median survival times in the range of 3-7 years (Pound, Partin et al. 1999). Treatment of metastatic prostate cancers is generally based on ablation of growth-promoting androgens via surgical or medical castration, since early prostate cancers are typically androgen-dependent and, as such, susceptible to growth arrest/apoptosis in response to the removal of androgens (Gleave, Miyake et al. 2005). Medical castration is based on hormonal manipulation using gonadotropin-releasing hormone (GnRH) agonists and/or anti-androgens. Although initially effective, androgen ablation frequently leads to the development of castration-resistant (androgen-independent) prostate cancer, which is generally also resistant to other available treatments. At this stage treatment options are limited and may include second round hormone therapy or chemotherapy. The chemotherapeutic agent docetaxel is the only agent proven to prolong the lives of men with castration-

resistant disease, although the improvements are typically modest with a median extension of survival time of 2.9 months (Tannock, de Wit et al. 2004; Berthold, Pond et al. 2008; Antonarakis, Carducci et al. 2009). Castration resistance commonly marks the end-stage of prostate cancer and is the major obstacle toward improvement of patient survival.

Development of castration-resistant prostate cancer is characteristically associated with marked increases in resistance to apoptosis, a major death pathway for drug action (Raffo, Perlman et al. 1995; Miyake, Nelson et al. 2000; Rocchi, So et al. 2004; Gleave, Miyake et al. 2005). Apoptosis resistance, resulting from up-regulation of anti-apoptotic genes and their products, is thought to be a key contributor to the development of castration resistance, as well as general resistance to anti-cancer treatments. As such it forms a major hurdle in effective therapy (Makin and Dive 2001; Gleave, Miyake et al. 2005). Elucidating the role of anti-apoptotic genes and their products in prostate cancer progression is therefore likely to lead to improvements in the treatment of refractory disease.

1.2 Apoptosis and cancer

Apoptosis is a cell suicide program that functions to eliminate malfunctioning or unwanted cells. It is essential in tissue homeostasis, preventing the accumulation of cells which could prove detrimental to tissue function and overall health. Apoptosis can be initiated via two different pathways, the intrinsic pathway which is initiated by the release of cytochrome c from the mitochondrial

intermembrane space and the extrinsic pathway which is initiated by extracellular receptor-ligand binding (i.e., Fas to Fas-Ligand).

Activation of the intrinsic pathway for apoptosis (Fig. 1) is initiated by the release of cytochrome c from the mitochondrial intermembrane space. This pathway has also been called the stress-activated apoptotic pathway since its activating signals originate from within the cell in response to stresses such as hypoxia, DNA damage and growth factor withdrawal (Ashkenazi 2002). Release of cytochrome c is regulated by the activities of the Bcl-2 family of proteins which regulate the permeability of the outer mitochondrial membrane. Cellular stresses trigger apoptosis by promoting the translocation of pro-apoptotic Bcl-2 family members from the cytosol into the outer mitochondrial membrane, causing the release of cytochrome c (Zimmermann, Bonzon et al. 2001). Conversely, anti-apoptotic Bcl-2 family members inhibit the permeabilization of the outer mitochondrial membrane, thereby blocking cytochrome c release and inhibiting apoptosis (Zimmermann, Bonzon et al. 2001). Once released into the cytosol, cytochrome c binds with Apaf-1 to form the apoptosome (Zou, Li et al. 1999). The apoptosome is responsible for activating the caspases; a family of cysteine-aspartic acid-specific proteases, present in a pro-form which is activated via cleavage (Burz, Berindan-Neagoe et al. 2009). The apoptosome functions to activate the initiator caspase, caspase 9. Caspase 9 then proceeds to activate the executioner caspase, caspase 3; which then proceeds to activate additional executioner caspases (i.e., caspases 6, 7) (Burz, Berindan-Neagoe et al. 2009). The executioner caspases then cleave various substrates in the cell leading to its

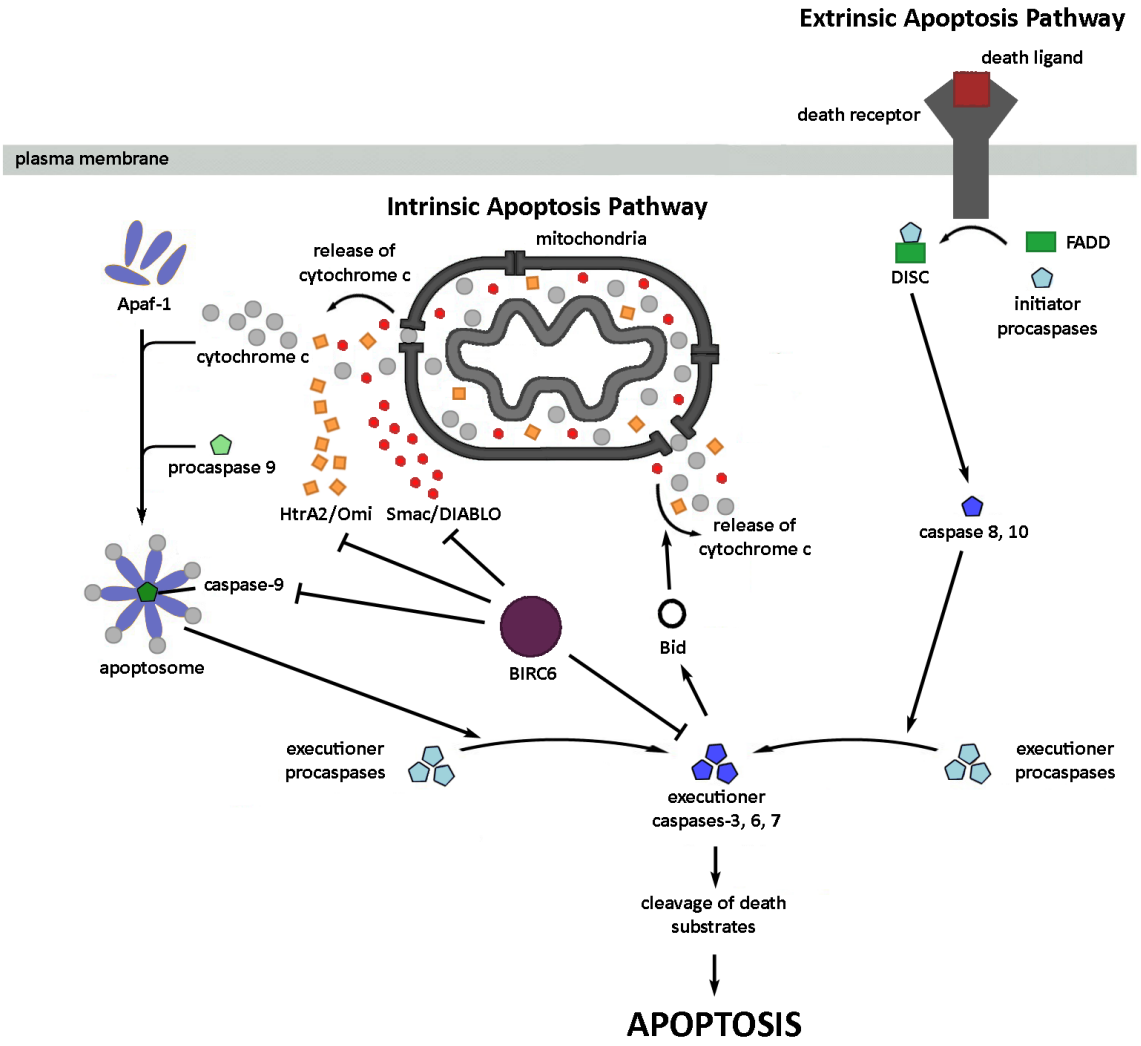

Figure modified from Weinberg 2007 (Weinberg 2007)

Figure 1. Intrinsic and extrinsic apoptosis pathways. Purple circle depicting BIRC6 and its role as an inhibitor of apoptosis.

controlled disassembly and death (Zimmermann, Bonzon et al. 2001).

The extrinsic apoptotic pathway (Fig. 1), also known as the receptor-activated apoptotic pathway, is initiated by the binding of ligands (i.e., TNF-α, TRAIL, FasL) to death receptors (i.e., TNFR1, DR4, DR5, Fas) at the cell's surface (Ashkenazi and Dixit 1998). Receptor-ligand binding results in the activation of death domains in the receptors and the recruitment and activation of Fas-associated death domain (FADD) protein. The initiator caspase, procaspase 8 then binds to FADD, forming the death-inducing signalling complex (DISC), which activates caspase 8 (Kischkel, Hellbardt et al. 1995). Caspase 8 then proceeds to cleave and activate the executioner caspases 3, 6 and 7, which are common to both the intrinsic and extrinsic apoptotic pathways. As with the intrinsic pathway, activation of the executioner caspases results in the controlled disassembly and death of the cell (Zimmermann, Bonzon et al. 2001). Once activated, the caspases also activate Bid, a protein which amplifies the apoptotic signals by causing the mitochondrial release of cytochrome c, thereby concurrently activating the intrinsic apoptotic pathway (Li, Zhu et al. 1998; Gross, Yin et al. 1999). Experimentally, apoptosis can be readily detected by Western blot analysis for expression of activated caspases and their early cleavage products, i.e., cleaved caspase-3 and cleavage products of poly ADP-ribose polymerase (PARP), a substrate of caspase-3 (Duriez and Shah 1997).

In cancer, elevated expression of anti-apoptosis genes/gene products has been demonstrated to promote tumourigenesis and resistance to therapy (Li, Marani et al. 2001; Nijhawan, Fang et al. 2003; Amantana, London et al. 2004;

Yamanaka, Rocchi et al. 2005). The ability of cancer cells to resist apoptosis represents a major impediment to the development of effective anti-cancer therapies, since the majority of current anti-cancer therapies execute their cytotoxicity at least in part through the induction of apoptosis (Li, Feng et al. 2001; Makin and Dive 2001; Ikuta, Takemura et al. 2005). Elevated expression of proteins such as heat shock protein 27 (Hsp27) in prostate cancer provide evidence for the critical role of apoptosis inhibitors in cancer cell survival and treatment resistance. Hsp27 expression is elevated in castration-resistant prostate cancers and studies have shown that anti-sense oligonucleotide or siRNA targeting of Hsp27 in androgen-independent PC3 prostate cancer cells was capable of inducing apoptosis and sensitizing the cells to the chemotherapeutic paclitaxel both *in vitro and in vivo* (Rocchi, So et al. 2004). Thus, the identification of anti-apoptotic genes associated with prostate malignancy and especially castration-resistant disease are likely to offer novel targets for prostate cancer therapy, with the potential to control disease progression and treatment resistance.

1.3 The Inhibitors of Apoptosis Protein (IAP) family

The first members of the Inhibitors of Apoptosis Protein (IAP) family were characterized as baculovirus gene products with anti-apoptotic function believed to have been utilized by the viruses to prevent a defensive apoptotic response in host insect cells, enabling viral propagation (Harvey, Soliman et al. 1997; Uren, Coulson et al. 1998). Several homologous proteins have now been identified in

organisms ranging from drosophila to humans. Eight human IAPs have so far been identified, namely Neuronal Apoptosis Inhibitory Protein (BIRC1/NAIP), Cellular-IAP1 (BIRC2/HIAP2/c-IAP1), Cellular-IAP2 (BIRC3/HIAP1/c-IAP2), X-linked IAP (XIAP/BIRC4), Survivin (BIRC5), Apollon (BIRC6), Livin (BIRC7/KIAP/ML-IAP) and IAP-like Protein-2 (BIRC8/ILP-2) (Rothe, Pan et al. 1995; Roy, Mahadevan et al. 1995; Liston, Roy et al. 1996; Ambrosini, Adida et al. 1997; Chen, Naito et al. 1999; Vucic, Stennicke et al. 2000; Richter, Mir et al. 2001). The IAP family members share in common the presence in the proteins of one to three copies of a Baculoviral IAP Repeat (BIR) domain. Members of the family are categorized into type 1 and type 2 IAPs. Type 2 IAPs play a role in cell mitosis and include Survivin (Ruchaud, Carmena et al. 2007) and BIRC6 (Pohl and Jentsch 2008). The other six human IAPs belong to the type 1 group, and have roles in apoptosis and immunity (Robertson, Croce et al. 2006). The IAPs have been demonstrated to bind to and inhibit a variety of pro-apoptotic factors, involved in intracellular apoptotic signalling, thereby effectively suppressing apoptosis induced by a wide range of effectors, including chemotherapeutics and irradiation (LaCasse, Baird et al. 1998). The BIR domain, an approximately 70 amino acid zinc-binding domain, is essential for interaction of the IAPs with the pro-apoptotic factors, which include the caspases.

