University of Alberta

Clinical Significance and Cross Talk of Wnt Canonical Pathway in Cancer

by

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I dedicate this thesis to my husband and two daughters for all their love and support

ABSTRACT

Wnt signalling is of great biological importance as it has been implicated in development and cancer. The Wnt canonical pathway (WCP) is the best characterized signalling pathway. Based on my preliminary studies in my first and second years I found that the WCP is not linear and that it is interacting with many other signalling proteins. Thus, I hypothesized that WCP is cross talking with oncogenic networks in lymphoid and solid tumors. Through my work in this thesis I examined different models of cross talk between WCP and other signalling pathways implicated in cancer pathogenesis.

The first objective of this thesis examined the biological and clinical significance of the WCP member pGSK-3β, which also acts as a key member in the PI3K/Akt pathway. pGSK-3β was shown to be expressed in two cancer models; breast cancer and mantle cell lymphoma (MCL). In MCL, pGSK-3β expression was shown to correlate to WCP activation, however, in breast cancer it correlated to PI3K/Akt activation. Importantly, pGSK-3β expression correlated with a worse clinical outcome in breast cancer and MCL patients.

The second objective of this thesis examined the regulation of β-catenin (WCP member) by signal transducer and activator of transcription 3 (STAT3) in breast cancer. STAT3 was shown to regulate β-catenin at the transcriptional level, as STAT3 binds to the promoter of β-catenin.

Moreover, STAT3 was shown to correlate with nuclear β-catenin expression in patient samples.

The third objective of this thesis was to study the regulatory role of β-catenin on a disintegrin and metalloproteinase 10 (ADAM10) in mantle cell lymphoma. However, ADAM10 was shown to regulate the TNFα/NFκB signalling pathway.

The fourth objective of this thesis examined the cross talk between the WCP and NPM-ALK the major oncogenic protein in ALK^+ALCL. In ALK^+ALCL, cross talk between WCP and NPM-ALK through casein kinase 2α was identified.

Overall, the identification of the cross talk between WCP and various signalling pathways in different cancer models furthers our current understanding of the importance of the WCP and about the complexity of signalling networks in cancer. These findings provide a framework for the development of novel anti-cancer targeting strategies.

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LIST OF ABBREVIATIONS

ADAM10 - A disintegrin and metalloproteinase 10

ALK – Anaplastic lymphoma kinase

ALK+ALCL - Anaplastic lymphoma kinase positive anaplastic large cell lymphoma

ALL - Acute lymphoblastic leukemia

AML - Acute myeloid leukemia

ANOVA - Analysis of variance

APC - Adenomatous polyposis coli

BCL - B-cell leukemia

βTrCP - Beta transduction repeat-containing protein

BRG1 - Brahma-related gene 1

CamKII - Calcium-calmodulin-dependent kinase II

CBP - cAMP response-element binding proteins

*CCND1- cyclin D1*gene

CK1 - Casein kinase 1

CK2 - Casein kinase 2

CLL - Chronic lymphatic leukemia

CML - Chronic myelogenous leukemia

COX-2 - Cyclooxygenase-2

CRC - Colorectal cancer

CRD - Cysteine rich domain

CT - Cycle threshold

CTD - C-terminal domain

DEP - Dvl, Egl-10, Pleckstrin

DFS - Disease free survival

DIX - Dishevelled, Axin

Dkk - Dickkopf

DLBCL - Diffuse large B-cell lymphomas

DMSO - Dimethyl sulfoxide

Dvl - Dishevelled

EGF - Epidermal growth factor

EGFR - Epidermal growth factor receptor

ER - Estrogen receptor

ES - Embryonic stem

FAP - Familial Adenomatous Polyposis

FDA - Food and Drug Administration

FL – Follicular lymphoma

Fz - Frizzled

GBP - GSK-3β binding protein

GSK-3β - Glycogen synthase kinase-3β

H_2O_2 - Hydrogen peroxide

HDACs - Histone deacetylases

HER2 - Human Epidermal growth factor receptor 2

HSCs - Hematopoietic stem cells

JAK – Janus kinase

IgH - Immunoglobulin heavy chain

JNK - c-Jun N-terminal kinase

Lgs - Legless

LOH - Loss of heterozygosity

LRP5/6 - Low density lipoprotein-related proteins 5 and 6

LSAB - Labeled streptavidin-biotin

MAPK - Mitogen-activated protein kinase

MCL - Mantle cell lymphoma

MMP-7 - Matrix metalloprotease-7

MMTV - Mouse mammary tumor virus *Int-1*(for ‘integration)

mTOR - Mammalian target of rapamycin

MZL - Marginal zone lymphomas

NFAT - Nuclear factor associated with T cells

NFκB - Nuclear factor kappa B

NLK - Nemo-like kinase

NPM – Nucleophosmin

NPM-ALK – Nucleophosmin-anaplastic lymphoma kinase

NRs - Nuclear receptors

NSAIDS - Non Steroidal anti-inflammatory drugs

NSCLC - Non small cell lung cancer

OS - Overall survival

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PCP - Planar cell polarity

PCR - Polymerase chain reaction

PDZ - Postsynaptic density 95, Discs Large, Zonula occludens-1

pGSK-3β - Phosphorylated GSK-3β

PI - Propidium iodide

PI3K - Phosphatidylinositol 3 kinase

PKC - protein kinase C

PPARγ - Peroxisome proliferator-activated receptor γ

PR - Progesterone receptor

qRT-PCR - Quantitative reverse transcriptase-polymerase chain reaction

RT – Reverse transcription

RYK - Related to tyrosine kinase

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Ser/Thr - Serine/threonine

SFRPs - Secreted Frizzled-related proteins

siRNA - Short interfering RNA

STAT - Signal transducers and activators of transcription

STAT3 - Signal transducer and activator of transcription-3

STAT3C – Constitutively active STAT3

TBB - 4,5,6,7-Tetrabromobenzotriazole

TBS - Tris buffered saline

TCF/LEF - T-cell factor/Lymphocyte enhancer factor

TNFα - Tumor necrosis factor alpha

TRE – Tetracycline response element

tTA – Tetracycline-controlled transactivator

VEGF - Vascular endothelial growth factor

WCP - Wnt canonical pathway

Wg - Wingless

WIF-1 - Wnt inhibitory factor-1

Wls/Evi - Wntless/Evenness interrupted

❖ Chapter 1

General Introduction

1.1. Introduction

The development of cancer in humans has been described as a multistep process. Moreover, transgenic mouse models have emphasized the importance of multiple rate limiting events in tumorgenesis (Kinzler and Vogelstein, 1996; Renan, 1993). The main hallmarks in cancer pathogenesis have been attributed to disruption in one or more of six capabilities within normal cells: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replication, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000) (Figure 1.1).

Signalling pathways are important for the cellular communication during development and in mediating homeostasis in adult tissues. Wnt signalling pathway is one of the best characterized signalling pathways implicated in both development, as well as in diseases especially cancer (Clevers, 2006). Aberrant activation of the Wnt pathway has been shown in various cancers including colorectal, breast, pancreatic, prostate, hepatocellular and thyroid cancers (Abraham et al., 2002; Garcia-Rostan et al., 1999; Morin et al., 1997; Nagahata et al., 2003; Voeller et al., 1998; Wong et al., 2001). The mechanism which the Wnt pathway plays in cancer development has been attributed to its activation of a wide array of target genes. Through activating these target genes, Wnt regulates various cellular processes contributing to the features of a malignant phenotype as described by Hanahan and Weinberg (Ilyas, 2005; Lustig and Behrens, 2003) (Figure 1.2).

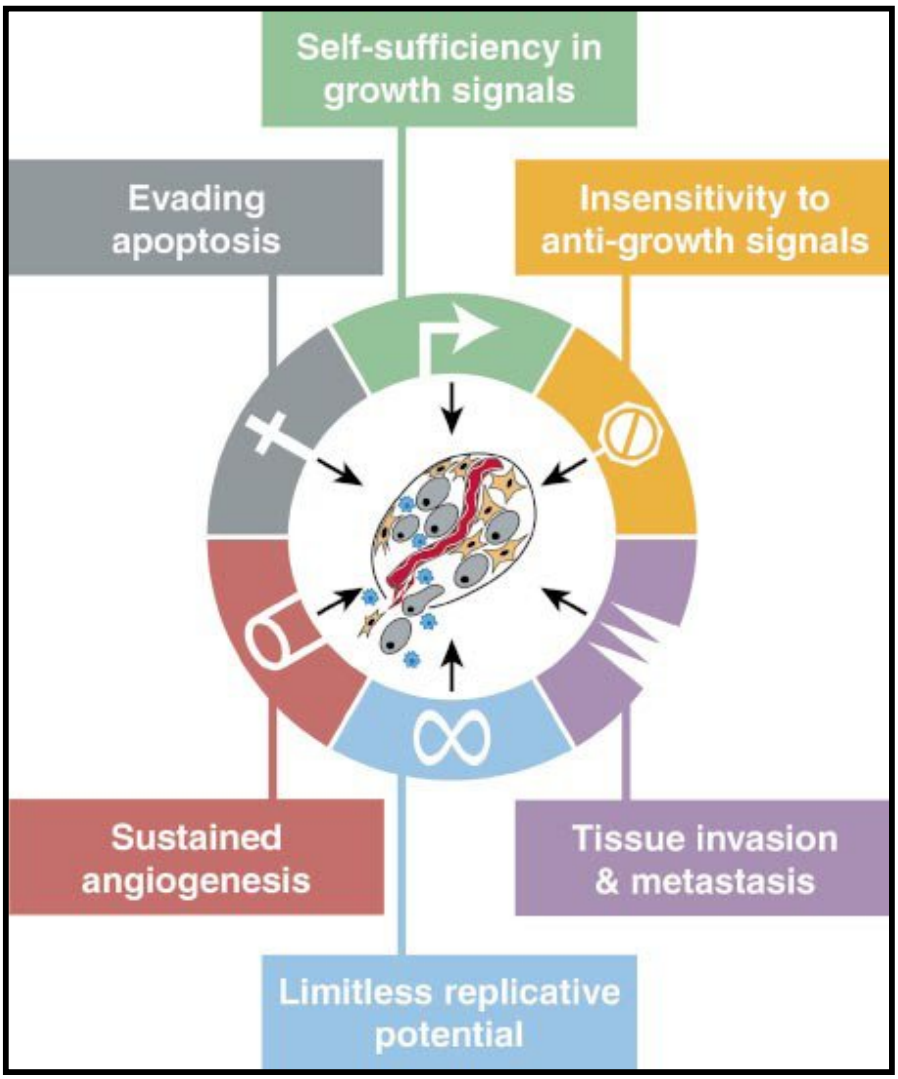

Figure 1.1. The main hallmarks in cancer pathogenesis

(Reprinted from Cell, Vol. 100, Hanahan, D., and Weinberg, R.A., The hallmarks of cancer, 57-70, Copyright 2000, with permission from Elsevier)