In humans, the anti-apoptotic function of the IAPs is antagonized by SMAC/DIABLO and HtrA2/OMI, mammalian homologues of a Drosophila family of IAP antagonists (Reaper family), which are thought to bind to IAPs and displace active caspases (Martins 2002). Upon activation of the intrinsic

apoptosis pathway, SMAC/DIABLO and HtrA2/OMI are released into the cytoplasm, along with cytochrome c, where they counteract the anti-apoptotic function of the IAPs, allowing the caspases to trigger downstream apoptotic events. Over-expression of HtrA2/OMI has been demonstrated to sensitize cells to apoptosis (Suzuki, Imai et al. 2001; Hegde, Srinivasula et al. 2002; Martins, Iaccarino et al. 2002), whereas RNAi knockdown of HtrA2/OMI in cultured cells reduces cell death (Martins, Iaccarino et al. 2002). Htra2/OMI induces apoptosis both in a caspase–independent manner via its protease activity and caspase-dependent manner via the interference of caspase-IAP interaction (Hegde, Srinivasula et al. 2002). Similarly, over-expression of SMAC/DIABLO can increase a cell's sensitivity to apoptotic stimuli (Du, Fang et al. 2000).

Many pro- and anti-apoptotic proteins exist, and it is likely that the balance of the various pro- and anti-apoptotic proteins expressed in a cell determine its sensitivity or insensitivity to apoptosis. Based on their function, the IAPs represent a model family of proteins whose expression, if found elevated in prostate cancer, could very likely play an important role in swaying the balance towards apoptosis insensitivity and lead to disease progression and treatment resistance.

1.4 Baculoviral IAP Repeat-Containing 6 (BIRC6)

Of the IAPs, BIRC6 has in general received relatively little attention. At 528 kDa, the BIRC6 protein is an unusually large member of the IAP family. It consists of a single N-terminal BIR domain and a unique C-terminal ubiquitin-

conjugating (UBC) domain (Fig. 2); the latter has chimeric E2/E3 ubiquitin ligase activity and also anti-apoptotic activity (Bartke, Pohl et al. 2004). Through its BIR domain, BIRC6 is capable of binding to and inhibiting active caspases, including caspases-3, 6, 7 and 9 (Bartke, Pohl et al. 2004) and, through its UBC domain, BIRC6 facilitates proteasomal degradation of pro-apoptotic proteins caspase-9 (Hao, Sekine et al. 2004), SMAC/DIABLO (Hao, Sekine et al. 2004; Qiu and Goldberg 2005) and HTRA2/OMI (Bartke, Pohl et al. 2004; Sekine, Hao et al. 2005). Such interactions have been shown to underlie BIRC6's ability to inhibit the caspase cascade and ultimately apoptosis (Fig. 1). Interestingly, the anti-apoptotic activity of BIRC6 is antagonized by the blocking of BIRC6-caspase binding by SMAC/DIABLO, and cleavage of BIRC6 by the proteases HtrA2, caspases 3, 6, 7 and 9, and to a lesser extent caspase 8 (Bartke, Pohl et al. 2004; Sekine, Hao et al. 2005). The reciprocal inhibitions of the anti-apoptotic BIRC6 protein and the pro-apoptotic proteins, SMAC/DIABLO, HtrA2/OMI and the caspases, indicate that a cell's sensitivity to apoptosis is likely determined by the relative activities/expression levels of these proteins. Therefore, elevated expression of BIRC6 is likely to promote resistance of a cell to apoptotic triggers, and blocking activity/expression of BIRC6 can be expected to lead to an increase in a cell's sensitivity to apoptotic stimulation.

As a type 2 IAP, BIRC6 is also a critical regulator of cytokinesis and therefore plays an important role in cell proliferation (Pohl and Jentsch 2008). Cytokinesis is the final stage of cell division and begins with the contraction of a plasma-membrane-anchored actomyosin ring, which leads to the formation of a cleavage

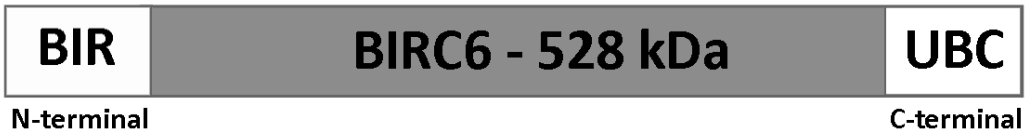

Figure 2. Domain structure of BIRC6.

furrow resulting in the formation of a narrow tubular intercellular bridge linking daughter cells (Eggert, Mitchison et al. 2006). This cell junction contains a group of tightly bundled anti-parallel microtubules which hold a circular structure called the midbody ring (Pohl and Jentsch 2008). During abscission, the final stage of cytokinesis, this junction is cleaved separating the two daughter cells. BIRC6 has been shown to be a major regulator of abscission, since the loss of BIRC6 expression in cells *in vitro* was demonstrated to cause defects in abscission leading to the formation of interconnected, syncitia-like cells and cytokinesis-associated apoptosis; defects in the formation of the midbody and midbody ring were also observed (Pohl and Jentsch 2008).

In transgenic mouse studies, the expression of wild type Birc6 (mouse homolog of BIRC6) has been found to be critical for viability. The generation of mice homozygous for a truncated form of Birc6 lacking the C-terminal end, including the UBC domain, has been found to result in embryonic lethality of these mice between 11.5 and 16.5 days of embryonic development (Hitz, Vogt-Weisenhorn et al. 2005; Ren, Shi et al. 2005). Furthermore, embryonic lethality was found to result from the progressive loss of the placental spongiotrophoblast layer in the developing placenta due to a lack of cell proliferation in this layer (Hitz, Vogt-Weisenhorn et al. 2005). In another study, embryonic lethality resulting from the expression of truncated Birc6 protein was reported to be due to the activation of apoptosis in the placenta and yolk sac of mouse embryos, indicating a role for Birc6 as an inhibitor of apoptosis (Ren, Shi et al. 2005). These studies demonstrate that Birc6 is an important inhibitor of apoptosis and

regulator of cell division in mice and is vital for normal mouse embryonic development.

1.5 BIRC6 expression in cancer

Recent evidence supports a widespread role for BIRC6 in conferring apoptosis resistance to cancer cells. BIRC6 protein expression has been detected in a variety of cancer cell lines and *in vitro* studies have shown that BIRC6 functions as an apoptosis inhibitor in glioma (SNB-78) (Chen, Naito et al. 1999), lung cancer (H460) (Ren, Shi et al. 2005), cervical cancer (Hela) (Qiu, Markant et al. 2004; Qiu and Goldberg 2005; Chu, Gu et al. 2008; Pohl and Jentsch 2008), fibrosarcoma (HT-1080) (Hao, Sekine et al. 2004; Chu, Gu et al. 2008), osteosarcoma (U2OS) (Pohl and Jentsch 2008) and breast cancer cells (MCF-7 (Chu, Gu et al. 2008), ZR75.1, MDA-MB-231 (Lopergolo, Pennati et al. 2009)). In breast and lung cancer cells, loss of BIRC6 expression has been demonstrated to trigger apoptosis through the stabilization of p53 and activation of caspase 3 (Ren, Shi et al. 2005; Lopergolo, Pennati et al. 2009).

BIRC6 expression in patients' cancer samples has been observed for colorectal cancer (Bianchini, Levy et al. 2006) and childhood *de novo* acute myeloid leukemia (AML) (Sung, Choi et al. 2007). In the latter, elevated expression of *BIRC6* mRNA was associated with poor prognosis as indicated by elevated leukocyte numbers, extramedullary disease, patients with unfavourable French-American-British classification (FAB) subtype M7, unfavourable response

to chemotherapy and poor 3-year relapse-free survival rates (Sung, Choi et al. 2007).

1.6 Expression of IAPs in prostate cancer

In vitro studies have demonstrated apoptosis-inhibitory activity of c-IAP2 (McEleny, Watson et al. 2002) and XIAP (Amantana, London et al. 2004) in prostate cancer cells; as well, elevated expression of cIAP-1, c-IAP2, XIAP, SURVIVIN (Krajewska, Krajewski et al. 2003) and Livin (Song, Hong et al. 2008) has been reported in patients' prostate cancer specimens, suggesting a role for these IAPs in prostate cancer. A role for BIRC6 in this disease, however, has not been reported.

1.7 Autophagy and cancer

Apoptosis is often associated with autophagy, a cellular process involving the degradation of a cell's own components through the lysosomal machinery (Bursch 2001). It involves the packaging of proteins and organelles within autophagosomes, followed by fusion of the latter with lysosomes leading to the degradation of the proteins and organelles. Autophagy can be essential for viability, differentiation and development of cells in mammals, and defects in autophagy have been associated with cancer (Tanida, Ueno et al. 2004; Corcelle, Puustinen et al. 2009; Chen and Debnath 2010). Autophagy is a survival pathway activated in response to stresses including nutrient deprivation,

metabolic stress and anti-cancer drugs (Chen and Karantza-Wadsworth 2009). Activation of autophagy (Fig. 3) begins when the mammalian target of rapamycin (mTOR) is inhibited by signals generated in response to cellular stress (Chen and Karantza-Wadsworth 2009). Following the inhibition of mTOR, activation of a protein complex involving Beclin-1/hVps34 results in the production of phosphatidylinositol 3-phosphates and marks the beginning of autophagosome formation (Pattingre, Espert et al. 2008). Autophagy-related gene (Atg) proteins, Atg12-Atg5 and Atg 8 (LC3) are then recruited to a developing double membrane structure called the isolation membrane. Cytosolic proteins and organelles are then sequestered by the isolation membrane, culminating in the formation of autophagosomes. The development of the mature autophagosome is marked by the covalent binding of LC3B-I to phosphatidylethanolamine of the autophagosome membrane, forming LC3B-II, followed by the recycling of Atg proteins to the cytosol (Pattingre, Espert et al. 2008). The final step in autophagy involves the fusion of autophagosomes with lysosomes to form autophagolysosomes. This last step results in the degradation of the autophagolysosomal contents including LC3-II (Pattingre, Espert et al. 2008).