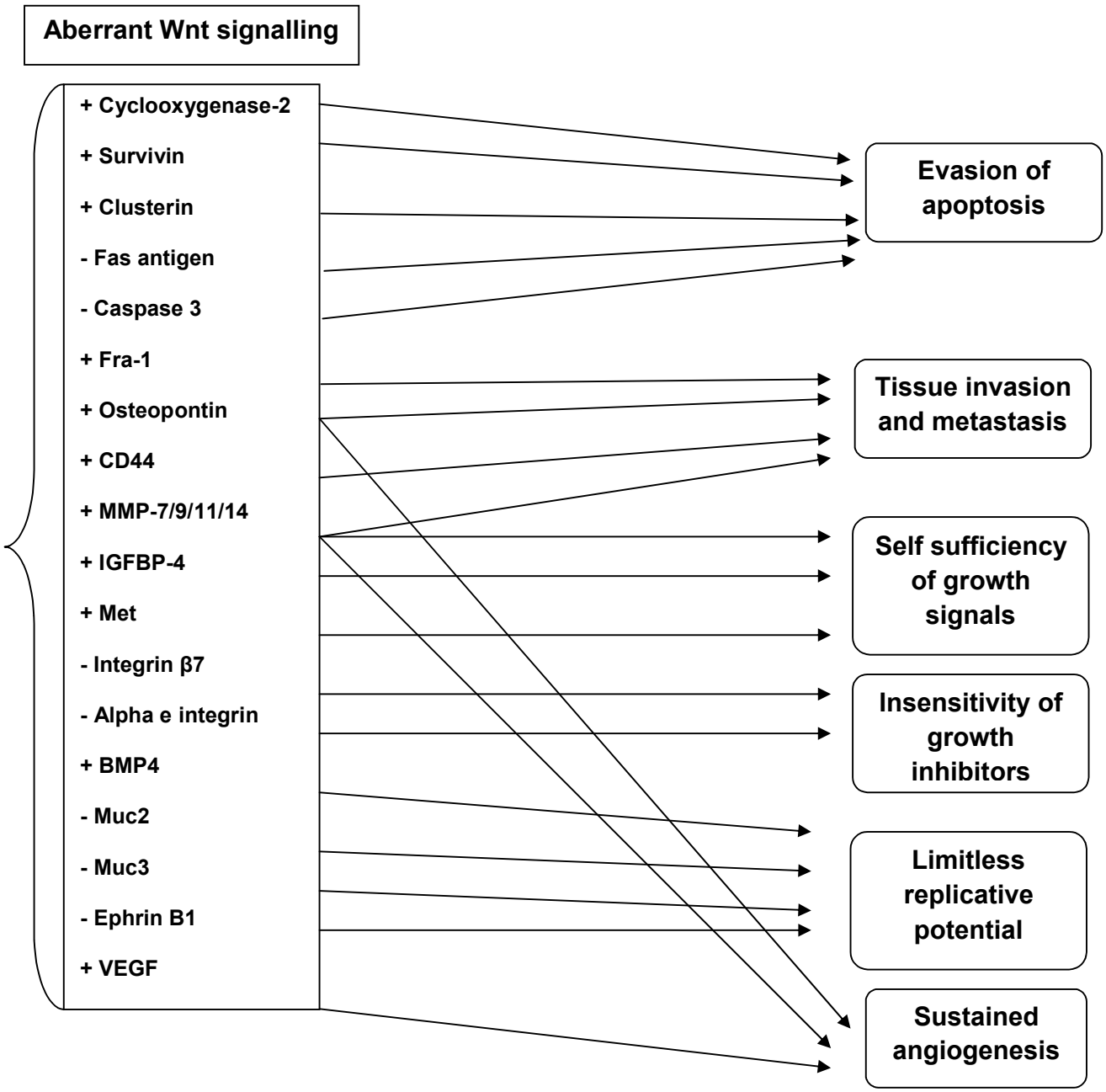

Figure 1.2. Mechanistic basis of neoplastic changes induced by aberrant Wnt signaling. Inappropriate elevation of β-catenin results in either upregulation or downregulation of large number of genes. These genes affect a large number of processes which characterize a malignant tumor. A single target gene may affect a variety of processes (i.e. MMPs) (modified from Ilyas, 2005)
(+) indicate upregulation, (-) indicate downregulation

There is mounting evidence that Wnt signalling is biologically important in pathogensis of cancer; however in the era of translational research the clinical importance of Wnt signalling in cancer has only been partially elucidated. Also, the regulatory mechanisms of Wnt signalling and its cross talk with other signalling pathways in cancer need further investigation. Therefore, further work is required to increase our understanding of the regulation of Wnt signalling and to underlie its clinical importance in cancer.

1.2. Wnt signalling pathway

The discovery of the Wnt signalling pathway came from the genetic studies in *Drosophila,* which later was found to be highly conserved throughout the animal kingdom. The identification of Wnt signalling came through extensive search for the preferential site of integration of the mouse mammary tumor virus (MMTV) in virally induced breast tumors. Scientists identified the integration site of the virus in the promoter of a gene which they called *Int-1* (for 'integration) (Nusse et al., 1984; Nusse and Varmus, 1982). Furthermore, forced expression of the Int-1 protein in transgenic mice led to the development of mammary tumors (Tsukamoto et al., 1988). When sequence analysis was done for the *Int-1* gene it was shown to be the orthologue of the *Drosophila* segment polarity gene *Wingless* (*Wg*) (Rijsewijk et al., 1987; van 't Veer et al., 1984). The name Wnt came from the amalgam of *Wg* and *Int-1* genes.

Mammals have 19 Wnt genes, the transforming ability in cell culture and knockout mouse models were done for most of them (Chien et al., 2009; Miller, 2002; Nusse, 2001) (Table 1.1). Wnt proteins are mainly defined by sequence rather than function. Wnts are secreted glycoproteins which contain a signal sequence and share the presence of 22 cysteine

Table 1.1. List of Wnts, their transforming ability and knockout mouse phenotypes

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Official designation	Transformation of C57MG breast cells	Knockout mouse phenotype
Wnt1	Strong	Midbrain and hindbrain defects
Wnt2	Strong	Abnormal placental development
Wnt3	Strong	Abnormal placental development
Wnt3a	Strong	Truncated A/P axis
Wnt4	No	No renal tubules or Mullerian ducts
Wnt5a	No	Truncated A/P axis, defects in lung and pancreas morphogenesis
Wnt5b	No	Viable
Wnt6	Weak	Viable
Wnt7a	Weak	A/P axis patterning defects; female and male reproductive tract abnormalities
Wnt7b	No	Abnormal placental development
Wnt8a	ND	Viable
Wnt8b	ND	Viable
Wnt9a	ND	Skeletal and synovial abnormalities
Wnt9b	Weak	Urogenital defects
Wnt10a	ND	ND
Wnt10b	ND	Accelerated myoblast differentiation
Wnt11	Yes	Renal and ureteral defects
Wnt16	ND	Viable

ND: not determined

residues which may help in the formation of intramolecular disulphide bonds (Du et al., 1995; Mason et al., 1992). Although they are secreted, scientists had difficulty in purifying them. This was attributed to the fact that they are palmitoylated which makes them hydrophobic (Willert et al., 2003). Most of the studies trying to delineate the mechanism regulating the Wnts secretion and transport from Wnt producing cells were done on the *Wg* gene in *Drosophila*. Upon their secretion from the endoplasmic reticulum, Wnts are lipid modified by a protein called Porcupine (Tanaka et al., 2002). Another transmembrane protein, Wntless/Evenness interrupted (Wls/Evi), was shown to be important in promoting Wnt secretion from the Wnt producing cells (Banziger et al., 2006). Different molecules have been suggested to enhance Wnt transport such as lipoprotein particles and a cluster of proteins called the retromer complex (Panakova et al., 2005; Prasad and Clark, 2006) (figure 1.3).

Regarding Wnt receptors, different receptor types have been suggested to bind Wnts which dictate whether canonical or non-canonical pathways are being activated (Mikels and Nusse, 2006). Two important Wnt signalling pathways are currently characterized: The canonical Wnt pathway (β-catenin dependent) and the non canonical Wnt pathway (β-catenin independent). Accordingly, Wnt ligands have been classified into canonical Wnts (Wnt1, 2, 3, 3a, 8a, 8b, 10a and 10b) activating the canonical pathway, and non canonical Wnts (Wnt4, 5a, 5b, 6, 7a and 7b), activating the non canonical pathway. Wnt11 was shown to be an activator for both the canonical and the non canonical pathways (Staal et al., 2008; Tao et al., 2005).

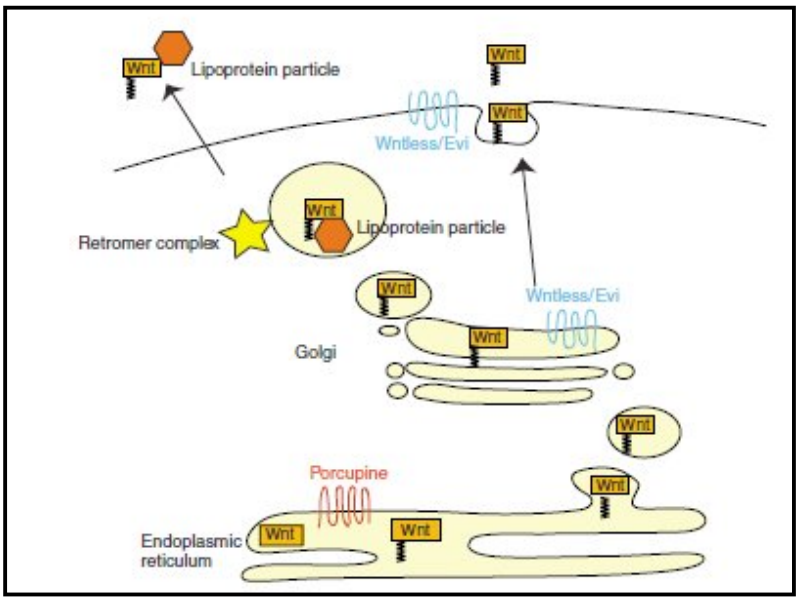

Figure 1.3. Wnt proteins secretion and transport. In Wnt producing cells, Wnt proteins become palmitoylated in endoplasmic reticulum by Porcupine. Further transport and secretion is controlled by Wls/Evi, which is present in the Golgi and/or plasma membrane. Retromer complex acts on the Wnt producing cells to generate forms which can be transported to outside the producing cells.

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1.2.1. Wnt Canonical signalling

(A) Overview of the Wnt canonical pathway (WCP)

In the absence of Wnt signalling, β-catenin is attached to the cell membrane where it is associated with E-cadherin in adherens junctions. As shown in Figure 1.4, the rest of cytosolic β-catenin is destroyed by a multiprotien complex consisting of adenomatous polyposis coli (APC) protein, axin, casein kinase 1α (CK1α) and glycogen synthase kinase-3β (GSK-3β). CK1α starts the phosphorylation of β-catenin at residue 45 followed by phosohorylation by GSK-3β at serine/threonine (Ser/Thr) residues 41,37,33. This phosphorylation targets β-catenin for ubiquitination by beta transduction repeat-containing protein (βTrCP) and its degradation by the proteasome pathway. When Wnt is activated, signalling is initiated by binding of Wnt ligands to Frizzled (Fz) receptors and low density lipoprotein-related proteins 5 and 6 (LRP5/6) which acts as coreceptors. This binding induces phosphorylation of Dishevelled (Dvl) proteins. Recent studies indicate that LRP5/6 interacts at the same time with Axin with its cytoplasmic tail, thus disrupting the protein destruction complex leading to accumulation of a pool of cytoplasmic β-catenin which is not phosphorylated, which then translocates to the nucleus where it activates the T-cell factor/lymphocyte enhancer factor (TCF/LEF) in the nucleus, leading to activating downstream target genes (Clevers, 2006; Gordon and Nusse, 2006; Logan and Nusse, 2004).

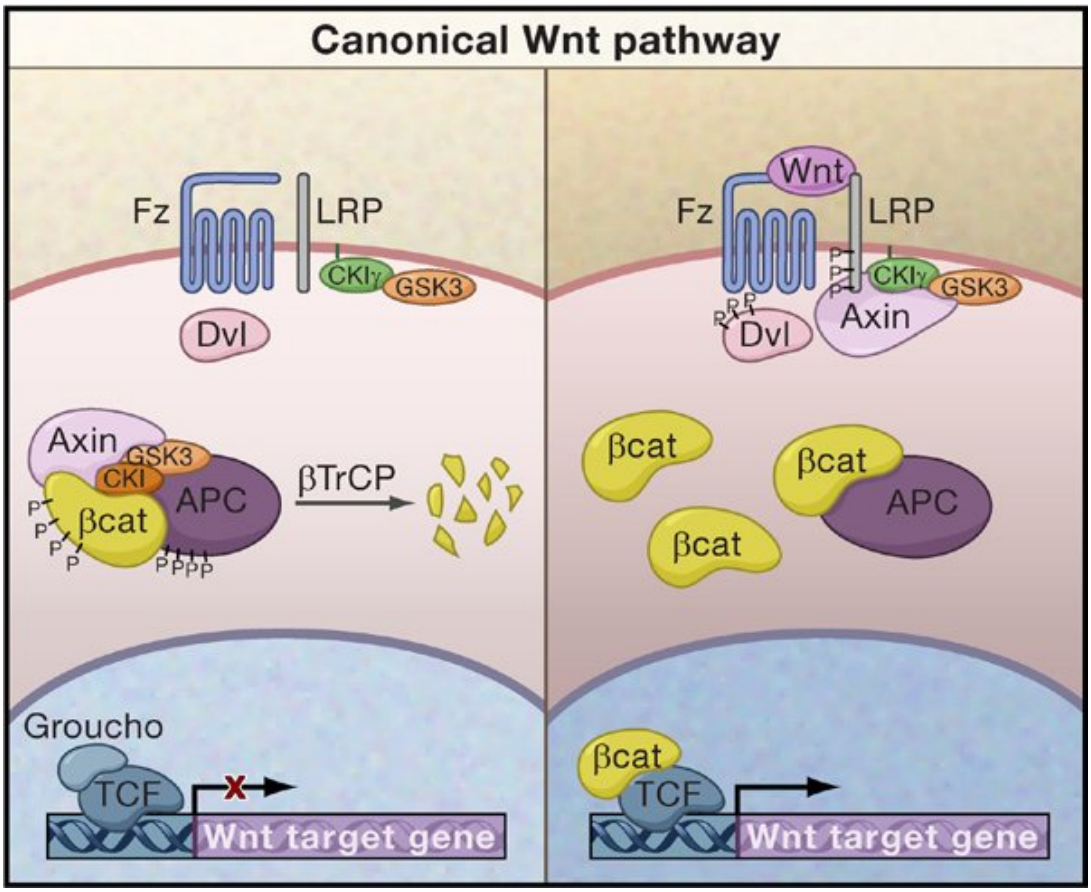

Figure 1.4. Overview of the Wnt canonical pathway signalling

(Reprinted from Cell, Vol. 127, Clevers H., Wnt/β-Catenin Signaling in Development and Disease, 469-480, Copyright 2006, with permission from Elsevier)