The role of autophagy in the development of cancer and its treatment is unclear, since there is evidence that autophagy can support both promotion and suppression of cancer growth (Corcelle, Puustinen et al. 2009; Chen and Debnath 2010). Inhibition of autophagy by the disruption of essential autophagy genes (e.g., *atg 5*, *Beclin-1*) has been shown to promote tumorigenesis and as such demonstrates a tumour-suppressive function for autophagy (Liang, Jackson

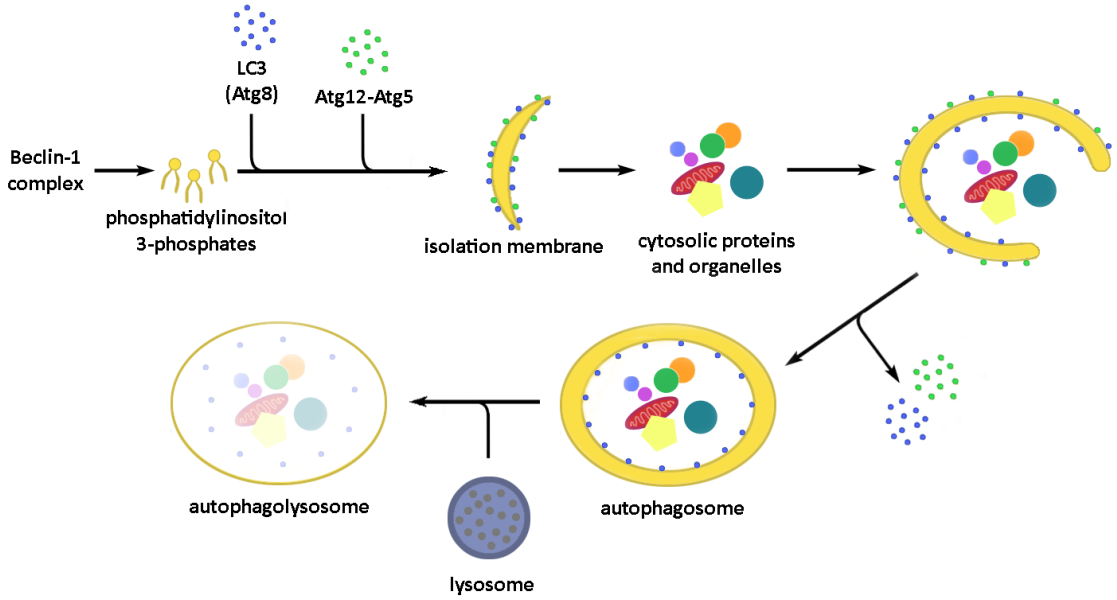

Figure modified from Pattingre *et al.* 2008 (Pattingre, Espert et al. 2008)

Figure 3. Autophagy pathway. Showing (1) the initiation of autophagosome formation, (2) sequestering of cytosolic proteins and organelles by the isolation membrane and the development of the mature autophagosome, (3) fusion of autophagosome with lysosome forming the autophagolysosome and the degradation of its contents.

et al. 1999; Qu, Yu et al. 2003; Yue, Jin et al. 2003; Iqbal, Kucuk et al. 2009). However, there is increasing evidence that autophagy can act as a survival mechanism of cells in response to a wide range of stresses, including nutrient starvation, hypoxia and treatment with anti-cancer agents (Corcelle, Puustinen et al. 2009; Chen and Debnath 2010). In fact, targeting autophagy has been shown to sensitize a variety of cancers to treatment, including prostate cancer (Apel, Herr et al. 2008; Kim, Coates et al. 2009; Vazquez-Martin, Oliveras-Ferraros et al. 2009).

In order to detect autophagic activity in cultured cells, Western blot detection of LC3B-II is often used, since LC3B-II is specifically associated with autophagosomes and levels of LC3B-II have been demonstrated to closely correlate with the number of autophagosomes within cells (Kabeya, Mizushima et al. 2000; Mizushima and Yoshimori 2007; Rubinsztein, Cuervo et al. 2009; Parikh, Childress et al. 2010). However, since LC3B-II is degraded upon autophagosome-lysosome fusion, LC3B-II levels offer only a snap shot of the number of autophagosomes in cells at one time point and does not necessarily indicate an upregulation or downregulation of the autophagic process in its entirety (Mizushima and Yoshimori 2007; Rubinsztein, Cuervo et al. 2009). Detection of other critical autophagy proteins like Beclin-1 can offer further insight into the activation of autophagy within these cells. The initiation of autophagosome formation involves a Beclin-1/Vps34 protein complex. Accordingly, Beclin-1 has been shown to be essential for autophagosome formation and thus autophagy (Liang, Feng et al. 2007; Levine, Sinha et al. 2008;

Pattingre, Espert et al. 2008; Kim, Coates et al. 2009; He and Levine 2010). Currently there is no evidence suggesting a role for IAPs in the regulation of autophagy in humans.

1.8 Rationale, hypothesis and objectives

Rationale:

Considering the lack of effective treatment options for men with advanced prostate cancer, the rationale for this study is to identify a role for BIRC6 in the survival of prostate cancer cells and in the development and progression of prostate cancer, with the purpose of identifying a novel potential target for the treatment of this disease.

Hypothesis:

The hypothesis for this thesis is that BIRC6 plays an essential role in the survival of prostate cancer cells and its expression is important in the progression of prostate cancer.

The following objectives have been designed in order to test the hypothesis:

The first objective is to investigate the expression of BIRC6 protein in benign and malignant prostate cell lines and clinical prostate samples, as well as clinical castration-resistant prostate cancers, in order to determine if prostate cancer, and in particular advanced disease, is associated with elevated BIRC6 expression.

The second objective is to investigate the role of BIRC6 in prostate cancer cell survival by knocking down *BIRC6* expression using siRNAs and determining the effects on cell viability, apoptosis and autophagy.

The third objective is to induce apoptosis in prostate cancer cells using doxorubicin and to determine the effect of apoptosis induction on the expression of BIRC6.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

Chemicals, solvents, and solutions were obtained from Sigma-Aldrich Canada Ltd, Oakville, ON, Canada, unless otherwise indicated.

2.2 Clinical prostate cancer tissues

Specimens were obtained from patients, with their informed consent, following a protocol approved by the University of British Columbia - British Columbia Cancer Agency Research Ethics Board (REB NUMBER: H04-60131). Gleason-graded tissue microarrays (TMAs) were used consisting of 35 benign prostate specimens, 6 prostatic-intraepithelial neoplasia specimens and 167 radical prostatectomy prostate cancer specimens. The prostate cancer specimens consisted of 74 Gleason score 6, 23 Gleason score 7, 43 Gleason score 8, 2 Gleason score 9, and 15 Gleason score 10 prostate cancer specimens that had not been subjected to neo-adjuvant hormone therapy and 10 prostate cancer specimens which had been subjected to neo-adjuvant hormone therapy and had progressed to castration-resistant prostate cancer. The latter 10 specimens had Gleason scores of 8 (n=6) and 10 (n=4) prior to therapy. Samples for TMA construction had been selected randomly from collections at the Vancouver Prostate Centre, Vancouver General Hospital (supplied by the Department of Pathology, University of British Columbia, Vancouver, BC, Canada). Human

prostate tissue samples were fixed in 10% neutral-buffered formalin and embedded in paraffin. Samples with sufficient amounts of carcinoma or benign prostatic epithelium were identified via Hematoxylin and Eosin (H&E) staining of tissue sections. For TMA construction, tissue cores were taken from each tissue sample using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). Following immunohistochemical staining for BIRC6, each array spot was given a score based on staining intensity.

2.3 Immunohistochemistry for BIRC6 protein expression

Serial sections (5 µm thick) were cut from TMAs on a microtome, mounted on glass slides, de-waxed in xylene and then hydrated using graded alcohol solutions and distilled water. Sections were subjected to antigen retrieval by boiling for 10 min in antigen-unmasking solution (Vector Laboratories Inc.; Burlingame, CA). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min followed by washing with phosphate buffered saline (PBS; pH 7.4). SuperBlock blocking buffer (Thermo Scientific; Rockford, IL) in tris buffered saline (TBS; pH 7.4) was applied to the sections for 60 min to block nonspecific sites. The sections were then incubated with rabbit polyclonal anti-BIRC6 antibody (Novus Biologicals, Littleton, CO) recognizing epitopes between residue 4775 and 4829 (C-terminus) of BIRC6 (Swiss-Prot entry Q9NR09; GeneID 57448) at a 1:100 dilution at 4°C overnight. After washing and further incubation with goat anti-rabbit secondary antibodies for 30 min at room temperature, the sections were washed in PBS (five 5-min washes), and then

incubated with avidin-biotin complex (Vector laboratories, Foster City, CA) for 30 min at room temperature. Following 25 min of washing in PBS (five 5-min washes), immunoreactivity was visualized using 3', 3'-diaminobenzidine (DAB) in PBS and 3% H_2O_2, generating a brown stain for regions positive for BIRC6 protein expression. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

To confirm the specificity of the anti-BIRC6 antibody, clinical ovarian cancer specimens were used as positive control tissues, as suggested by the manufacturer (Novus Biologicals) and strong positive BIRC6 staining in these tissues was observed (Figure 4A). In addition, a blocking peptide (Novus Biologicals) was used to confirm that staining in prostate cancer tissues was specific for BIRC6. Incubation of prostate cancer tissue sections with the anti-BIRC6 antibody and a BIRC6 blocking peptide (Novus Biologicals) at a ratio of 1:10 led to complete absence of BIRC6 staining indicating high specificity of the antibody (Figure 4B).

2.4 Scoring of BIRC6 protein expression

BIRC6 staining in tissue samples was evaluated by a pathologist and given a score of 0, 1, 2 or 3, representing no, weak, moderate or strong BIRC6 staining intensities, respectively. Mean staining intensities were then calculated for sections of benign prostate tissues, prostatic intraepithelial neoplasia tissues, sections of Gleason score 6, 7, 8, 9 and 10 prostate cancer tissues and castration-resistant prostate cancer tissues. Percent positivity (as a measure of

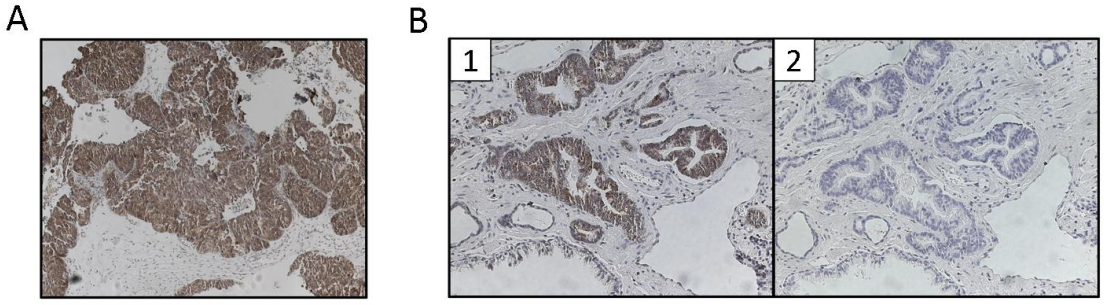

Figure 4. Immunohistochemistry positive control tissues. A, BIRC6 expression (brown stain) in patients' high grade serous ovarian carcinoma positive control tissue at 100× magnification. **B**, BIRC6 expression (brown stain) in serially cut clinical prostate cancer tissue sections (1) without blocking peptide and (2) with blocking peptide at 100× magnification.

staining frequency) was calculated for each group based on the number of sections with staining intensities of ‘1’ or higher.

2.5 Prostate cancer cell lines

Six human prostate cancer cell lines (LNCaP; PC3; PC3-M; DU145; C42; VCaP), two benign prostatic cell lines (BPH1, RWPE1) and two positive control cell lines (cervical and ovarian cancer) known to express BIRC6 (OVCAR-8, HeLa) were used (Chen, Naito et al. 1999). All cancer cell lines were cultured using RPMI-1640 medium. BPH1 cells were cultured using DMEM and RWPE1 cells were cultured using Keratinocyte-SFM (Gibco-BRL; Burlington ON, Canada). All media were supplemented with fetal bovine serum (10%), penicillin G (100 IU/mL) and streptomycin (100 µg/mL). Cells were cultured in a humidified incubator at 37°C and 5% CO_2. All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

To determine the effect of inducing apoptosis in LNCaP cells on BIRC6 expression, cells were seeded into 6-well plates at a density of 600,000 cells per well, incubated for 24 h to allow cell attachment and then further incubated with doxorubicin (1 µg/mL) for 24 h.

2.6 Western blotting

Cell lysates were prepared using cell lysis buffer (1% NP-40, 0.5% sodium deoxycholic acid) supplemented with a protease inhibitor cocktail (Roche, Basel,

Switzerland); total lysate protein was determined using the BCA protein assay (Pierce, Rockford, IL). For the detection of BIRC6 (528 kDa), 10 µg whole cell lysate was run on a two-part (5% and 12.5%) sodium dodecyl sulfate (SDS) polyacrylamide gel at 80 volts for 2.5 h. Gels were cut, and BIRC6 was electrotransferred to a polyvinylidene fluoride (PVDF) membrane at 25 volts for 3 h in transfer buffer (Tris 25 mM, Glycine 191.5 mM, Methanol 10%, SDS 0.05%) using a semi-dry transfer apparatus. To ensure equivalent loading and transfer of protein, actin (42 kDa) was electrotransferred to PVDF membrane separately at 100 volts for 1 h at room temperature with transfer buffer [Tris (25 mM), Glycine (191.5 mM), Methanol (15%)] using standard wet transfer. Nonspecific binding was blocked with Tris buffered saline + Tween 20 (TBS-T buffer [0.5 mmol/L Tris-HCl, 45 mmol/L NaCl, 0.075% Tween 20 (pH 7.4)]) containing 5% bovine serum albumin (BSA). BIRC6 membranes were first probed with rabbit polyclonal anti-BIRC6 antibody at 1:500 dilution (Novus Biologicals) and actin membranes with rabbit anti-actin polyclonal antibody (Sigma-Aldrich) at 1:2000 dilution overnight at 4°C. Membranes were then washed, probed with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Pierce) and developed. The chemoluminescent signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). OVCAR-8 (Chen, Naito et al. 1999) and Hela (Bartke, Pohl et al. 2004) cells were used as positive controls.

For the detection of full length PARP (116 kDa), cleaved PARP (89 kDa), full length caspase-3 (35 kDa), cleaved caspase-3 (17 and 19 kDa), LC3B (LC3B-I, 16 kDa and LC3B-II, 14 kDa) and Beclin-1 protein expression, the above

conditions were used with the following adjustments. Five to 15 µg of whole cell lysate was run on 10% or 12.5% SDS polyacrylamide gels. PARP, caspase-3, LC3B, Beclin-1 and the appropriate loading control proteins were electrotransferred to PVDF membranes at 100 volts for 80 min using standard wet transfer. Membranes were probed for PARP, caspase-3, LC3B and Beclin-1 using rabbit anti-PARP and anti-caspase-3 antibodies (Cell Signaling; Beverly MA) at 1:1000 dilution, rabbit anti-LC3B antibodies (Abcam, Cambridge, MA) at 0.85:1000 dilution and rabbit anti-Beclin-1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 1:1000 dilution with an overnight incubation at 4°C. Depending on the size of the target protein, actin (42 kDa) or vinculin (117 kDa) were used as controls to ensure equivalent loading and transfer of protein. For the detection of Vinculin, membranes were incubated with mouse anti-Vinculin antibody (Sigma-Aldrich) at a 1:3000 dilution overnight at 4°C and probed with a secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce).

2.7 Small interfering RNA (siRNA) and cell transfection

Custom siRNAs synthesized by Dharmacon (Lafayette, CO) and known to target *BIRC6* had the following sequences: BIRC6 siRNA-1, sense, 5'-GUU UCA AAG CAG GAU GAU G-dTdT-3' (Ren, Shi et al. 2005) and BIRC6 siRNA-2, sense, 5'-CUC AGG AGA GUA CUG CUC A-dTdT-3' (Pohl and Jentsch 2008). A third, non-targeting siRNA (siGENOME Non-Targeting siRNA #3 D-001210-03-05, Dharmacon) was used as an experimental control. siRNAs were diluted in 1× siRNA buffer (Dharmacon). To examine the effect of the siRNAs on BIRC6

protein, LNCaP cells were plated in 6-well plates in antibiotic-free RPMI-1640 medium supplemented with fetal bovine serum (10%). After 24 h, the cells were transfected using 100 nM siRNA in lipofectamine 2000 reagent (Invitrogen; Burlington, ON) following the manufacturer's instructions. Briefly, lipofectamine 2000 was diluted in Opti-MEM (Invitrogen) and incubated at room temperature for 5 min. In a separate tube, siRNA was diluted in Opti-MEM. The diluted lipofectamine 2000 was added to the diluted siRNA and the complex incubated for 20 min at room temperature. This complex was then gently added to each well (500 µL). After 30 h, the transfection mixture was removed and fresh antibiotic-free RPMI-1640 media supplemented with fetal bovine serum (10%) was added. Vehicle control (lipofectamine 2000) and non-targeting siRNA were applied to replicate cell cultures. After the transfection, Western blotting and MTT cell proliferation assays were performed to determine the effect of loss of BIRC6 protein expression on LNCaP cells.

2.8 MTT cell proliferation assay

Cells were seeded at a density of 25,000 cells per well in a 24-well dish and transfected with siRNAs as described above (scaled down to appropriate volumes for 24-well dishes). The siRNA-2 sequence was used for all MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] viability assay experiments. At 0, 24, 48 and 72 h following transfection, 50 µL of MTT (5 mg/mL) was added to each well and cultures were incubated in a humidified incubator at 37°C and 5% CO_2 for 4 h. The colour of the media changed from yellow to dark

purple as viable cells broke down and converted the tetrazolium substrate to its formazan product. SDS solution (20%) was then added (500 µL) to each well with a further incubation overnight at room temperature in the absence of light. Samples (100 µL) were then transferred to 96-well plates for measurement of absorbance at 570 nm using a micro-plate reader.

2.9 Statistical analysis

The Student's *t*-test was used to determine the significance of differences in mean BIRC6 staining intensities of clinical prostate cancer samples and differences in cell viability of siRNA-treated cells. Results with a *P*-value <0.05 were considered significant.

CHAPTER 3: RESULTS

3.1 Elevated BIRC6 protein expression in human prostate cancer cell lines

Western blotting revealed strong BIRC6 protein expression in all prostate cancer cell lines examined, i.e. PC3, PC3M, DU145, LNCaP, C42, VCaP (Fig. 5). In contrast, only low levels of BIRC6 protein expression were detected in BPH1 and RWPE1 benign prostate cell lines. Moderate to strong BIRC6 expression was detected in OVCAR8 and Hela as reported by others (Chen, Naito et al. 1999; Bartke, Pohl et al. 2004). The differential in BIRC6 expression between benign and malignant prostate cells indicates that BIRC6 likely plays a role in the development of prostate cancer.

3.2 Elevated BIRC6 protein expression in clinical prostate cancer tissues

3.2.1 BIRC6 protein expression in Gleason scored prostate cancer tissues

Clinical prostate tissue sections, morphologically categorized into benign tissue and cancers of Gleason score 6, 7, 8 and 9+10, were stained and scored for BIRC6 expression. As shown in Figure 6, positive cytoplasmic staining for BIRC6 was, in general, low in benign epithelium, substantially more intense in well differentiated Gleason grade 3 and strongest in poorly differentiated Gleason grade 4 prostate cancer tissues. Gleason grade 5 tissues generally expressed low levels of BIRC6 staining in comparison with the other prostate cancer tissues examined, similar to benign tissues. Sections containing both benign tissue and

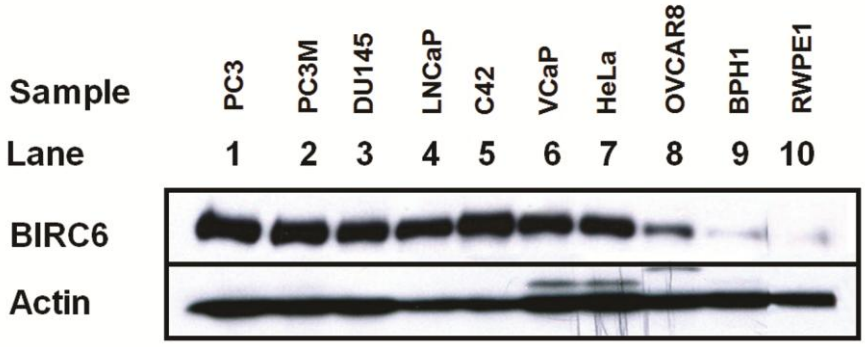

Figure 5. BIRC6 protein expression in prostate cancer cell lines. BIRC6 protein expression is elevated in prostate cancer cells in comparison with benign prostate cells as shown by Western blot analysis. BIRC6 protein expression in malignant prostate cells (Lanes 1-6), positive control malignant cervical and ovarian cells (Lanes 7, 8) and benign prostate cells (Lanes 9, 10). The results are representative of three independent experiments.

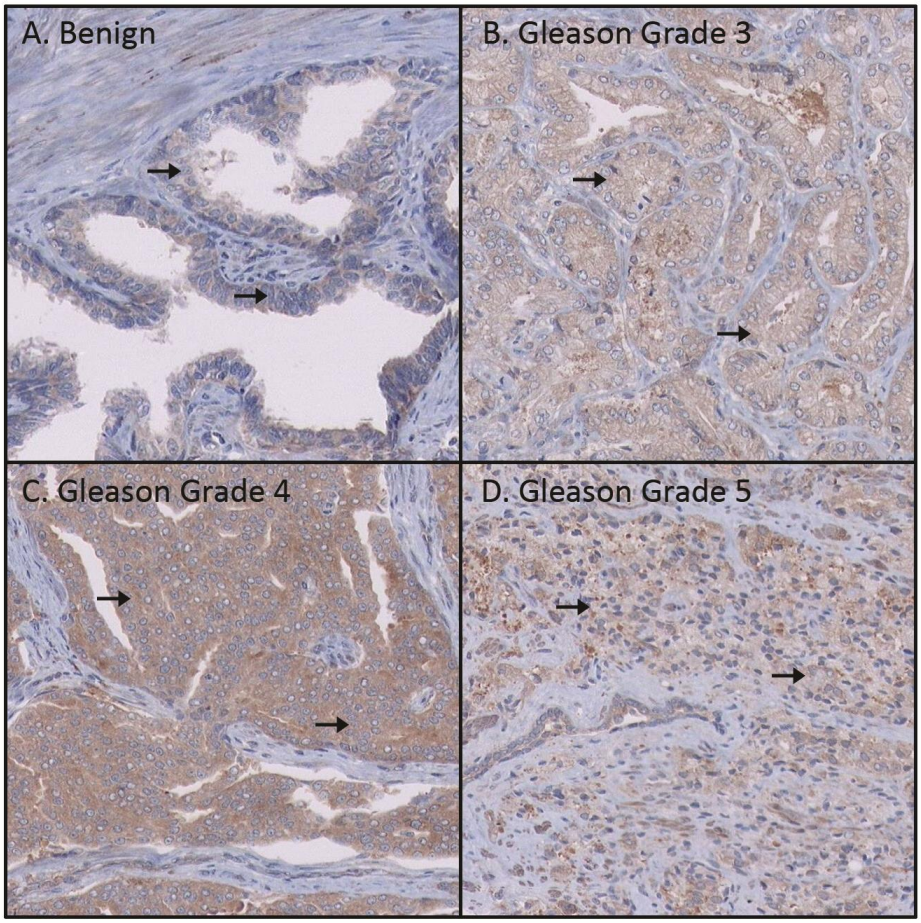

Figure 6. BIRC6 protein expression in patients' prostate cancer tissues. BIRC6 protein expression (brown stain) in TMA sections of prostate tissue samples. A, black arrows showing weak cytoplasmic BIRC6 staining in benign prostatic luminal cells (score '1'). B, black arrows showing moderate cytoplasmic BIRC6 staining in Gleason grade 3 prostate cancer cells (score '2'). C, black arrows showing strong cytoplasmic BIRC6 staining in Gleason grade 4 prostate cancer cells (score '3'). D, black arrows showing weak cytoplasmic BIRC6 staining in Gleason grade 5 prostate cancer cells (score '1'). All images taken at 200× magnification.

cancer tissue (Gleason grade 3) showed strong positive BIRC6 staining in the malignant epithelium and absent or weak expression in the benign epithelium (Fig. 7). Mean staining intensities of prostatic intraepithelial neoplasia (PIN) were slightly elevated compared to benign epithelium, although not significantly (data not shown). In the Gleason scored tissues (Fig. 8), BIRC6 expression was low for benign tissues, but substantially higher in the Gleason score 6, 7 and 8 cancer tissues, with a peak value in Gleason score 7 cancers. BIRC6 expression in Gleason score 9+10 specimens was similar to that of benign tissues. The mean staining intensities for each group were 1.00±0.80 S.D. for benign epithelium and 1.53±0.92 S.D. (P=3.24×10^{-3}) for Gleason score 6, 2.22±0.90 S.D. (P=4.45×10^{-6}) for Gleason score 7 and 1.60±0.82 S.D. (P=1.63×10^{-3}) for Gleason score 8 prostate cancer tissues. The intensity of BIRC6 expression in Gleason score 9+10 prostate cancer tissues was statistically no different from that of benign tissues (0.71±0.47 S.D.; P=0.10). In addition, the low intensity of BIRC6 expression observed in Gleason score 9+10 prostate cancer tissues was reflective of the large proportion of typically weak BIRC6 expressing Gleason grade 5 cancers found in this group.