(B) Members of the WCP

1) Receptors:

As shown in figure 1.4, WCP signalling is initiated when Wnt ligands bind to Fz receptors. Fz are seven transmembrane proteins, 10 family members of which have been identified in humans (Bhanot et al., 1996). They have a long N-terminal cysteine rich domain (CRD) where Wnt ligands bind.They also have at the C-terminal a consensus PDZ (Postsynaptic density 95, Discs Large, Zonula occludens-1) domain binding motif. In cell culture, overexpression of Fz does not activate WCP unless the Wnt ligand is present (Dann et al., 2001; Rulifson et al., 2000). In addition to Fz receptors the Wnt ligands bind to LRP5/6 complexes (which are single pass transmembrane proteins) where they act as co-receptors with the Fz. The cytoplasmic domain of the LRP5/6 receptor contains five phosphorylated motifs with flanking Ser/Thr clusters, which are a target for phosphorylation by casein kinase1γ (CK1γ). Upon Wnt-Fz binding, LRP5/6 becomes phosphorylated by CK1γ which allows axin binding to the LRP5/6 tail (Davidson et al., 2005; Zeng et al., 2005). LRP6 mutant mice shows abnormality with striking resemblance to defects in Wnt1, Wnt3a and Wnt7a knockout mice (Pinson et al., 2000; Tamai et al., 2000). LRP5/6 importance is emphasized by the finding that the extracellular inhibitor of the WCP Dickkopf (Dkk) binds to it (Glinka et al., 1998).

2) Dishevelled:

Study of Dvl in model systems identified it as an intermediate positive regulator of the WCP, positioned downstream of receptors and upstream of β-catenin (Noordermeer et al., 1994). Three Dvl forms are identified in humans: Dvl1, 2 and 3. They share a high sequence homology (Lijam and Sussman, 1995). Dvl has three conserved domains which are: DIX

(Dishevelled, Axin), PDZ and DEP (Dvl, Egl-10, Pleckstrin) domains. The DIX domain is the one responsible for the interaction with axin and mutations in this domain diminishes Wnt signalling (Boutros and Mlodzik, 1999). Upon Wnt stimulation, Dvl is recruited to and bound to Fz by its PDZ domain. Dvl provides a platform for axin and GSK-3β binding (Cliffe et al., 2003). At the same time Wnt stimulation causes phosphorylation of Dvl; however currently the exact role of this phosphorylation is not completely understood. Dvl phosphorylation has been reported to occur by several kinases including CK1ε, CK1δ and casein kinase 2 (CK2) (Bryja et al., 2007; Willert et al., 1997).

3) Axin:

Axin was identified as a negative regulator of the WCP in murine models as its mutation caused axis duplication (Zeng et al., 1997). Similarly, overexpression of axin led to a decrease in nuclear β-catenin and its transcriptional activity (Behrens et al., 1998). Axin serves as a scaffolding protein in the so called “destruction complex”, it binds APC, GSK-3β, CK1α and β-catenin, where it facilitates the phosphorylation of β-catenin by GSK-3β (Peifer and Polakis, 2000). Axin has three regions: regulators of G protein signalling region which binds APC, a central region which binds β-catenin, GSK-3β and CK1α and a C-terminal DIX domain which is related to Dvl (Ikeda et al., 1998). The importance of axin as a putative tumor suppressor gene was highlighted by studies showing its allelic inactivation in human hepatocellular carcinoma (Satoh et al., 2000).

4) GSK-3β:

GSK-3 is a serine/threonine kinase deriving its name from its substrate glycogen synthase which is a key enzyme involved in glycogen synthesis (Embi et al., 1980). GSK-3 is ubiquitously expressed and has two isoforms

encoded by two different genes: GSK-3α, a 51 kDa protein, and GSK-3β, a 47 kDa protein with an 85% amino acid identity to GSK-3α (Woodgett, 1990). Serine phosphorylation of the N-terminal at S21 for GSK-3α, and S9 for GSK-3β, has an inhibitory effect, while tyrosine phosphorylation of the C-terminal at Y279 for GSK-3α, and Y216 for GSK-3β causes activation of the enzyme (Stambolic and Woodgett, 1994; Wang et al., 1994). GSK-3β has some major differences from other enzymes. First, it is constitutively active and is inactivated in response to cellular signalling (Frame et al., 2001). Second, phosphorylation of substrates by GSK-3β leads to their inactivation (Doble and Woodgett, 2003). Third, GSK-3β needs a priming phosphorylation of its substrates at phosphorylated Ser/Thr residues, with a consensus recognition sequence Ser/Thr-(X-X-X)-pSer/pThr, X being any amino acid and pSer/pThr being the phosphorylated Ser/Thr which serves as a priming phosphate. Multiple subsequent phosphorylations are carried by GSK-3β as the phosphorylated primed site of the substrate interacts with the three positive residues in GSK-3β, Arg 96, Arg 180 and Lys 205, which form a binding pocket for the priming phosphate (Dajani et al., 2001).

Early studies in *Drosophila* identified β-catenin as a GSK-3β substrate where GSK-3β mediated phosphorylation triggers β-catenin destabilization (Peifer et al., 1994). In the WCP, GSK-3β forms a part of the 'destruction complex' together with APC, axin and CK1α, where GSK-3β phosphorylates the N terminal Ser/Thr of β-catenin leading to its proteasomal degradation. CK1α primes β-catenin at Ser 45 which is followed by Ser/Thr 41, 37, 33 phosphorylations by GSK-3β (Hagen et al., 2002). Binding of Wnt ligands induces dissociation of the destruction complex with the escape of β-catenin from phosphorylation (Jope and Johnson, 2004; Patel et al., 2004). Another function of GSK-3β, which has

been identified in the WCP, is that GSK-3β can phosphorylate LRP6 which leads to its activation and promotes the engagement of LRP6 with the scaffolding protein axin (Zeng *et al.*, 2005). Although this activator function of GSK-3β is opposite to its inhibitory function on β-catenin, it has been suggested that different pools of GSK-3β have different functions where cytosolic GSK-3β phosphorylates β-catenin and antagonizes the WCP signalling, whereas the plasma membrane bound GSK-3β phosphorylates LRP6 and activates the WCP signalling (Zeng et al., 2005)

5) APC:

APC is an important inhibitor of the WCP, where germ line mutations of *APC* gene were shown as a cause of familial adenomatous polyposis (FAP), which is characterized by multiple polyps in the intestine (Groden et al., 1991). The APC protein has binding sites for various proteins including axin, β-catenin, microtubules and cytoskeletal regulating proteins (Bienz, 2002). APC inhibits β-catenin mediated transcription through different mechanisms. First, APC together with axin and GSK-3β forms the destruction complex which binds β-catenin promoting its phosphorylation and subsequent proteasomal degradation (Rubinfeld et al., 1993). Second, APC has a nuclear export signal which enables it to capture and shuttle nuclear β-catenin from the nucleus to the cytoplasm (Rosin-Arbesfeld et al., 2003). Third, APC itself can bind to nuclear β-catenin sequestering it away from TCF (Neufeld et al., 2000).

6) β-catenin:

β-catenin is the central player in the WCP signalling. The protein is formed of central 12 repeat regions called the armadillo repeats. The crystal structure reveals that each of these repeats is formed of three α-helices which are tightly packed (Huber et al., 1997). The N-and C-terminals of β-

catenin are important for its interaction with the transcription regulators and protein-protein interaction. Different proteins interact with various β-catenin domains (Choi et al., 2006; Takemaru et al., 2008).

β-catenin has multiple functions being involved both in cell adhesion and in signalling in the WCP. β-catenin at the cell surface acts as a cell adhesion molecule at the adherens junction where it binds E-cadherins to α-catenin which mediates the actin filaments assembly (Nelson and Nusse, 2004). In the absence of Wnt signalling, cytoplasmic β-catenin is bound by the destruction complex where CK1α starts the phosphorylation of β-catenin N-terminal at residue 45 followed by phosohorylation by GSK-3β at Ser/Thr residues 41,37,33. The phosphorylated S33 and 37 form docking sites for the E3 ubiquitin ligase βTrCP promoting β-catenin proteasomal degradation (Liu et al., 2002). The binding of Wnt ligands to Fz and LRP5/6 receptors leads to dissociation of the destruction complex with axin binding to the Dvl through its DIX domain, leading to the escape of β-catenin from being phosphorylated and its subsequent nuclear translocation (Hoppler and Kavanagh, 2007).

In the nucleus, β-catenin forms a complex with the TCF/LEF family of transcription factors driving gene expression (Stadeli et al., 2006; Willert and Jones, 2006). In the absence of β-catenin, TCF/LEF is bound by the transcriptional repressor Groucho which interacts with histone deacetylases (HDACs) to compress chromatin and inhibit transcription. Nuclear β-catenin competes with Groucho for TCF binding (Courey and Jia, 2001; Daniels and Weis, 2005). As Shown in figure 1.5, several nuclear factors which interact with β-catenin have been identified (Mosimann et al., 2009). Among them p300 and cAMP response-element binding proteins (CBP), which have histone acetyltransferase activity,

interact with the C-terminal domain (CTD) of β-catenin enhancing transcriptional activity (Hecht et al., 2000; Takemaru and Moon, 2000). Mixed lineage leukaemia proteins are one of the factors shown to interact with the CTD of β-catenin. They have histone methyltransferase activity (Milne et al., 2005). Another β-catenin CTD interacting factor is Parafibromin which forms a part of the polymerase-associated factor 1. It interacts with the RNA polymerase II important for transcriptional elongation (Mosimann et al., 2006). Another factor which interacts with nuclear β-catenin is Brahma-related gene 1 (BRG1) which binds to β-catenin at the armadillo repeats 7-12 (Barker et al., 2001). BRG1 belongs to the SWI2/SNF2 family protein of ATPases which help gene transcription by the disassembling of histone octamers (Roberts and Orkin, 2004). Chibby is another nuclear protein interacting with the CTD of β-catenin. It is an inhibitor of the Wnt signalling as it competes with TCF/LEF for β-catenin binding (Takemaru et al., 2003). BCL9 is another important factor which binds to the N-terminal domain of β-catenin and acts as an activator of the Wnt signalling, it is the homologue of the *Drosophila* gene *Legless* (*Lgs*) (Kramps et al., 2002). BCL9 belongs to B-cell leukemia (BCL) genes family involved in pathogenesis of lymphomas and leukemia (Rowley, 2001). BCL9 serves as an adaptor molecule to recruit Pygo to β-catenin. Pygo is a protein which has a PHD finger found in factors responsible for chromosomal remodelling. In *Drosophila* studies Pygo and Lgs were shown to be important for Wnt signalling, however their exact biological functions in vertebrates remain to be investigated (Kramps et al., 2002).