With regard to the frequency of BIRC6 expression, Gleason score 6, 7 and 8 malignant epithelia more frequently expressed BIRC6 (staining intensity score ≥ 1) than benign prostate epithelium (Fig. 8). All malignant tissues showed a high frequency of BIRC6 expression coupled to high BIRC6 intensity, except for Gleason score 9+10 tissues which showed a high frequency of BIRC6 expression coupled to a low average intensity. Taken together, the data (Figs 6-8)

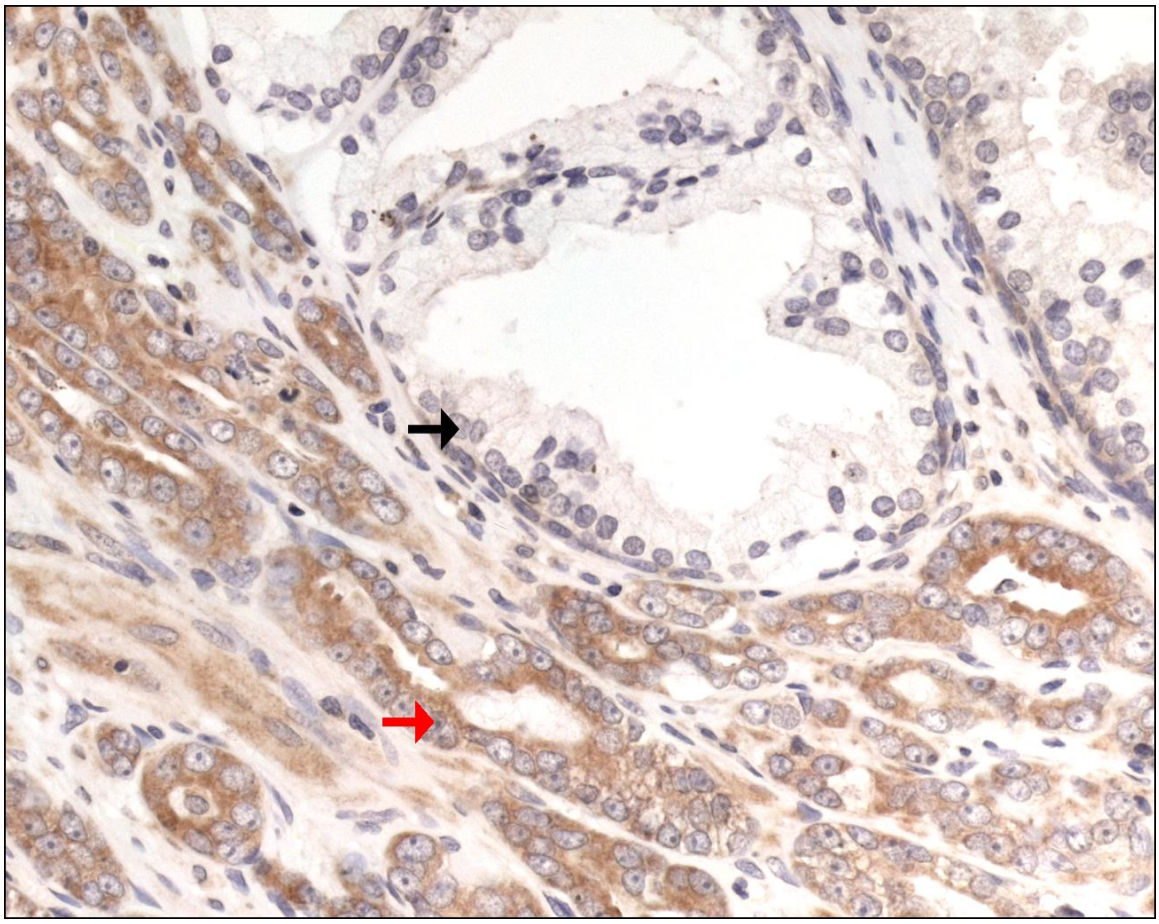

Figure 7. Differential expression of BIRC6 protein in benign and malignant prostate epithelium. Black arrow, showing absence of BIRC6 expression in benign prostate epithelium and red arrow, showing moderate BIRC6 expression in Gleason grade 3 malignant epithelium. Image taken at 400× magnification.

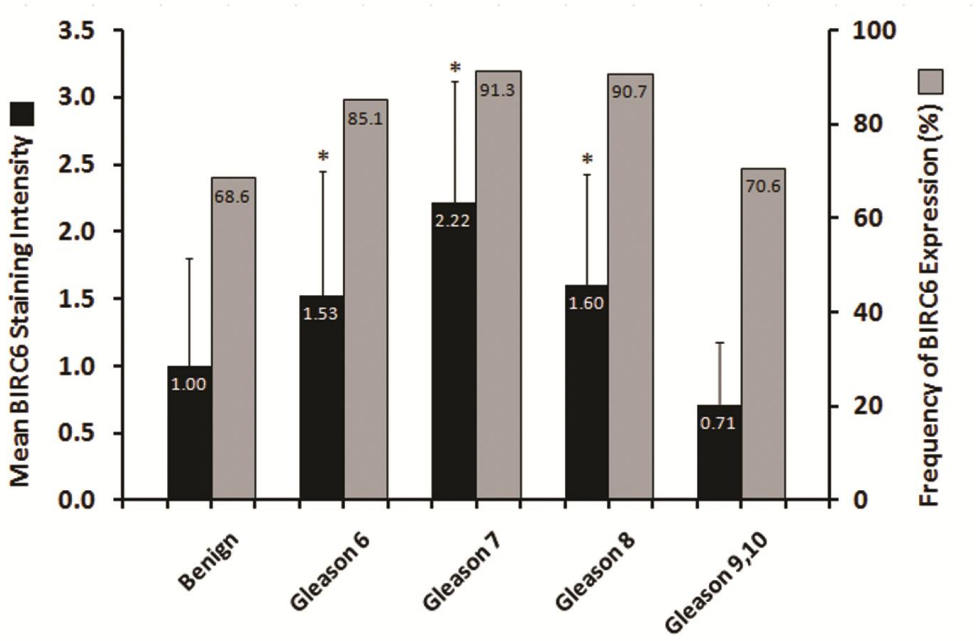

Figure 8. Summary of BIRC6 protein expression in Gleason scored prostate cancer tissues. Black bars showing mean BIRC6 staining intensity in benign prostate epithelium (n=35) and Gleason score 6 (n=74), 7 (n=23), 8 (n=43) and 9+10 (n=17) patient prostate cancer tissues. Grey bars showing frequency of BIRC6 expression in each group by percent positivity, as defined by weak, moderate or strong staining intensities (scores ≥ '1'). Elevated staining intensities were observed in Gleason score 6 (**P*=3.24×10^{-3}), 7 (**P*=4.45×10^{-6}) and 8 (**P*=1.63×10^{-3}) prostate cancer tissues as compared to benign prostate epithelium. Error bars depict standard deviations.

indicate that prostate cancer progression from benign to Gleason score 8 prostate cancers is associated with elevations in BIRC6 protein expression.

3.2.2 BIRC6 protein expression in castration-resistant prostate cancers

Tissue sections from patients' prostate cancers (Gleason score 8 and 10) which had progressed to castration resistance following neo-adjuvant hormone therapy (n=10) were stained and scored for BIRC6 expression and compared with sections of high grade prostate cancers (Gleason score 8, 9, 10) which had not been subjected to neo-adjuvant hormone therapy (n=60). As shown in Figure 9, the mean staining intensity for BIRC6 was significantly higher in castration-resistant prostate cancers than in the untreated cancers (2.30±0.67 S.D. and 1.35±0.84 S.D. respectively; $P=1.38\times10^{-3}$). The frequency of BIRC6 expression was very high in both types of tissue. The data suggest that the development of castration-resistant prostate cancer is associated with elevations in BIRC6 protein expression.

3.3 Reduction of BIRC6 expression in LNCaP prostate cancer cells

3.3.1 Effect on cell viability

It has been reported that reduction of BIRC6 expression leads to an increased rate of apoptosis (e.g., in breast cancer cells) and that p53 plays a role in this process (Ren, Shi et al. 2005; Lopergolo, Pennati et al. 2009). We therefore used the LNCaP cell line, known to express wild type p53 (Planchon,

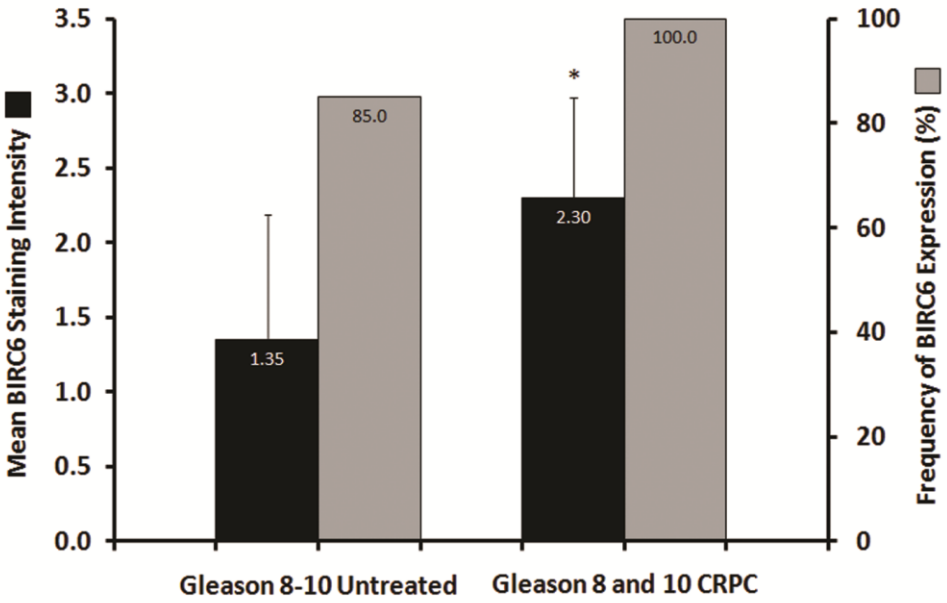

Figure 9. Summary of BIRC6 protein expression in castration-resistant prostate cancer tissues. Black bars showing mean BIRC6 staining intensity in tissues of untreated high grade (Gleason score 8-10) prostate cancers (n=60) and in tissues of castration-resistant prostate cancers (CRPC) which had developed from Gleason score 8 and 10 cancers following neo-adjuvant hormone therapy (n=10). Grey bars showing frequency of BIRC6 expression in each group by percent positivity, as defined by weak, moderate or strong staining intensities (scores ≥ '1'). Castration-resistant prostate cancer tissues showed a significantly higher mean BIRC6 staining intensity than tissues from untreated high grade prostate cancers (*P=1.38×10^{-3}); both types of tissue highly expressed BIRC6. Error bars depict standard deviations.

Wuerzberger et al. 1995), for studying the effect of reducing BIRC6 expression on prostate cancer cell viability. As shown in Figure 10, incubation of LNCaP cells transfected with *BIRC6*-targeting siRNA-1 and -2 (Lanes 3, 4, 7, 8, 11, 12) resulted in substantial loss of BIRC6 protein, compared to cells treated with lipofectamine only (Lanes 2, 6, 10), lipofectamine + non-targeting siRNA (Lanes 5, 9, 13) or no treatment (Lane 1). The effect was already apparent after the 30 h of pre-incubation (at h 0) and became more prominent at 24 h and 48 h of further incubation. It may be noted that cells transfected with lipofectamine only or with lipofectamine + non-targeting siRNA showed a small increase in BIRC6 expression, presumably due to the vehicle. Since the knockdown of BIRC6 protein expression was greater in the siRNA-2-treated cells in comparison with the siRNA-1-treated cells at 48 h, all subsequent knockdown experiments were conducted using siRNA-2.

Following the transfection period, the siRNA-2-transfected cultures showed a marked reduction in cell proliferation relative to the non-targeting siRNA-treated cultures (Fig. 11). Thus the relative cell numbers in the siRNA-2 cultures were significantly lower than those in the non-targeting siRNA-treated cultures by 83.0% (P=2.52×10^{-3}), 62.0% (P=6.08×10^{-4}) and 53.5% (P=2.00×10^{-5}) at 24, 48 and 72 h, respectively. The reduction of cell proliferation by non-targeting siRNA is likely due to non-specific toxicity. Similar results were obtained by cell counting using trypan blue exclusion (data not shown). siRNA-2-treated cells were found to form syncytia-like structures in which clusters of cells were joined by

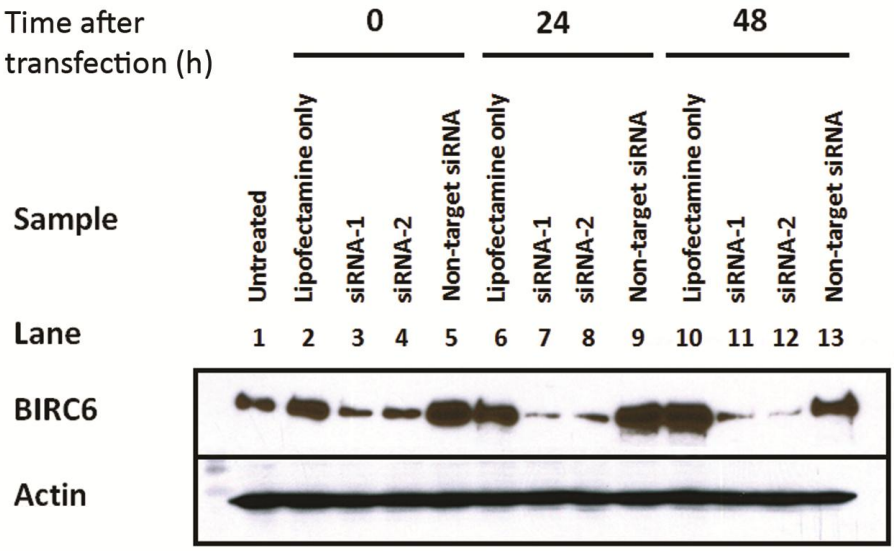

Figure 10. siRNA reduction of BIRC6 protein expression in LNCaP prostate cancer cells. Treatment of LNCaP cell cultures with siRNAs targeting *BIRC6* leads to reduction of BIRC6 protein expression as shown by Western blot analysis. BIRC6 protein expression in untreated LNCaP cells (Lane 1; at 48 h) and in LNCaP cells incubated with lipofectamine only (Lanes 2, 6, 10), siRNA-1 targeting *BIRC6* (Lanes 3, 7, 11), siRNA-2 targeting BIRC6 (Lanes 4, 8, 12) or non-targeting siRNA (Lanes 5, 9, 13) for 0, 24 and 48 h following transfection. The results are representative of three independent experiments.

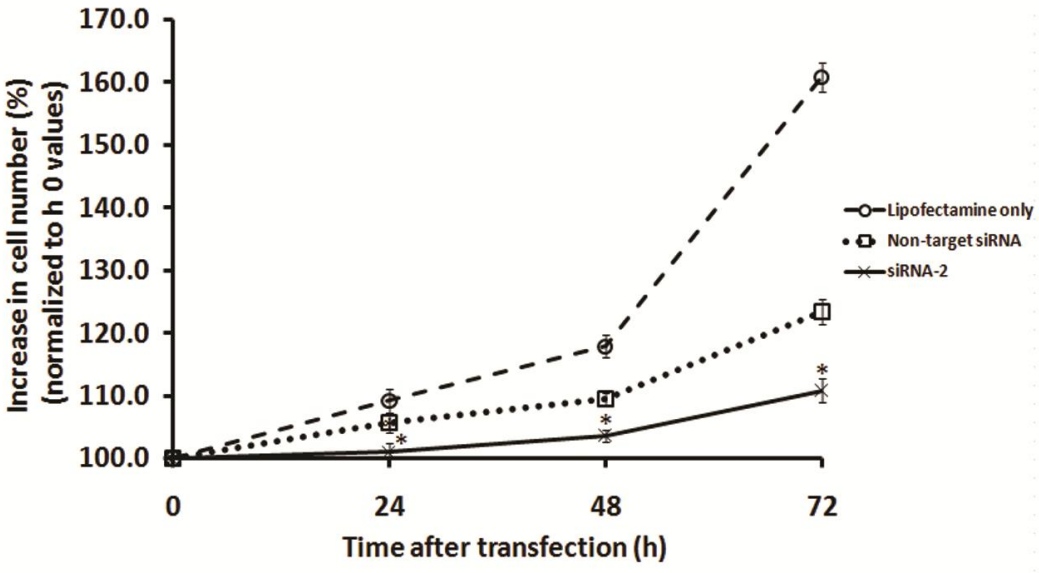

Figure 11. Proliferation of BIRC6 protein-depleted LNCaP prostate cancer cells. Treatment of LNCaP cell cultures with siRNA-2 targeting BIRC6 leads to reduced cell proliferation as shown by MTT assay. Cultures were treated for 30 h with lipofectamine only or with lipofectamine plus either non-targeting siRNA or siRNA-2 targeting *BIRC6* and then incubated for 24, 48 and 72 h in fresh media. The relative cell numbers in the siRNA-2 cultures were significantly lower than those in the non-targeting siRNA treated cultures by 83.0% (**P*=2.52×10^{-3}), 62.0% (**P*=6.08×10^{-4}) and 53.5% (**P*=2.00×10^{-5}) at 24, 48 and 72 h, respectively. Error bars depict standard deviations. The results are representative of two independent experiments.

long spindle-like projections (Fig. 12). These syncytia-like structures were not observed in the untreated and non-targeting siRNA control cultures.

3.3.2 Effect on apoptosis and autophagosome formation

As shown in Figure 13, incubation of LNCaP cells transfected with siRNA-2 (Lane 3) led to marked changes in expression of apoptosis markers. Thus an increase in cleaved caspase-3 (17 and 19 kDa), loss of full length PARP (116 kDa) and an increase in a cleaved PARP product (89 kDa) were observed in comparison with LNCaP cells transfected with non-targeting siRNA (Lane 4). Decreases in full length PARP were also observed in controls containing lipofectamine or non-targeting siRNA (Lanes 2 and 4). However, the loss of full length PARP in these controls was not coupled to a significant increase in cleaved caspase-3 indicating that it did not involve activation of caspase-3. The results show that reduction of BIRC6 protein expression in siRNA-2-treated LNCaP cells results in an increase in apoptosis.

As shown in Figure 14, transfection of LNCaP cells with siRNA-2 (Lane 3) led to marked changes in LC3B-II protein expression. Thus a decrease in LC3B-II protein (14 kDa; the lipidated form of LC3-I) was observed with no effect on the levels of LC3B-I protein (16 kDa) when compared with controls (Lanes 1, 2, 4). Similarly, there was a marked decrease in Beclin-1 (60 kDa) protein expression, when compared with controls (Lanes 1, 2, 4). The results suggest that reduction of BIRC6 protein expression in siRNA-2-treated LNCaP cells inhibits the initiation

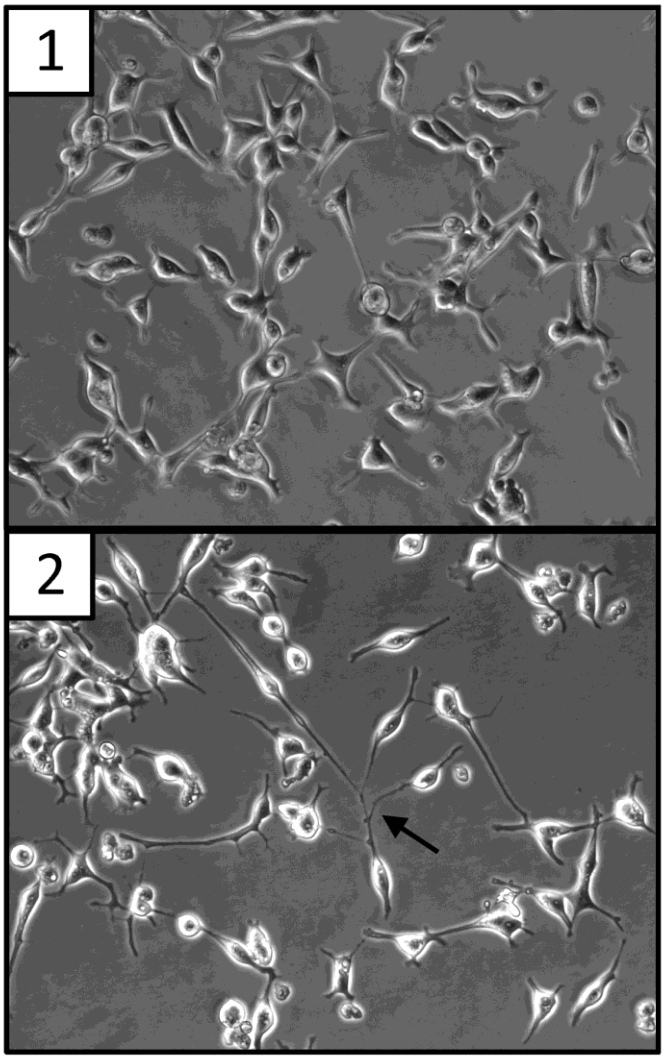

Figure 12. Morphology of BIRC6 protein-depleted LNCaP prostate cancer cells. Morphology of LNCaP prostate cancer cells transfected with (1) non-targeting siRNA and (2) siRNA-2 targeting BIRC6, at 200× magnification. Black arrow showing syncitia-like cells interconnected by filamentous projections in siRNA-2-treated cultures.

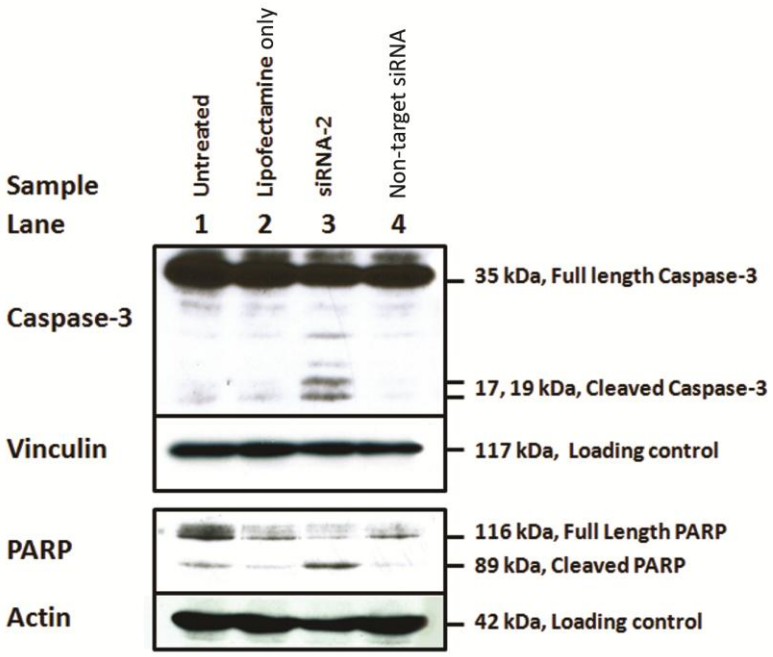

Figure 13. Expression of apoptosis proteins in BIRC6 protein-depleted LNCaP prostate cancer cells. Treatment of LNCaP cells with siRNA-2 targeting *BIRC6* leads to activation of caspase-3 (as shown by appearance of cleaved caspase-3) and degradation of its death substrate, PARP (as shown by loss of full length PARP and appearance of a cleaved PARP product). Untreated LNCaP cells (Lane 1); LNCaP cells incubated with lipofectamine only (Lane 2), siRNA-2 targeting *BIRC6* (Lane 3) or non-targeting siRNA (Lane 4) for 96 h following transfection. The results are representative of two independent experiments.