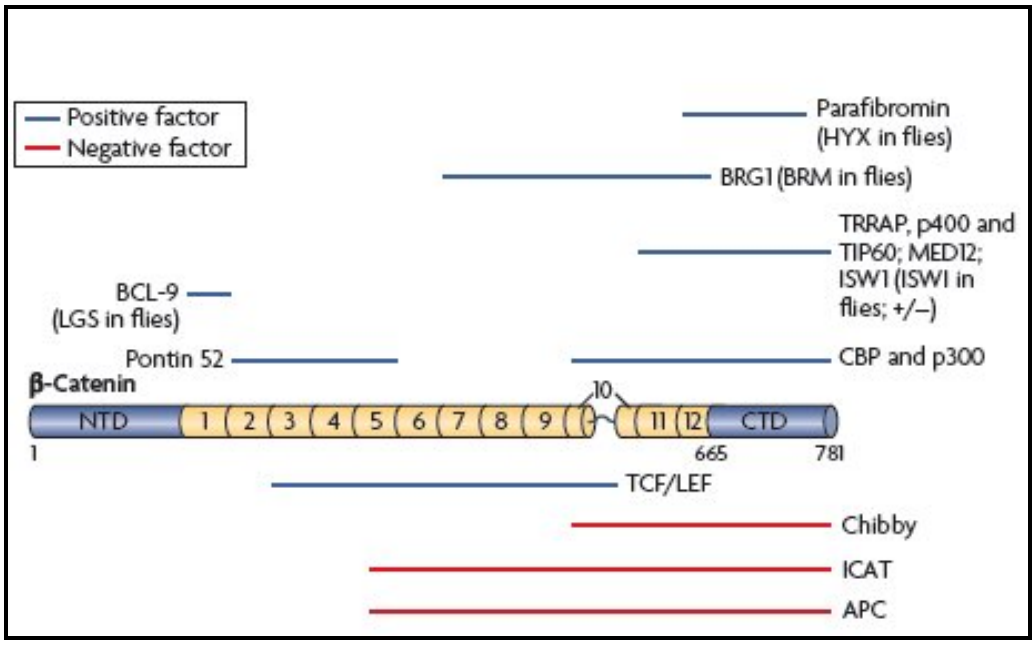

Figure 1.5. Nuclear β-catenin interactions

[Blue bars indicate positive β-catenin interactors and red bars indicate negative negative interactors]

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Several mechanisms determining the switch between the adhesive and transcriptional function of β-catenin have been proposed (Bienz, 2005). One mechanism identifies BCL9 as the main player, where tyrosine phosphorylation of β-catenin at Y142 by tyrosine kinases induces BCL9 binding to β-catenin (Brembeck et al., 2004). Given that α-catenin and BCL9 have same binding sites on β-catenin, it has been proposed that BCL9 removes α-catenin and binds in its place as shown in figure 1.6. Another proposed mechanism suggests the presence of two forms of β-catenin, one that has adhesive function formed of β-catenin and α-catenin dimers. In presence of Wnt signalling conformational changes are induced in the CTD of β-catenin generating another monomeric form of β-catenin which selectively binds TCF/LEF leading to target gene transcription (Gottardi and Gumbiner, 2004).

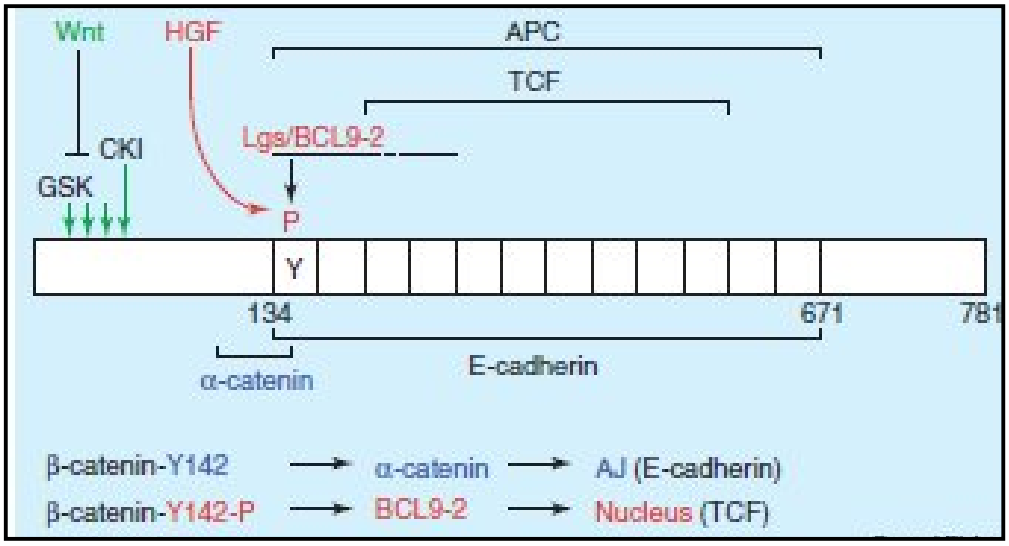

Figure 1.6. β-catenin choice of binding partners in Wnt signalling and cell adhesion. Phosphorylation of tyrosine 142 is suggested to switch the function of β-catenin from cell adhesion to Wnt signalling.
(Reprinted from Current Biology, Vol. 15, Bienz M., β-Catenin: A Pivot between Cell Adhesion and Wnt Signalling, R64-67, Copyright 2005, with permission from Elsevier)

7) Casein kinases in WCP:

Several members of the casein kinase family i.e. CK1 and CK2 have been shown to be involved in modulating the WCP signalling. CK1 is a Ser/Thr kinase which has seven members in mammals α, β, γ1, γ2, γ3, δ and ε (Hanks and Hunter, 1995). CK1 involvment in WCP signalling came from the observation that, in *Xenopus* embryos CK1 can induce axis duplication and stabilization of β-catenin (Sakanaka et al., 1999). Different members of the CK1 family have distinct functions in the WCP. CK1α is involved in the priming phosphorylation of β-catenin at Ser 45 in the destruction complex (Liu et al., 2002). CK1ε is involved in phosphorylation of Dvl, where it was suggested that this phosphorylation leads to recruitment of a GSK-3β binding protein (GBP) to the destruction complex inhibiting GSK-3β activity (Lee et al., 2001). CK1 γ is involved in the phosporylation of the LRP5/6 tail enhancing its interaction with axin during WCP activation (Davidson et al., 2005).

CK2, another member of the casein kinase family, has been identified as a positive regulator of the WCP signalling (Seldin et al., 2005). CK2 is a tetrameric holoenzyme composed of two catalytic alpha and /or alpha` (α and/or α`) subunits and two regulatory beta (β) subunits (Traugh et al., 1990). Overexpression of CK2 in *Xenopus* embryo resulted in ectopic axis formation (Dominguez et al., 2004). CK2 was shown to bind to and phosphorylate Dvl (Willert et al., 1997). CK2 was also shown to phosphorylate β-catenin at Thr393 making it more resistant to proteasomal degradation and increasing its transcriptional activity (Song et al., 2003).

8) Wnt target genes:

Wnt target genes are diverse regulating several aspects from cell differentiation, proliferation, to cell cycle regulation and apoptosis, a selected list of Wnt target genes is shown in table 1.2. Vlad et al. have classified Wnt target genes to three levels (figure 1.7): 1- Primary level, where TCF/LEF regulate effectors with direct biologic function such as matrix metalloprotease-7 (MMP-7), transcription factors such as c-myc and regulators of pathways such as vascular endothelial growth factor (VEGF). 2- Secondary level, which includes genes regulated by transcription factors such as p21 and target pathways. Of note, Wnt pathway itself is the most regulated pathway indicating feedback regulation which is positive as in case of Fz receptors upregulation, or negative as in the case of Dkk1 upregulation. 3- Tertiary levels, which contain effectors of target pathways regulated by Wnt (Vlad et al., 2008). However, given this diversity of Wnt genes, it should be noted that not all genes are activated in the same cell, but only some genes are activated in specific cells, indicating cell type specificity of Wnt signalling (Logan and Nusse, 2004).

Table 1.2. Selected list of Wnt target genes with their corresponding biochemical function and regulation trend:
(Reprinted from Cellular Signalling, Vol. 20, Vlad A et al., The first five years of the Wnt targetome, 795-802, Copyright 2008, with permission from Elsevier)

Function	Target gene	Trend
Cell cycle kinase regulators	Cyclin D1 p21	Up Down
Cell adhesion proteins	L1CAM, , Nr-CAM, connexin-30 E-cadherin, periostin	Up Down
Receptors	CD44, Fz7, EGF, Met, Ret, retinoic acid receptor gamma	Up
Factor synthases	COX-2, NOS-2	Up
Hormone, growth factors	BMP4, Dkk1, gastrin, FGF20/18/9/4, IGF-I/II, IL-6, jagged, nanog, SFRP, VEGF,	Up
Transcription regulators	c-myc, c-Jun, Sox-2, SALL4, TCF, LEF, Fra-1, Twist	Up
Proteases, protease inhibitors, protease receptors	CD44, MMP-7, stromelysin-1, survivin, ADAM10	Up
Matrix proteins	Fibronectin, keratin	Up
Other	MDR1, βTrCP	Up

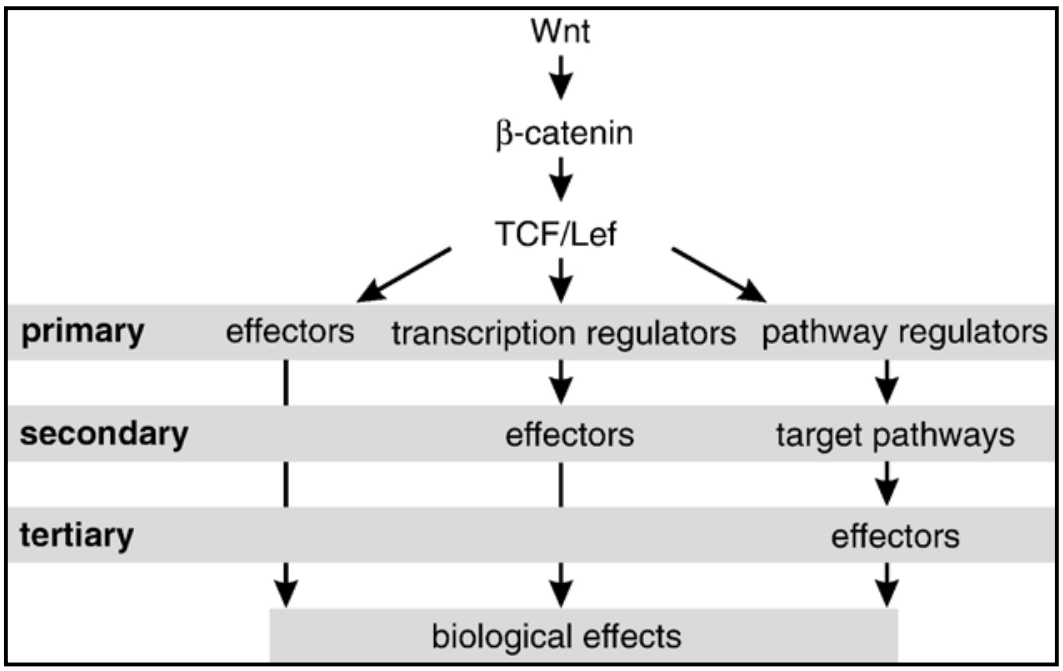

Figure 1.7. Three levels of Wnt target genes.

(Reprinted from Cellular Signalling, Vol. 20, Vlad A et al., The first five years of the Wnt targetome, 795-802, Copyright 2008, with permission from Elsevier)

1.2.2. Non-canonical Wnt signalling

The discovery of non canonical Wnt signalling came from the experiments in *Xenopus* embryos which showed that some Wnts as Wnt1 and Wnt8 caused axis duplication through activation of WCP (β-catenin dependent pathway). On the other hand other Wnts as Wnt4, Wnt 5a and Wnt11 caused defects in convergence and extension of the body axis without an effect on cell fate (Du et al., 1995; Moon et al., 1993b). Many non-canonical Wnt signalling pathways have been identified todate some of which use the same receptors used in WCP while others use alternative Wnt receptors (van Amerongen and Nusse, 2009). Although, these non-canonical Wnt pathways were primarily identified by their ability to regulate morphogenetic processes, recently more evidence suggests that components of these pathways can promote malignant progression. For example, Wnt5a expression was associated with tumor cells proliferation in NSCLC, and stimulated migration and invasiveness in gastric cancer (Huang et al., 2005; Kurayoshi et al., 2006). The following are known non-canonical Wnt signalling pathways known:

A) Planar cell polarity signalling:

Planar cell polarity (PCP) is the process in which cells orient themselves relative to the plane of the tissue in which they reside. PCP pathway is the best characterized non-canonical Wnt signalling pathway. In vertebrates, PCP signalling is mediated by non-canonical Wnts such as Wnt5a which do not signal through β-catenin (Heisenberg et al., 2000; Moon et al., 1993a). In mice, Wnt5a knockout causes defects in convergence extension movements (Qian et al., 2007). PCP signalling is mediated through Fz receptors and Dvl leading to activation of downstream effectors involved in cellular polarity. DEP domain of Dvl plays an important role in activation of small GTPases as Rac and Rho (Boutros and Mlodzik, 1999).

Dvl forms various complexes with Rac which leads to activation of downstream targets as c-Jun N-terminal kinase (JNK). Other complexes are reported between a) Dvl and Rho, and b) Dvl and RING finger protein XRNF185, however, many aspects of the exact mechanism of action of these complexes are still enigmatic (Habas et al., 2001; Rosso et al., 2005; Sugimura and Li).

B) Wnt-Calcium signalling pathway:

In this pathway binding of the Wnt to Fz receptors induces activation of heterotrimeric G proteins, which stimulates the release of calcium from intracellular stores (figure 1.8) (Semenov et al., 2007). Calcium release results in activation of intracellular calcium sensitive enzymes such as calcineurin, calcium-calmodulin-dependent kinase II (CamKII) and protein kinase C (PKC) (Kuhl et al., 2000; Sheldahl et al., 1999). Calcineurin is a protein phosphatase which leads to activation of calcium dependent molecules as the transcription factor nuclear factor associated with T cells (NFAT) (Saneyoshi et al., 2002). CamKII in turn can lead to activation of nemo-like kinase (NLK) and TAK1 kinases which signal in the mitogen-activated protein kinase (MAPK) pathway (Ishitani et al., 2003). PKC regulates the small GTPase Cdc42 which has the ability to remodelling the actin cytoskeleton (Choi and Han, 2002). However, it is controversial whether Wnt-calcium signalling pathway does in fact need Wnt binding. Also, conflicting data exist regarding the involvement of Dvl in this pathway activation; as a Dvl mutant which lacks the DIX domain increases intracellular calcium influx in *Xenopus* embryos and loss of Dvl results in a decrease of membrane translocation of ectopically expressed PKC (Sheldahl et al., 2003).

Cross talk between the WCP and the calcium signalling pathway has been suggested and data implicated the Wnt-calcium pathway in antagonizing the WCP. Treatment of NIH3T3 cells with ionomycin led to inhibition of WCP (Maye et al., 2004). In *C.elegans*, studies showed that activated MAPK can phosphorylate β-catenin/TCF complex leading to its inhibition (Ishitani et al., 1999). Similarly, dominant negative TAK1 was shown to inhibit β-catenin dependent transcription (Ishitani et al., 2003). In *Xenopus* embryos, dominant negative NFAT was shown to stabilize β-catenin and a constitutively active NFAT inhibited Wnt induced axis duplication (Saneyoshi et al., 2002).

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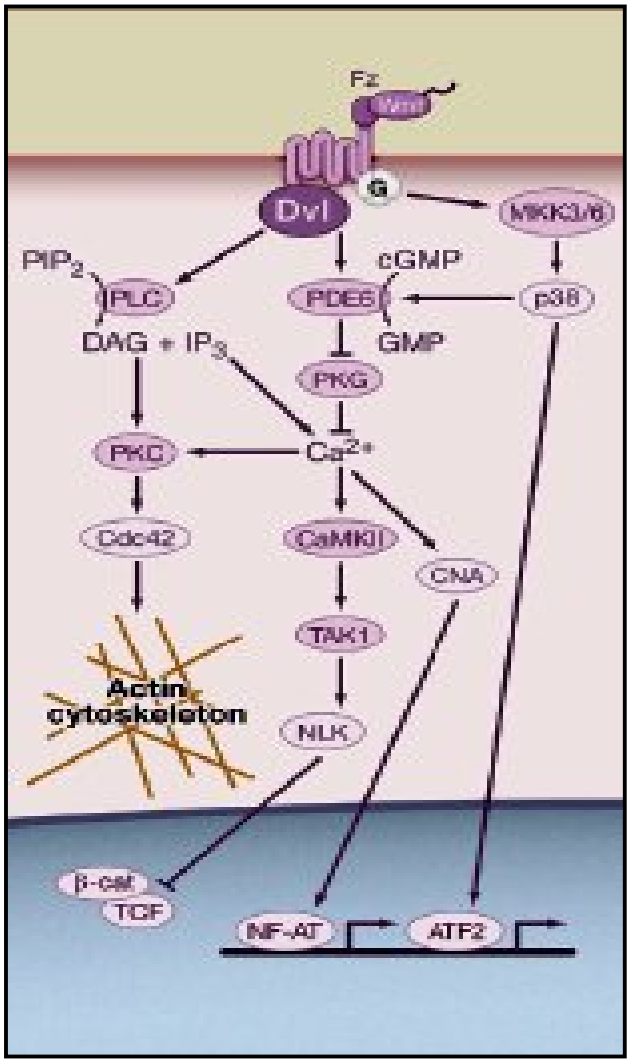

Figure 1.8. Non canonical Wnt signalling: calcium signalling pathway.

(Reprinted from Cell, Vol. 131, Semenov MV et al., SnapShot: Noncanonical Wnt Signaling Pathways, 1378.e1-1378.e2, Copyright 2007, with permission from Elsevier)

C) Other non-canonical Wnt signalling pathways:

1- Wnt-RYK signalling:

RYK is a tyrosine kinase-like receptor which can bind Wnt ligands and mediates Wnt-induced repulsion of axons and cell migration (Schmitt et al., 2006). RYK is related to the *Drosophila* homologue Derailed which functions in a Src-dependent manner (Wouda et al., 2008).

2- Wnt-ROR2 signalling:

ROR2 is a single pass receptor tyrosine kinase, which contains a CRD and binds to Wnt5a. It can also bind Filamin A which is an actin binding protein promoting filopodia formation (Oishi et al., 2003). In *Xenopus* embryos, ROR2 inhibited WCP and influenced convergence and extension movements by activation of JNK (Yamamoto et al., 2008).

3- Wnt-mTOR signalling:

The mammalian target of rapamycin (mTOR) is an important translation regulator implicated in tumorigenesis. Wnt through inhibiting GSK-3β mediated phosphorylation of tuberous sclerosis complex 2, a tumor suppressor which negatively regulates mTOR, can activate mTOR mediated translation. Fz, LRP5/6, Dvl and axin but not β-catenin are required for mTOR activation by Wnt (Inoki et al., 2006).

4- Wnt-GSK-3β-microtubule signalling:

Wnt signalling through Dvl stabilizes microtubules by inhibiting GSK-3β which phosphorylates the microtubule associated protein 1B. This pathway is involved in axonogenesis and is a transcriptional independent mechanism (Ciani et al., 2004).

1.3. Wnt inhibitors

Inhibitors of Wnt signalling range fall into two main groups: 1- secreted Frizzled-related proteins (SFRPs) and Wnt inhibitory factor-1 (WIF-1) which bind Wnt ligands and/or Fz receptors, 2- Dickkopf (Dkk) and Sclerostin which bind to LRP5/6 receptors. Also, new inhibitors have been identified such as Shisa which can entrap Fz in the endoplasmic reticulum (MacDonald et al., 2009) (figure 1.9).

A) SFRPs:

SFRPs are a family of proteins that have structural similarity to Fz receptors. Their N-terminal contain a CRD with ten cysteine residues forming disulphide bridges, which is similar to CRD of Fz receptors (Roszmusz et al., 2001). They comprise 5 members SFRP1 to SFRP5, sequence comparison shows that SFRP1, SFRP2 and SFRP5 are closely related while SFRP3 and SFRP4 cluster together (Bovolenta et al., 2008). SFRPs were shown to interact physically with Wnt proteins because of their homology to the Wnt binding region of the Fz receptors (Lin et al., 1997). Evidence of SFRPs inhibition of Wnt signalling came from studies showing that SFRP1 and 2 could inhibit Wnt3a signalling and β-catenin transcriptional activity in cell culture (Wawrzak et al., 2007). Also, some reports suggest that the CRD of SFRP can dimerize with other SFRPs and may also bind to Fz receptors itself as an alternative mechanism of inhibiting Wnt signalling through formation of non-functioning receptor complexes (Bafico et al., 1999). Loss of SFRPs expression was implicated in cancer pathogenesis indicating their important role as possible tumor suppressors. The loss of SFRPs expression in cancer cells can be attributed to two different mechanisms; either epigenetic inactivation or allelic loss.

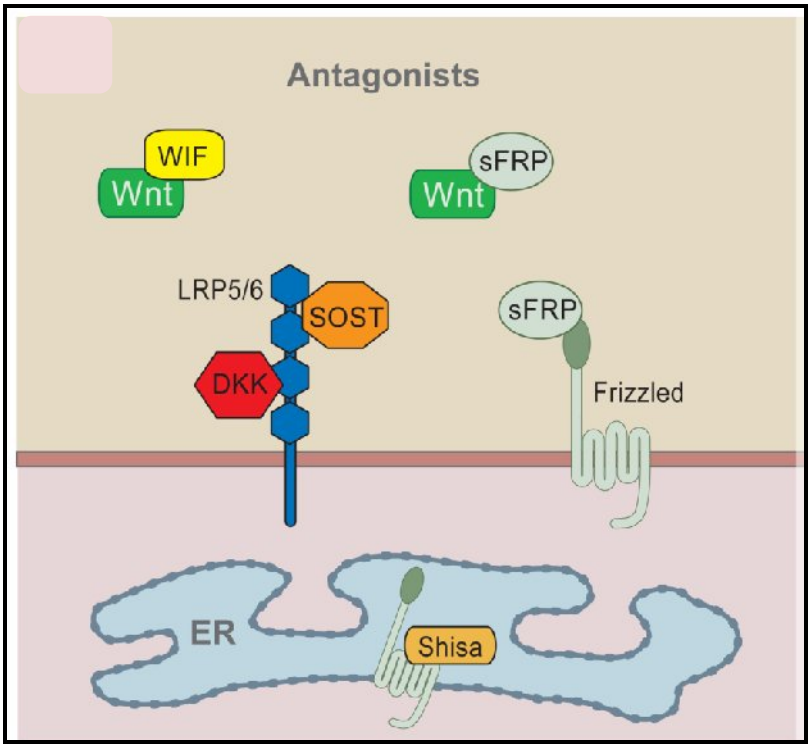

Figure 1.9. Secreted Wnt antagonists. WIF-1 and SFRP bind directly to secreted Wnts and/or Fz. Dkk and SOST proteins bind LRP5/6 preventing Fz-LRP5/6 complex formation. Shisa proteins trap Fz in endoplasmic reticulum.

(Reprinted from Developmental Cell, Vol. 17, MacDonald BT et al., Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases, 9-26, Copyright 2009, with permission from Elsevier)

Epigenetic inactivation of SFRPs due to promoter methylation was shown for SFRP1, SFRP2, SFRP4 and SFRP5 as they contain rich CpG islands. Promoter methylation of SFRPs were shown in mesothelioma, cervical, breast and colorectal cancers (Caldwell et al., 2004; Chung et al., 2009; Lee et al., 2004; Veeck et al., 2008; Zhou et al., 1998). The other mechanism of SFRP inactivation is loss of heterozygosity (LOH), *SFRP1* gene was shown to lie at the chromosome locus 8p which is commonly associated with deletions (Armes et al., 2004). LOH of SFRPs was reported in breast, lung and colorectal cancers (Leach et al., 1996; Ugolini et al., 1999). Furthermore, SFRP5 promoter methylation was an independent risk factor for reduced overall survival (OS) in breast cancer patients (Veeck et al., 2008).

B) WIF-1:

WIF-1 is another secreted protein which has the ability to bind to Wnt ligands inhibiting Wnt signalling. Unlike SFRPs WIF-1 does not have a CRD and the exact mechanism of its binding to the Wnt ligands is not completely understood. WIF-1 has N-terminal signal sequence, a WIF domain which is highly conserved across species, and five epidermal growth factor (EGF)-like repeats (Hsieh et al., 1999). The WIF domain is similar to that found in the RYK (related to tyrosine kinase) receptor tyrosine kinase, which is one of the receptors implicated in non-canonical Wnt signalling (Patthy, 2000). Several reports implicate WIF-1 as a tumor suppressor where it is inactivated through promoter methylation in different cancers as astrocytoma, mesothelioma, lung cancer, hepatocellular carcinoma, and breast cancer (Ai et al., 2006; Deng et al., 2010; Kohno et al., 2010; Mazieres et al., 2004; Yang et al., 2010). Downregulation of WIF-1 protein expression was found in breast cancer, prostate cancer, non small cell lung cancer (NSCLC) and urinary bladder

cancer as compared to their normal epithelial counterparts (Wissmann et al., 2003). Furthermore, downregulation of WIF-1 correlated with high tumor grade in urinary bladder cancer and WIF-1 methylation was an independent poor prognostic factor for disease free survival (DFS) in acute promyelocytic leukemia (Chim et al., 2006; Wissmann et al., 2003)

C) Dkk:

Dkk1 was the first member of the Dkk family to be identified by its ability to block Wnt signalling. Four isoforms are found in humans Dkk1-4 which contain two CRDs i.e. an N-terminal and C-terminal domain which are conserved between family members (Glinka et al., 1998). Only Dkk1 and Dkk4 were reported to suppress Wnt-induced secondary axis duplication in *Xenopus* embryos (Krupnik et al., 1999). Dkk1 inhibits Wnt signalling by binding to the Wnt co-receptor LRP5/6 (Mao et al., 2001).

Dkk have been implicated in several human diseases. Knockdown of Dkk2 in mouse models led to blindness due to keratinization of the epithelium of the retina secondary to activation of the WCP (Mukhopadhyay et al., 2006). Dkk loss have also been implicated in cancer as in epithelial ovarian cancer Dkk3 loss of expression was reported in 66% of patients, similarly epigenetic inactivation of Dkk3 promoted growth in lung cancer (You et al., 2011; Yue et al., 2008). Inactivation of Dkk3 by promoter methylation was an independent prognostic factor for poor OS and short DFS in breast cancer patients and as poor outcome factor in gastric cancer patients (Veeck et al., 2009; Yu et al., 2009).