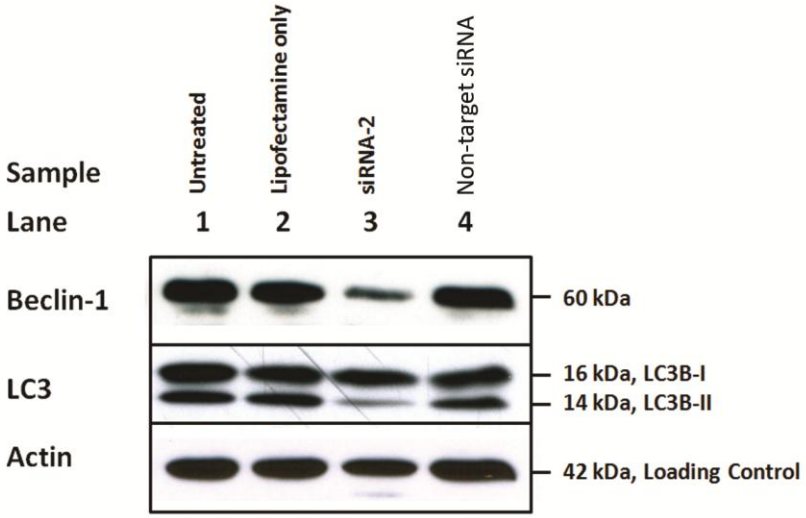

Figure 14. Expression of autophagosome proteins in BIRC6 protein-depleted LNCaP prostate cancer cells. Treatment of LNCaP cells with siRNA-2 targeting *BIRC6* leads to decreases in Beclin-1 expression and reduction of LC3B-II. Untreated LNCaP cells (Lane 1); LNCaP cells incubated with lipofectamine only (Lane 2), siRNA-2 targeting *BIRC6* (Lane 3) or non-targeting siRNA (Lane 4) for 113 h following transfection. The results are representative of two independent experiments.

of autophagy leading to lower numbers of autophagosomes within cultured LNCaP cells.

3.4 Effect of doxorubicin on BIRC6 protein expression and apoptosis in LNCaP cells

Doxorubicin, a drug used for therapy of prostate cancer (Petrioli, Fiaschi et al. 2008), has been reported to trigger apoptosis along with a marked accumulation of p53 (Lorenzo, Ruiz-Ruiz et al. 2002). To investigate the effect of doxorubicin on BIRC6 expression and apoptosis in prostate cancer cells, wild-type p53-expressing LNCaP cells were incubated for 24 h with or without doxorubicin (1 µg/mL). As shown by Western blot analysis, the treatment with doxorubicin resulted in a very marked reduction of BIRC6 expression (Fig. 15A). In addition there was a reduction in full length PARP protein (116 kDa) expression and an increase in a cleaved PARP product (89 kDa), indicative of apoptosis. Furthermore, there was a reduction in cell density with evidence of cell deterioration (Fig. 15B).

3.5 Summary of results

BIRC6 has been reported to play a significant role in apoptosis resistance of a variety of cancers (Qiu, Markant et al. 2004; Ren, Shi et al. 2005; Chu, Gu et al. 2008; Pohl and Jentsch 2008; Lopergolo, Pennati et al. 2009). In the present study we investigated whether it also has a role in apoptosis resistance of prostate cancer, as this process may underlie the development of castration

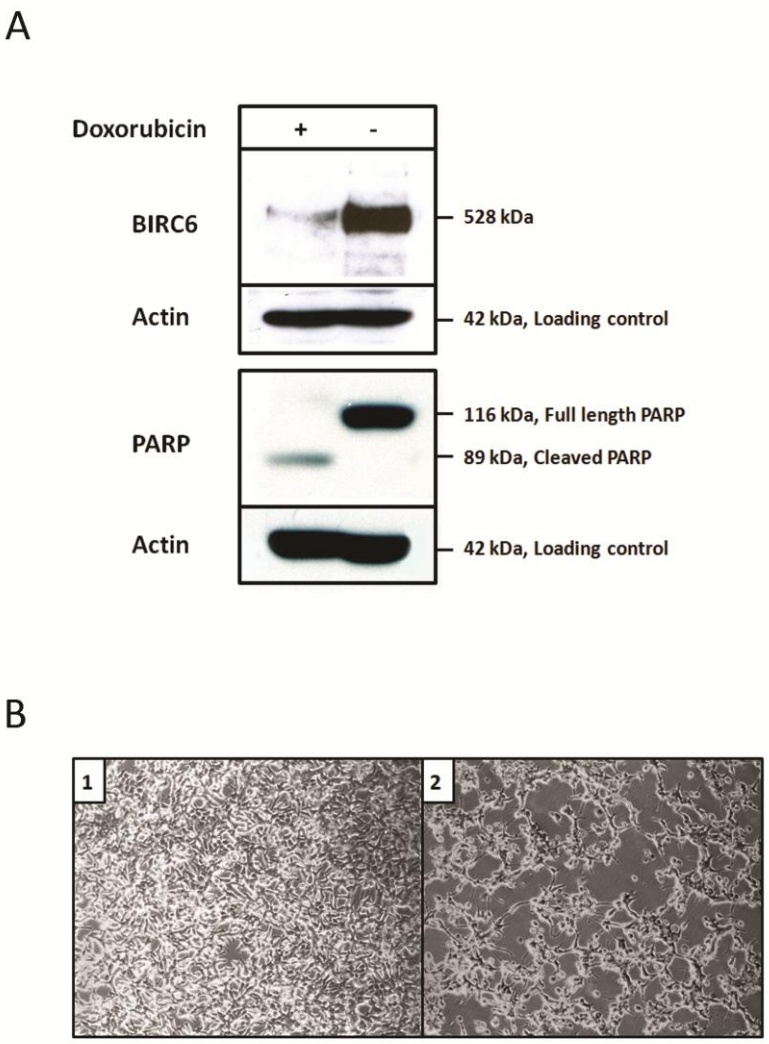

Figure 15. Effect of doxorubicin-induced apoptosis on BIRC6 protein expression in LNCaP prostate cancer cells. A, incubation (24 h) of LNCaP cells with doxorubicin (1 µg/mL) leads to reduction of BIRC6 protein expression and apoptosis (loss of full length PARP and appearance of cleaved PARP) as indicated by Western blot analysis. **B**, LNCaP cells incubated for 24 h (1) without doxorubicin and (2) with doxorubicin (1 µg/mL) at 100x magnification. The results are representative of two independent experiments.

resistance. In contrast to earlier reports (Chen, Naito et al. 1999), our study established that the BIRC6 protein is markedly expressed by a variety of conventional prostate cancer cell lines as distinct from benign prostate cell lines (Fig. 5), indicating that BIRC6 could have a significant role in prostate cancer. Support for this suggestion comes from elevated expression of BIRC6 observed in clinical prostate cancer samples (Figs 6-9).

CHAPTER 4: DISCUSSION

4.1 Role of BIRC6 protein in the survival of prostate cancer cells

Functioning of BIRC6 protein was found to be critical for the survival of LNCaP cells, which were used as a model system since they express wild-type *p53*, a gene reported to be involved in apoptosis resulting from knockdown of BIRC6 expression (Qiu, Markant et al. 2004; Ren, Shi et al. 2005). Thus, specific reduction of BIRC6 expression in LNCaP cells by targeting siRNA led to a marked inhibition of cell proliferation (Figs 10, 11), which notably was coupled to a marked increase in the expression of apoptosis markers, i.e. activated caspase-3 and a cleaved PARP product (Fig. 13). The reduction in population growth induced by non-targeting siRNA is likely due to non-specific toxicity as reported by others (Fedorov, Anderson et al. 2006); importantly, it was not associated with an increase in apoptosis marker expression (Fig. 13). The results are consistent with reports of a critical role for BIRC6 in the survival of other types of cancer cells (Qiu, Markant et al. 2004; Ren, Shi et al. 2005; Chu, Gu et al. 2008; Pohl and Jentsch 2008; Lopergolo, Pennati et al. 2009). For instance, elevated levels of BIRC6 have been linked to apoptosis resistance in the SNB-78 glioma cell line (Chen, Naito et al. 1999) and over-expression of BIRC6 in human fibrosarcoma cells supports resistance to anti-cancer drugs and death receptor ligation (Hao, Sekine et al. 2004). Furthermore, down-regulation of BIRC6 expression in SNB-78 cells was shown to sensitize the cells to apoptosis induced by cisplatin and camptothecin (Chen, Naito et al. 1999). It is therefore

conceivable that the elevated expression of BIRC6 in castration-resistant prostate cancer (Fig. 9) may be responsible, at least in part, for the therapy resistance of refractory disease.

4.2 Phenotype of BIRC6 protein-depleted LNCaP cells

BIRC6 belongs to the type 2 group of IAPs and hence has been shown to play a role in cell division. It has been reported that BIRC6 is important for cytokinetic abscission and that depletion of BIRC6 in cells *in vitro* leads to defects in abscission and to the formation of interconnected, syncitia-like cells and cytokinesis-associated apoptosis (Pohl and Jentsch 2008). When BIRC6 expression in LNCaP prostate cancer cells was reduced, the same striking, syncitia-like morphology was observed, in which multiple cells were interconnected by long filamentous projections (Fig. 12). Since these BIRC6-depleted LNCaP cell cultures also showed increases in apoptosis, it is possible that the apoptosis resulted from a BIRC6-induced interference with cytokinesis. The data indicate that BIRC6 is involved in abscission in prostate cancer cells and as such may play an important role in prostate cancer cell proliferation. However, further work is required to confirm this conclusion.

4.3 Potential role for BIRC6 protein in the regulation of autophagy

The specific reduction of BIRC6 expression in LNCaP cells leading to a decrease in the expression of LC3B-II and Beclin-1 in these cells (Fig. 14), suggests that there is a novel role for BIRC6 in the regulation of autophagy. The

reduced expression of LC3B-II indicates that loss of BIRC6 expression results in a lower number of autophagosomes. However, based on these data alone, it is not possible to conclude that the loss of BIRC6 expression inhibits autophagosome formation since the autophagosome levels in a cell are affected by both the rate of formation and degradation (Mizushima and Yoshimori 2007; Rubinsztein, Cuervo et al. 2009). Accordingly, the observed reduction in LC3B-II expression in BIRC6-depleted LNCaP cells could be due to either a decrease in autophagosome formation or to an increase in autophagosome degradation.

To provide further insight into regulation of autophagy by BIRC6, the BIRC6-depleted LNCaP cells were also examined for changes in the levels of Beclin-1 (Fig. 14). This protein is involved in both the signaling pathway activating autophagy and in the initial step of autophagosome formation, which involves its interaction with hVps34, a class III phosphatidylinositol 3-kinase (PI3K) (Kabeya, Mizushima et al. 2000; Klionsky, Abeliovich et al. 2008; Eskelinen and Saftig 2009). In mammalian cells enhanced expression of Beclin-1 has been shown to increase their autophagic response (Liang, Jackson et al. 1999) and the suppression of Beclin-1 has been shown to impair autophagy and sensitize cells to starvation-induced apoptosis (Boya, Gonzalez-Polo et al. 2005). The reduced expression of Beclin-1 in the BIRC6-depleted LNCaP cells suggests that the lower numbers of autophagosomes in these cells (i.e., observed decrease in LC3B-II expression) is likely due to the inhibition of autophagy initiation and autophagosome formation. These data suggest that the loss of BIRC6

expression in LNCaP prostate cancer cells leads to inhibition of autophagy and that BIRC6 protein may be a positive regulator of autophagy.