D) Sclerostin:

Sclerostin is a secreted protein product of *SOST* gene which is mutated in patients with sclerosteosis (an autosomal recessive disease characterized

by overgrowth of bone tissue) (Balemans et al., 2001). It was found that sclerostin is a Wnt inhibitor which binds to LRP5/6 receptors disrupting Fz-LRP5/6 interaction. Furthermore, abnormalities seen in sclerosteosis are similar to the increased bone mass density seen in gain of function mutation in *LRP* gene (Semenov et al., 2005). Currently, antibodies against sclerostin are being tested as a potential therapy for osteoporosis (Rachner et al., 2011).

E) Shisa:

Shisa is an antagonist for both Wnt and fibroblast growth factor signalling. Shisa function by trapping of Fz in endoplasmic reticulum inhibiting their release. It was previously identified as head inducer in *Xenopus* and its mouse homologue was identified (Furushima et al., 2007).

1.4. Wnt pathway in development

Wnt signalling has been shown to be an important mediator of stem cell renewal as well as development in various tissues such as hematopoietic cells and mammary tissues (Nusse, 2008).

1.4.1. Wnt in haematopoiesis

Haematopoietic stem cells (HSCs) are cells which give rise to blood elements (Baum et al., 1992). Self renewal of HSCs was found to involve the activation of β-catenin, as overexpression of β-catenin was able to reconstitute the haematopoietic system in lethally irradiated mice. Ectopic expression of Wnt pathway inhibitors such as axin inhibited HSCs growth *in vitro* and reduced *in vivo* marrow reconstitution. The same results were obtained using purified Wnt3a protien (Reya et al., 2003; Willert et al., 2003).

In T-cells, Wnt signalling was required for differentiation of thymocytes, but it had no role in biology of mature T cells (Schilham et al., 1998; Verbeek et al., 1995). Mouse deficient in Wnt1 and Wnt4 had a reduced thymic cellularity especially those of immature ones. Also, transgenic mice with increased axin had reduced thymic cellularity and apoptosis (Hsu et al., 2001; Mulroy et al., 2002). In B-cells, Wnt3a was shown to stimulate pro-B cells (Reya et al., 2000). Different B-cell progenitor maturation stages showed differential expression of the Wnt receptors and coreceptors, suggesting that canonical Wnt signalling regulates early B-cell lymphopoiesis (Dosen et al., 2006; Wang, 2004).

1.4.2. Wnt in breast development

Wnt signalling has been implicated in different stages of mammary development from prenatal stage to pregnancy changes. Formation of the

mammary rudiments required the Wnt component LEF1, where mice deficient in LEF1 failed to develop mammary buds. Also, in mice transgenic for the Wnt antagonist Dkk1 mammary buds were absent (Andl et al., 2002; van Genderen et al., 1994).

In postnatal development, at puberty several members of the WCP are expressed. Ectopic expression of Wnt1 in transgenic mouse model led to ductal outgrowth. Mice which expressed mutated stabilized β-catenin developed lobuloalveolar hyperplasia similar to that seen in MMTV-Wnt1 transgenic mice (Imbert et al., 2001). Also, it has been shown that the tumors induced in transgenic mice by Wnt pathway have early developmental markers which suggest that Wnt induces cancer from progenitor cells. On the other hand, the small tumors which are induced in MMTV-Wnt1 or with β-catenin mutation imply that to progress to carcinoma other additional oncogenic changes are required (Li et al., 2003). In pregnancy, presence of either ectopic Wnt1 or Wnt10a induced alveolar hyperplasia in virgin mice similar to that found in mice at mid pregnancy. Wnt4 overexpression led to ductal hyperbranching and it was found to be increased in pregnancy (Brisken et al., 2000; Lane and Leder, 1997).

1.5. Wnt pathway in disease

Wnt regulates several aspects of development and is important for tissue homeostasis. Accordingly, it is not surprising that deregulation of the components of Wnt signalling can lead to development of human diseases (Clevers, 2006). Table 1.3 shows a list of diseases associated with deregulation of different members of the Wnt signalling (Barker and Clevers, 2006; Luo et al., 2007). One of the important consequences of Wnt deregulation is the development of cancer and a growing list of cancers has been linked to the deregulation of the Wnt pathway. We will discuss three important cancers related to deregulation of the Wnt pathway; colorectal cancer as it represents the paradigm of Wnt deregulation, breast cancer and hematopoietic malignancies as they are related to scope of the current thesis.

Table 1.3. Diseases linked to aberrant Wnt activation

(Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery. Barker N and Clevers H. Mining the Wnt pathway for cancer therapeutics. 5, 997-1014, copyright 2007)

Pathway component	Observed alteration	Associated disease/cancer
Wnt ligands	Increased expression	Barrett's esophagus, rheumatoid arthritis and schizophrenia Colon cancer, breast cancer, melanoma, head & neck cancer, NSCLC, gastric cancer and mesothelioma
Frizzled receptors	Increased expression	Rheumatoid arthritis Colon cancer, breast cancer, head & neck cancer, gastric cancer, synovial sarcomas
Dishevelled family members	Increased expression	Mesothelioma, NSCLC, and cervical cancer
APC	Loss-of-function mutations/reduced expression	Barrett's oesophagus Colon cancer
β-catenin	Gain-of-function mutations	Colon cancer, gastric cancer, hepatocellular cancer, hepatoblastoma, Wilm's tumour, endometrial ovarian cancer, adrenocortical tumours and pilomatricoma
Axin 1	Loss-of-function mutations	Hepatocellular cancer and hepatoblastomas
SFRP family members	Reduced expression	Barrett's oesophagus Colon cancer, breast cancer, gastric cancer, mesothelioma, NSCLC and leukaemia
WIF family members	Reduced expression	Colon cancer, breast cancer, prostate cancer, lung cancer, bladder cancer and mesothelioma
LRP5	Gain-of-function mutations	Increased bone density

1.5.1. Wnt and Colorectal cancer:

Colorectal cancer (CRC) represents the paradigm for the accumulation of molecular and genetic events underlying tumors formation and progression (Fearon and Vogelstein, 1990). The discovery that the *APC* gene mutation was the culprit in the hereditary syndrome termed FAP opened the door towards the importance of the Wnt signalling in the pathogenesis of CRC (Powell et al., 1992). FAP patients develop large number of polyps in the colon in their early adulthood due to inheriting of one defective allele of the *APC* gene. In patients with FAP, some of the polyps, which are clonal growths, develop inactivation of the second allele of the *APC* gene, through a truncating mutation or through LOH leading to CRC development. Most (~60%) of the mutations in the *APC* gene occur in a region referred to as the mutation cluster region, resulting in C-terminal truncation of the protein, which contains the domain needed for binding to β-catenin. This consequently induces stabilization and accumulation of nuclear β-catenin (Beroud and Soussi, 1996). Furthermore, β-catenin gene mutation was found in 10% of sporadic CRC, where the mutation affects the phosphorylation site required for its degradation (Morin et al., 1997). Mutations in the *β-catenin* and *APC* genes are mutually exclusive but they are not entirely equivalent, where β-catenin mutations are found mainly in microsatellite instability type of CRC (Fodde et al., 2001). However, although *APC* or *β-catenin* gene mutations are considered important events in development of CRC, alterations affecting other oncogenic molecules as K-ras and p53 are important for further tumor progression (Fearon and Vogelstein, 1990).

1.5.2. Wnt and breast cancer:

WCP has been shown to be important in normal breast development as well in the as pathogenesis of breast cancer (Smalley and Dale, 2001). Constitutive activation of WCP in breast cancer, has been attributed to one or more of the following mechanisms: 1) increased expression of Wnt ligands as Wnt3a,Wnt4,Wnt6, Wnt8a, Wnt9 and Wnt10b in breast cancer cells in comparison to normal mammary cells (Benhaj et al., 2006), 2) inactivation/gene silencing of negative regulators of WCP such as the SFRPs and WIF-1 (Ai et al., 2006; Klopocki et al., 2004; Wissmann et al., 2003), and, 3) upregulation of the *Dvl* genes and Fz receptors (Milovanovic et al., 2004; Nagahata et al., 2003). Moreover, no genetic mutations have been identified in the components of the WCP as APC, β-catenin or Axin (Ozaki et al., 2005). Correlating with this concept, β-catenin activation, as evidenced by its cytoplasmic and/or nuclear accumulation demonstrated by immunohistochemistry, has been reported in 60% of breast tumors (Lin et al., 2000; Ryo et al., 2001). Different Wnts (i.e. Wnt1 and Wnt3a) have been shown to transform mouse mammary epithelial cells *in vitro* (Wong et al., 1994). Also, *in vivo*, transgenic mice overexpressing kinase inactive GSK-3β under the control of MMTV developed mammary tumors overexpressing β-catenin and cyclin D1 (Farago et al., 2005). Inhibition of Wnt1 using monoclonal antibody or short inhibitory RNA (siRNA) has been shown to induce apoptosis in breast cancer cell lines (He et al., 2004; Wieczorek et al., 2008). Lastly, in several studies, gene methylation of SFRPs has been shown to be an independent prognostic factor for a shortened survival in breast cancer patients (Veeck et al., 2008; Veeck et al., 2006). Similarly, cytoplasmic and/or nuclear accumulation of β-catenin correlated with a poor prognosis in breast cancer patients (Lin et al., 2000).

1.5.3. Wnt and hematologic malignancies:

Wnt signalling has been shown to be important for HSCs self renewal, accordingly its role in hematologic malignancies has been under investigation. In acute lymphoblastic leukemia (ALL), up regulation of Wnt target genes was associated with nuclear localization of β-catenin in the cell lines. Furthermore, aberrant inactivation of Wnt antagonists was associated with decreased OS and treatment with Wnt inhibitors induced apoptosis in ALL cells (Roman-Gomez et al., 2007). In acute myeloid leukemia (AML), there were aberrant expression of various components of the Wnt signalling in primary AML cells (Simon et al., 2005). Expression of β-catenin in AML cells was an independent prognostic factor predicting event free survival and shortened OS (Ysebaert et al., 2006). Furthermore, abnormal methylation of Wnt antagonists in AML was associated with decrease relapse free survival (Valencia et al., 2009). Small molecule inhibitors for β-catenin induced apoptosis in AML blasts compared to peripheral blood mononuclear cells (PBMCs) (Minke et al., 2009). In chronic lymphatic leukemia (CLL), inhibitors of GSK-3β, enhanced survival of CLL cells, and treatment with R-etodolac (non steroidal anti-inflammatory drugs shown to inhibit Wnt activity) led to increased apoptosis in CLL cells (Lu et al., 2004). Treatment with siRNA against β-catenin inhibited the growth of multiple myeloma in a xenograft model (Ashihara et al., 2009).

In lymphomas, a study on cutaneous lymphomas found that there is β-catenin deregulation in 21% of primary cutaneous B-cell lymphomas and in 42% of primary cutaneous T-cell lymphoma (Bellei et al., 2004). A recent study from our laboratory showed constitutive activation of WCP in mantle cell lymphoma (MCL), where we showed expression of various Wnt ligands and nuclear accumulation of β-catenin in MCL cell lines and

patient samples (Gelebart et al., 2008). Recently, we have also reported that β-catenin is transcriptionally active in anaplastic lymphoma kinase positive (ALK+) anaplastic large cell lymphoma (ALK^+ALCL) cell lines, and this conclusion was supported by its nuclear localization and transcriptional activity. Also, we found β-catenin to be biologically significant in ALK^+ALCL, as down-regulation of β-catenin using siRNA significantly reduced the growth of ALK^+ALCL cells (Anand et al., 2011).

1.6. Wnt and cross talk with other signalling pathways in cancer

Cross talk between two signalling pathways has been attributed to one or more of the following mechanisms which are: a) physical interaction between components of the two pathways, b) one pathway component is an enzymatic or transcriptional target of the other pathway, c) one pathway competes with or modulates a key mediator of the other pathway (figure 1.10) (Guo and Wang, 2009). Several signalling pathways were shown to cross talk with the Wnt pathway; we will discuss two of the well characterized pathways.

1.6.1. Wnt and EGFR:

Epidermal growth factor receptor (EGFR) is a glycoprotein which has extracellular, transmembrane and intracellular domains, with its C terminal intracellular domain containing multiple phosphorylation sites. It belongs to the ErbB family of receptor tyrosine kinases, which are activated upon ligand binding phosphorylating itself and various effector molecules (Ullrich and Schlessinger, 1990; Wells, 1999). Numerous signalling pathways are activated downstream of EGFR including ras-MAPK, signal transducers and activators of transcription (STAT), heterotrimeric G proteins, phosphatidylinositol 3 kinase (PI3K) and the cytoplasmic tyrosine kinase src (Alroy and Yarden, 1997; David et al., 1996; Zwick et al., 1999). Deregulation of ErbB receptors have been implicated in development of different human cancers and are being used as a target for cancer therapy (Arteaga, 2003; Herbst and Shin, 2002).

The cross talk between Wnt and EGFR was shown in various cancer models, in breast cancer, ErbB was shown to associate with β-catenin (Schroeder et al., 2002) and Wnt was shown to mediate cell proliferation through the activation of the EGFR pathway (Schlange et al., 2007).

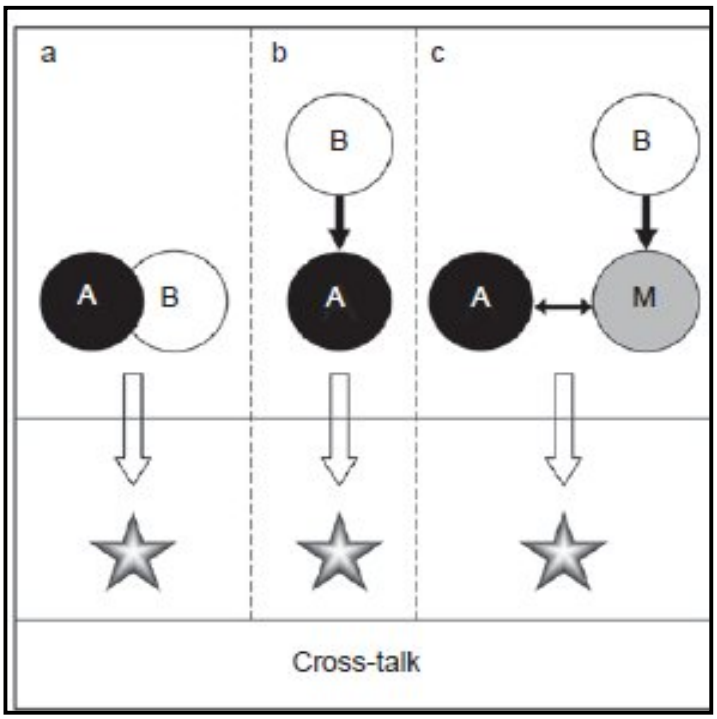

Figure 1.10. Models of cross talk between signalling pathways, where A and B represent two different signalling pathways. A and B must be connected in at least one of three ways: (a) components of the two pathways physically interact, (b) components of one pathway are the enzymatic or transcriptional target of the other and (c) one pathway modulates or competes for a key modulator (M) of the other pathway (arrows represent either positive or negative regulation)
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Furthermore, in mice with transforming growth factor-α (an EGFR ligand) mutations, latency of mammary tumors were further reduced when Wnt was induced using MMTV (Schroeder et al., 2000). In CRC, *K-ras* and *APC* mutations act synergistically in development of CRC (Janssen et al., 2006). In APC deficient mice there is an increase in EGFR activity in the intestinal enterocytes and adenomas (Moran et al., 2004). In NSCLC, a positive correlation was found between the nuclear accumulation of β-catenin and EGFR mutations (Suzuki et al., 2007). The points of possible convergence and cross talk between the two pathways are through a) Fz receptors which have structural similarity to G-protein coupled receptors (Prenzel et al., 1999), b) β-catenin where EGF stimulation can cause its tyrosine phosphorylation leading to its dissociation from E-cadherin at the membrane (Piedra et al., 2001).

1.6.2. Wnt and nuclear receptors:

Nuclear receptors (NRs) are a family of transcription factors which are regulated by ligands such as steroids, vitamin A, vitamin D, peroxisome proliferator-activated receptor γ (PPARγ) and thyroid hormones. They are divided into type I receptors which forms homodimers and type II receptors which forms heterodimers (Escriva et al., 2004). Cross talk between NRs and Wnt has been implicated in regulation of stem cell differentiation and cancer. Many of the NRs ligands such as PPARγ, glucocorticoids, progesterone and retinoic acid can induce the transcription of Wnt antagonists such as Dkk1 (Tulac et al., 2006; Verani et al., 2007). Also, retinoic acid was shown to redirect β-catenin to the membrane inducing a decrease in its nuclear levels which is the mainstay for WCP signalling (Byers et al., 1996). Furthermore, androgen receptors can directly bind to β-catenin in the nucleus competing with TCF/LEF binding (Mulholland et al., 2003). On the other hand, ectopic Wnt expression inhibits expression

of PPARγ (Kang et al., 2007). Although, in most cases these 2 pathways seem to be inhibiting each other, the context is mainly cell type specific as the interaction in some cases can lead to activation rather than inhibition (Aguilera et al., 2007).

1.7. Wnt pathway and cancer therapeutics

Given the importance of the WCP signalling in cancer development, several efforts have been made to target this pathway. The drugs targeting Wnt pathway range from new molecular compounds which are still under going trials to compounds which are already in the market and being used (Dihlmann and von Knebel Doeberitz, 2005) (figure 1.11).

A) Non specific Wnt inhibitors:

1. Non Steroidal anti-inflammatory drugs (NSAIDS)

NSAIDs are used clinically for treating different diseases; they also have been shown to have anticancer effects. Studies showed 45% reduction in CRC in patients taking NSAIDS. This action was attributed to inhibition of cyclooxygenase-2 (COX-2) activity in tumor cells. However, several studies on cell lines and animal models pointed to other possible mechanisms for their anti-neoplastic effect, as drugs lacking COX-2 activity can inhibit tumors growth and vice versa tumors lacking COX-2 activation are affected by COX-2 inhibitors (Jolly et al., 2002; Marx, 2001). Recently, it was suggested that NSAIDs act through inhibition of WCP, as they were found to restrict polyp formation in mouse model with FAP (Chan, 2003). Indomethacin was shown to arrest the growth of CRC at G_1 phase and to reduce nuclear β-catenin (Brown et al., 2001). Nitric oxide releasing Aspirin is a product with more improved effects than aspirin and reduced side effects as GIT ulceration and bleeding. *In vitro,* it is 2500-5000 times more potent than aspirin in inhibiting the growth of CRC cells; also it affected β-catenin in a concentration dependent manner (Nath et al., 2003). Celecoxib, a new selective COX-2 inhibitor reduced polyp formation by 28% in FAP patients. In 1999, Celecoxib was approved by the Food and Drug Administration (FDA) for the reduction and regression of polyps in FAP patients (Phillips et al., 2002; Steinbach et al., 2000).

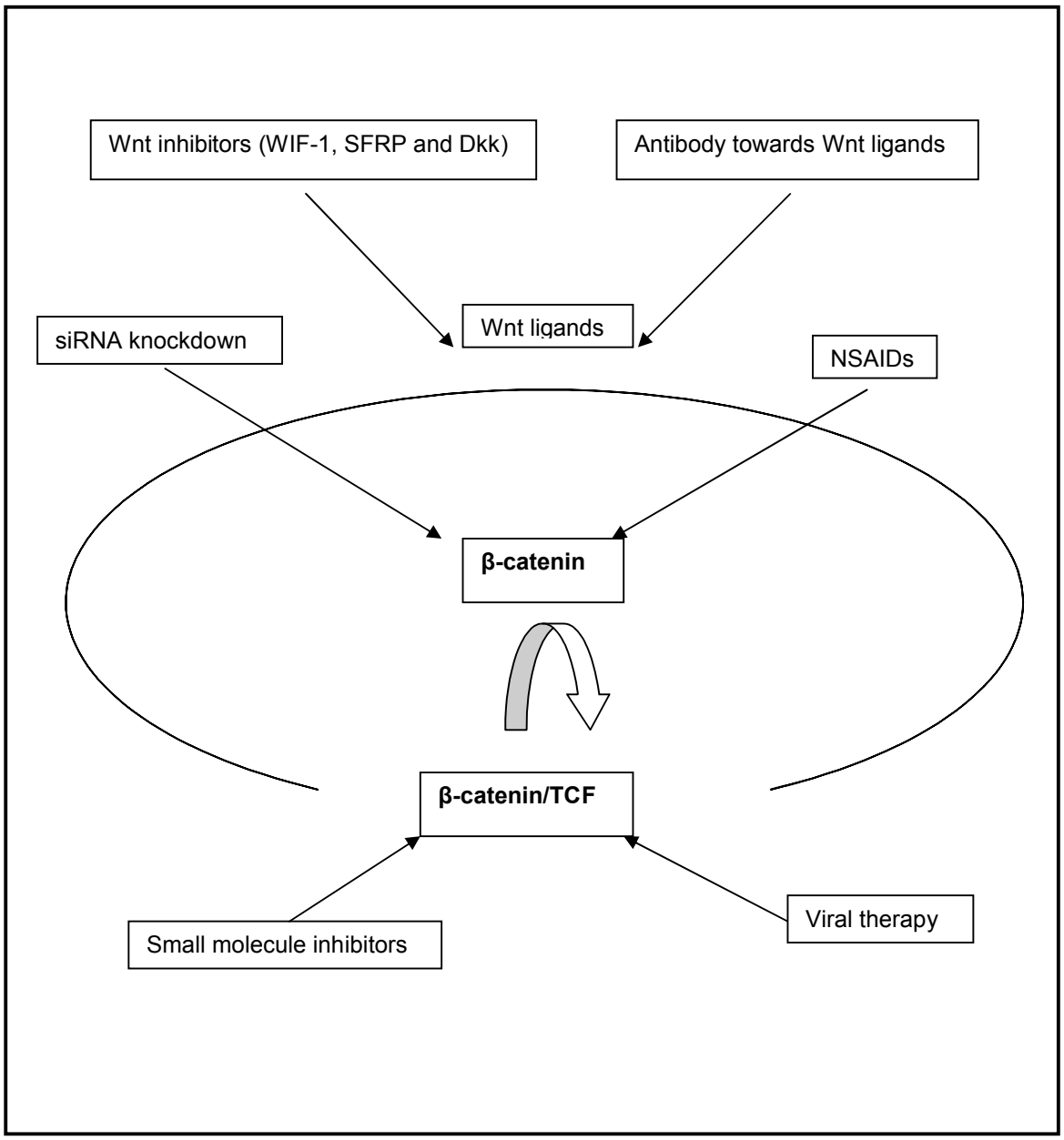

Figure 1.11. Potential strategies used for targeting the Wnt pathway

Exisulind is a NSAID which lacks COX-2 inhibitory activity; is thought to have anti-neoplastic effect secondsary to the inhibition of protein kinase G which can induce phosphorylation of β-catenin. However, in cell lines expressing a mutation of *APC* or *β-catenin* genes, exisulind still induced cell death (Goluboff, 2001). R-etodolac, another NSAID lacking COX-2 inhibitory activity induced downregulation of β-catenin at physiologic doses on hepatocellular carcinoma cell lines (Behari et al., 2007). Also, a novel etodolac analog SDX-308 induced inhibition of growth of multiple myeloma cells which was associated with the inhibition of the β-catenin/TCF pathway (Yasui et al., 2007).

2. Vitamins:

Vitamin A is converted in the body to different metabolites called retinoids. It was shown that agonists of retinoid X receptor induced degradation of β-catenin and repressed β-catenin mediated transcription. Studies indicate that this action is due to competition with β-catenin for binding site on TCF (Xiao et al., 2003). Also, Vitamin D was shown to increase E-cadherin expression in colon cancer cell lines and consequently inhibiting β-catenin signalling (Palmer et al., 2001).

3. Endostatin:

Endostatin inhibits blood vessel formation, proliferation of endothelium and suppresses tumor growth in animal models (O'Reilly et al., 1997). Endostatin was shown to inhibit WCP by stimulating the degradation of β-catenin (Hanai et al., 2002).

4. Natural products:

Folic acid reduced nuclear translocation of β-catenin in CRC cells (Jaszewski et al., 2004). Flavonoids are plant derivatives used to provide color to fruits and vegetables; Quercetin is the major representative of them. Quercetin was shown to have an antiproliferative effect on CRC and MCL which is mediated through the inhibition of WCP signalling (Gelebart et al., 2008; Park et al., 2005a). Curcumin, another natural plant product that exhibits anti-neoplastic activity, has also been shown to reduce β-catenin/TCF transcriptional activity (Park et al., 2005b).

B) Inhibitors of other signalling pathways

Gleevec (imatinib) is a small molecule inhibitor with activity against protein tyrosine kinases. It induced remission in patients with chronic myelogenous leukemia (CML) (Joensuu and Dimitrijevic, 2001). Studies have shown that β-catenin is not only regulated through phosphorylation at Ser/Thr residues, but it also can be affected by tyrosine phosphorylation through growth factors. Recently, β-catenin was found to be downregulated by Gleevec in Wnt1 induced cancer cells which led to the hypothesis that Gleevec has an impact on WCP (Zhou et al., 2003). In anaplastic thyroid tumors, Gleevec treatment resulted in reduction of β-catenin expression and its redistribution from the membrane to the nucleus (Rao et al., 2006).