With the increasing evidence that autophagy may serve as a survival mechanism of cells in response to stress, including anti-cancer therapeutics (Corcelle, Puustinen et al. 2009; Chen and Debnath 2010), BIRC6 may be a suitable target for the inhibition of autophagy-mediated cell survival and treatment resistance in prostate cancer cells. Targeting autophagy has already been shown to sensitize a variety of cancers to treatment, including prostate cancer (Apel, Herr et al. 2008; Kim, Coates et al. 2009; Vazquez-Martin, Oliveras-Ferraros et al. 2009). Treatment of prostate cancer cells deficient in argininosuccinate synthetase with siRNAs targeting *Beclin-1* or chloroquine (an autophagy inhibitor), has been reported to inhibit autophagy and increase the sensitivity of these cells to treatment with the anti-cancer agent ADI-PEG20, a pegylated arginine deiminase (Kim, Coates et al. 2009). In view of the above, it is proposed that targeting BIRC6 in prostate cancer can be used to inhibit autophagy, and thus, autophagy-mediated treatment resistance. This strategy represents a novel approach to sensitizing prostate cancer cells to therapy. However, further work is needed to determine the role of BIRC6 in autophagy and the effectiveness of targeting BIRC6 as a strategy to control autophagy-mediated treatment resistance.

4.4 Effect of doxorubicin induced apoptosis on BIRC6 expression

The finding that treatment of LNCaP prostate cancer cells with doxorubicin, an apoptosis inducer, results in a dramatic loss of BIRC6 expression (Fig. 15), is consistent with reports in which apoptosis induced in a variety of benign and malignant cell lines by topoisomerase inhibitors, etoposide and camptothecin, was associated with degradation of BIRC6 protein (Qiu, Markant et al. 2004). The authors of these studies conclude that degradation of BIRC6 appears to be a general event during initiation of apoptosis (Qiu, Markant et al. 2004). Based on our data, this may also be true for prostate cancer cells. However, our finding that specific siRNA-induced reduction of BIRC6 protein expression in LNCaP cells leads to apoptosis, as indicated by increased marker expression (Fig. 13), raises the possibility that the apoptotic effect of doxorubicin and perhaps of the topoisomerase inhibitors, is based at least in part, on a reduction of BIRC6 protein expression. This suggests a novel mechanism by which doxorubicin may induce apoptosis by triggering loss of BIRC6. As such, doxorubicin may, by reducing BIRC6 expression, be valuable as a sensitizer to apoptosis and be useful in combination therapy of prostate cancer.

4.5 Elevated BIRC6 protein expression in clinical prostate cancers

The increase in BIRC6 expression in Gleason scores 6-8 clinical prostate cancers (Fig. 8), including castration-resistant cancers (Fig. 9), suggests an important role for this protein in the development and progression of the disease.

In view of the pro-survival (anti-apoptotic) function for BIRC6 in prostate cancer cells (Figs 11, 13) and in other systems (Qiu, Markant et al. 2004; Ren, Shi et al. 2005; Chu, Gu et al. 2008; Pohl and Jentsch 2008; Lopergolo, Pennati et al. 2009), elevations in the expression of BIRC6 are expected to provide a cytoprotective advantage to prostate cancer cells and promote prostate cancer development and progression. The anti-apoptotic role of BIRC6 could likely be involved in the development of castration-resistant prostate cancer and underlie the therapy resistance of this advanced form of the disease.

While the large majority of prostate cancer tissues exhibited BIRC6 protein elevations, not all stages of the disease expressed elevated levels of the protein. Unexpectedly, the expression of BIRC6 over the course of prostate cancer progression reached peak levels in Gleason score 7 cancers, but had levels in Gleason score 9+10 prostate cancers which were similar to those of benign tissues (Fig. 8). On the other hand, the levels of BIRC6 protein were elevated in castration-resistant prostate cancers (Fig. 9). The reasons for these differences are not clear. However, the elevation of BIRC6 in castration-resistant cancers suggests that the protein provides a potential therapeutic target.

4.6 BIRC6 protein as a target for prostate cancer therapy

The role of BIRC6 as an inhibitor of apoptosis, regulator of cell division (Pohl and Jentsch 2008) and potential positive regulator of autophagy, suggests that targeting elevations in BIRC6 expression in prostate cancer cells could potentially block the survival, proliferation and treatment resistance mediated by

this protein and represent a promising new strategy for the treatment of prostate cancer (Fig. 16). Compounds with the ability to target BIRC6 may be useful as

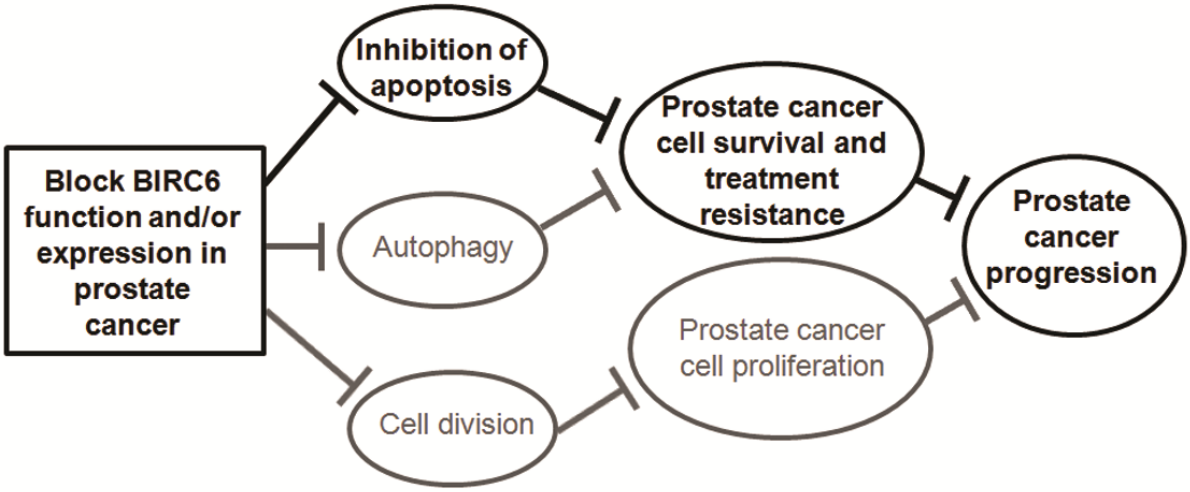

Figure 16: Potential model for the role of BIRC6 as a target for prostate cancer therapy. Grey lines showing hypothesized mechanisms of action and black lines showing mechanisms as supported by this study.

single therapeutic agents and/or for sensitizing prostate cancer cells to anti-cancer therapies. It may be noted that drugs targeting other IAP family members, e.g., XIAP and SURVIVIN, have shown promise for use as sensitizers in prostate cancer therapy. Anti-sense oligonucleotide inhibitors of XIAP led to sensitization of castration-resistant prostate cancer cells to cisplatin and TNF-related apoptosis-inducing ligand (TRAIL) (Amantana, London et al. 2004); in PC3 prostate cancer xenografts, they caused sustained tumour regression in combination with docetaxel (LaCasse, Cherton-Horvat et al. 2006). The treatment of mice with a small molecule inhibitor targeting survivin caused complete inhibition of the growth of orthotopically grafted PC3 prostate tumours (Nakahara, Takeuchi et al. 2007). These data, along with the data presented in this thesis, provide promising evidence to warrant a further study of BIRC6 as a target for prostate cancer therapy.

4.7 Significance

In conclusion, the present study indicates for the first time that the *BIRC6* gene and its product are potentially valuable targets for treatment of prostate cancers showing elevated BIRC6 expression. The function of BIRC6 as an inhibitor of apoptosis in LNCaP prostate cancer cells suggests that it may be important in maintaining low sensitivity of prostate cancer cells to apoptosis. Thus, elevated levels of the protein in prostate cancer cells are expected to provide a survival advantage, whereas lower levels or therapeutic reduction of the protein is expected to increase the sensitivity of prostate cancer cells to apoptosis and in

particular apoptosis induced by therapy. Notably, BIRC6 represents a promising new target for prostate cancer therapy, as it may have the potential to circumvent the therapy resistance of castration-resistant disease.

CHAPTER 5: CONCLUSIONS

The data generated in this thesis can be summarized by the following points:

1) BIRC6 expression is elevated in malignant prostate cells, indicating that BIRC6 is potentially important in the development of prostate cancer.

2) BIRC6 expression is elevated in castration-resistant prostate cancer, indicating that BIRC6 is potentially important in the development of this end-stage form of the disease.

3) Loss of BIRC6 expression in LNCaP prostate cancer cells results in a reduction in cell proliferation associated with an increase in apoptosis and a decrease in autophagosome formation, indicating that BIRC6 is likely an important survival factor in prostate cancer cells.

4) Based on the expression pattern and function of BIRC6, this protein represents a novel potential therapeutic target for the treatment of prostate cancer.

CHAPTER 6: FUTURE WORK

The future direction for this study will be to investigate the therapeutic potential of targeting BIRC6 in the treatment of prostate cancer and, particularly, to identify a role for this protein in therapy resistance and disease progression based on its function as an inhibitor of apoptosis and a potential positive regulator of autophagy.

In this study we found that elevations in BIRC6 protein expression were associated with clinical prostate cancers of Gleason score 6-8 and with castration-resistant prostate cancers. These associations suggest that the elevated expression of BIRC6 could potentially be valuable as a poor prognostic marker for prostate cancer disease progression. Using the clinical prostate cancer collection at the Vancouver Prostate Centre, Vancouver General Hospital (supplied by the Department of Pathology, University of British Columbia, Vancouver, BC, Canada), we plan to investigate whether BIRC6 is associated with clinical parameters such as elevated prostate-specific antigen (PSA) levels, metastases, reductions in time to disease recurrence and reductions in patient survival times. Using these collections, we will also be able to investigate whether BIRC6 expression is associated with resistance to chemotherapeutics such as docetaxel.

Treatment resistance has been shown to involve autophagy and inhibition of apoptosis, processes which might be blocked by the reduction of BIRC6 expression. To determine BIRC6's role in treatment resistance, BIRC6 will be

knocked-down and over-expressed in prostate cancer cell lines to determine whether treatment resistance is coupled to elevated BIRC6 expression and if treatment sensitivity is associated with reduced BIRC6 expression. We also plan to explore the involvement of both autophagy and apoptosis inhibition in treatment resistance by using the pan-caspase inhibitor Z-VAD-FMK and the autophagy inhibitor chloroquine. Using the models from these experiments we will also be able to identify the effectiveness of targeting BIRC6 in prostate cancer therapy and whether the efficacy of current anti-prostate cancer therapies can be enhanced by adjuvant targeting of BIRC6. We also plan to develop *BIRC6*-targeting antisense oligonucleotides (ASOs) and/or small molecule inhibitors and evaluate their effectiveness as single agents and in combination therapy using patient-derived prostate cancer xenograft mouse models.

We also plan to further investigate the role of BIRC6 in the regulation of autophagy by performing more comprehensive assays measuring autophagic flux, since our assays measured only the early stages of autophagy. The autophagic process can be further analyzed with the use of several autophagic inhibitors, such as bafilomycin, E64-d, pepstatin A, and chloroquine which block later stages in autophagy (Mizushima and Yoshimori 2007; Rubinsztein, Cuervo et al. 2009). In addition, the autophagic degradation of substrates such as p62 can be detected and also be used to measure autophagic flux.

In summary, the future work for this project is aimed at demonstrating that BIRC6 provides a new therapeutic target for the treatment of prostate cancer. If so, it could lead to urgently needed improvements in prostate cancer

management and patient survival.

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