C) Specific strategies targeting the Wnt pathway

1. Protein knockdown and siRNA targeting β-catenin:

In absence of the Wnt signalling, β-catenin is targeted for destruction through the ubiquitin system.This destruction is dependent on the Ser/Thr residues at the N-terminal of the β-catenin. In cancers with β-catenin mutation, this destruction is ineffective as the mutation usually affects the N-terminus which is the site for phosphorylation. Recent studies have

developed a mechanism to direct β-catenin for ubiquitination regardless of their state of phosporylation by developing chimeric F box proteins. One of these studies used a chimera consisting of β-catenin binding domain of E-cadherin fused to βTrCP ubiquitin protein ligase (Cong et al., 2003). Also, siRNA against β-catenin has been shown to inhibit tumor cell growth in CRC and ALK^+ALCL (Anand et al., 2011; Verma et al., 2003).

2. Antibodies towards Wnt ligands:

Wnt signalling pathway can be blocked by agents that selectively inhibit the binding of Wnt proteins to Fz receptors.Wnt1 signalling has an important role in carcinogenesis, as blocking of Wnt1 signalling by anti-Wnt1 antibodies induces apoptosis and inhibition of growth in tumor cells (You et al., 2006). Using anti-Wnt1 antibodies induced apoptosis in head and neck and breast cancer (Rhee et al., 2002; Wieczorek et al., 2008). Using anti Wnt2 antibodies induced apoptosis in lung cancer and melanoma (You et al., 2004a; You et al., 2004b).

3. Small molecule inhibitors:

Interaction of β-catenin with TCF/LEF plays a central role in WCP; Lepourcelet et al. have used high-throughput screening to test various compounds for their effect on Wnt signalling. Out of 7000 they examined, 8 compounds showed promising results with their IC50 lower than 10μM. Three of these compounds (PKF115-584, PKF222-185 and CGP049090) scored consistently in different assays including reporter gene assay, electrophoretic mobility shift assay and a GST-pulldown assay (Lepourcelet et al., 2004). Another group has identified a compound which can inhibit the interaction of β-catenin/TCF4 with the transcriptional coactivator CBP. The compound is called ICG-100, it induces apoptosis in CRC cells in a dose dependent manner with an IC50 of 8.07 μM (Eguchi

et al., 2005). Another small molecule which inhibits the interaction between the Dvl and Fz receptors is NSC668036. It was found to bind to the PDZ domain of Dvl inhibiting its interaction with Fz receptors. It blocked axis duplication in *Xenopus* embryo overexpressing Wnt3a (Shan et al., 2005). Another compound named FJ9 inhibited the interaction between Fz7 and PDZ domain of Dvl inducing apoptosis in human cancer cell lines and inhibiting tumor growth in a mouse xenograft model (Fujii et al., 2007). Other new small molecule inhibitors have been discovered that inhibit the activity of Porcupine which is essential for Wnt proteins secretion (Chen et al., 2009).

4. Viral based therapy:

Oncolytic viruses have been used to interfere with Wnt signalling by using adenoviral vectors which express cytotoxic genes under control of TCF promoters. Also, adenoviruses which selectively express Fas associated death domain have been developed (Chen and McCormick, 2001; Lipinski et al., 2004). An adenovirus with a TCF response element was used in anaplastic thyroid tumors which harbour a mutation of β-catenin and prolonging survival in mouse with tumors (Abbosh et al., 2007).

Despite the diversity of agents that can alter the Wnt pathway, currently Celecoxib is the only FDA approved drug for the treatment of patients with FAP (Phillips et al., 2002; Steinbach et al., 2000). Accordingly, until small molecule inhibitors of Wnt pathway are tested in clinical trials, drugs that are already available i.e. NSAIDs and vitamins can be used especially the newer generations with more efficiency and less side effects.

Challenges in developing drug targeted therapy to the WCP

Although several efforts have been made in trying to discover agents which could alter the activated WCP pathway in cancer, several challenges remains; The successful clinical application of Herceptin in patients with breast cancer with Human Epidermal growth factor receptor 2 (HER2) positivity and of Gleevec in patients with CML, points to important principle of targeting specific molecular pathways in cancer (Chang, 2007; Joensuu and Dimitrijevic, 2001). Accordingly, drugs that are used to inhibit the Wnt pathway should be only directed to tumors with known overactivity of WCP i.e. CRC. Drugs should confer a high selectivity, as β-catenin is an important factor for the adherens junction in normal cells (Daniels et al., 2001). Moreover, Wnt activity is also important for stem cell renewal in various tissues such as intestine, HSC and hair follicles (Huelsken et al., 2001; Reya and Clevers, 2005; Staal and Clevers, 2005). 3- Drug resistance is an important challenge facing drug development as Wnt pathway in interacting with other pathways as in case of CRC where *APC* gene mutation is the initiating event in CRC, but then other oncogenes as K-ras and p-53 are affected (Fearon and Vogelstein, 1990). Also, it is known that most cancers are heterogenous in their composition. A way to overcome this expected type of resistance is by combining drugs targeting different molecular pathways as using combination of Gleevec, Wnt inhibitors and other conventional chemotherapeutic agents.

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❖ Chapter 2

CLINICAL AND BIOLOGICAL SIGNIFICANCE OF GSK-3β INACTIVATION IN BREAST CANCER - AN IMMUNOHISTOCHEMICAL STUDY

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2.1. INTRODUCTION

Glycogen synthase kinase 3β (GSK-3β) was first identified as a serine/threionine kinase that inactivates glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). Functional aberrancies of GSK-3β have been implicated in the pathogenesis of various types of human disease including cancer (Doble and Woodgett, 2003). In colonic, ovarian and pancreatic adenocarcinomas, GSK-3β has been reported to promote cell growth and survival (Cao et al., 2006; Ougolkov et al., 2005; Shakoori et al., 2005). GSK-3β is known to be phosphorylated and inactivated by the phosphoinositide 3 kinase (PI3K/Akt) pathway (Cohen et al., 1997; Pap and Cooper, 1998). In addition, it is also known that GSK-3β is regulated by the Wnt canonical pathway (WCP). In the absence of stimulation of the WCP, GSK-3β forms the 'destruction complex' with various proteins including the adenomatous polyposis coli protein, axin, and casein kinase 1, thereby promoting the sequestration and proteasome degradation of cytosolic β-catenin. In the presence of stimulation of the WCP, the destruction complex falls apart, GSK-3β is phosphorylated/inactivated, and β-catenin accumulates in the cells and translocates to the nucleus, where β-catenin acts as a transcriptional co-activator by heterodimerizing with the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF). β-catenin has been shown to regulate the expression of a wide range of important genes including *c-myc* and *cyclin D1* (Clevers, 2006; Gordon and Nusse, 2006; Mikels and Nusse, 2006; Miller, 2002). GSK-3β can also mediate biological effects independent of β-catenin, as GSK-3β has been shown to phosphorylate a number of other substrates including eukaryotic initiation factor 2B (Jope and Johnson, 2004). The biological effects of GSK-3β appear to be cell-type specific; while functional GSK-3β promotes cell survival in ovarian and pancreatic cancer cells, it has been shown to promote apoptosis in cortical astrocytes following DNA damage *in vitro* (Cao et al., 2006; Ougolkov et al., 2005; Sanchez et al., 2003).

Recent studies suggest that PI3K/Akt is constitutively activated in a subset of breast cancer and that its activation can induce resistance to chemotherapy (Bose et al., 2006; Tokunaga et al., 2006). Thus, it is likely that GSK-3β is also inactivated in these tumors. The expression of the inactivated/phosphorylated form of GSK-3β (pGSK-3β) has not been extensively studied in breast cancer; we found only one paper describing the expression of pGSK-3β in breast cancer, in which approximately 60% of the tumors were scored positive by immunohistochemistry (Ding et al., 2007). However, the focus of this study was to establish the link between the expression of Mcl-1 and pGSK-3β, and the relationship between pGSK-3β and the activated form of Akt (pAkt) was not explored. Whether the pGSK-3β expression correlates with the nuclear localization of β-catenin was also not examined. Lastly, the relationship between pGSK-3β and various clinicopathologic parameters was not fully evaluated.

With this background, we assessed the activation status of GSK-3β in a series of breast carcinomas using immunohistochemistry to detect the expression of pGSK-3β, which is employed as a surrogate marker for the inactivation of GSK-3β. We assessed if pGSK-3β correlates with various clinical and pathologic parameters. In addition, we assessed if pGSK-3β correlates with the expression of pAkt, nuclear β-catenin, cyclin D1 and Ki67 (a cell proliferation marker).

2.2. MATERIALS AND METHODS

2.2.1. Patients and tissue specimens

With the approval of our institutional ethics review board, 72 consecutive, primary invasive breast carcinoma specimens resected in 1997 were retrieved from the laboratory at the Cross Cancer Institute in Edmonton, Alberta, Canada. All resection specimens had a minimum of 8 hours overnight fixation. These cases were constructed into tissue microarrays;

two cores from each case as well as cores from benign reactive tonsillar tissues (served as the controls) were included in the microarray tissue blocks. Morphologic features, including the histologic grade and the presence/absence of lymphatic invasion, were reviewed by one of the co-authors (JD). The hormone receptor expression status was determined using immunohistochemistry at the time of initial diagnosis. Information regarding treatment and clinical outcomes was available in 71 cases.

2.2.2. Immunohistochemistry

To detect pGSK-3β, β-catenin and Ki67, antigen retrieval was performed using microwave-treated citrate buffer (pH 6.0) for 20 minutes. To detect pAkt, Tris buffer (pH=11.0) and a pressure cooker were employed. To detect cyclin D1, antigen retrieval was performed using EDTA (pH=8.0). After antigen retrieval, tissue sections were incubated with 3% hydrogen peroxide (H_2O_2) for 10 minutes to block endogenous peroxidase activity. Subsequently, tissue sections were incubated overnight at 4˚C with a rabbit polyclonal antibody reactive with pGSK-3β (Ser 9)(1:100, Cell Signaling Technology, Danvers, MA), a rabbit polyclonal antibody reactive with pAkt (Ser473)(1:50 dilution, Cell Signaling), anti-cyclin D1 (1:75 dilution, Medicorp, Montreal, Quebec, Canada), anti- β-catenin (1:50 dilution, BD Transduction Laboratories, San Diego, CA) or anti-Ki67 (1:60 dilution, Dako Canada Inc., Mississauga, Ontario, Canada). Immunostaining was visualized using a labeled streptavidin-biotin (LSAB) method and DAB as a chromogen (Dako Canada). A case of mantle cell lymphoma positive for pGSK-3β, as previously shown by Western blots and immunohistochemistry, served as the positive control (Chung et al.; Gelebart et al., 2008). Benign tonsillar lymphoid tissues, as previously shown to be negative by immunohistochemistry and Western blots, served as the negative control.

2.2.3. Scoring of the markers and statistical analysis

Only invasive carcinomas were assessed. The score for pGSK-3β (or pAkt) for each case was the sum of the following: 3 points x % of tumor showing 3+ (strong) staining, 2 points x % of tumor showing 2+ (moderate) staining, 1 point x % tumor showing 1+ (weak). Only cytoplasmic staining was scored for both pGSK-3β and pAkt. Statistical analysis showed that ≥ 1.7 and >1.0 were the optimal cut-off points for pGSK-3β and pAkt, respectively. The score for cyclin D1 for each case was the sum of the following: 2 points x % of tumor showing 2+ (strong) staining, 1 point x % of tumor showing 1+ (moderate) staining. Statistical analysis showed that a score of 1.3 was the optimal cut-off. For β-catenin, only the nuclear staining was assessed, since nuclear localization of β-catenin likely reflects the transcriptionally active form of β-catenin. The nuclear staining of β-catenin was initially assessed as 3+ (strong), 2+ (moderate), 1+ (weak) and 0 (no nuclear staining) based on the majority of the tumor cells. A score of 1 or 0 was considered negative, and a score of 2+ or 3+ was considered positive. Ki67 scoring was achieved by quantifying the percentage of tumor cells showing positive nuclear staining; ≥ 24% was the optimal cut-off point based on statistical analysis for overall survival. Scoring for all of the markers was assessed independently by two of the authors (RL and HA) and major discrepancies between the two observers (generally <10% of the cases) were resolved using double headed microscope. Cut point analysis for overall survival was used to dichotomize continuous variables, including pGSK-3β, pAkt, cyclin D1 and Ki67, with the minimum p-value method applied to estimate the optimum division. A positive ER or PR result was considered where >1% of stained nuclei were found in the tumor (Yaziji et al., 2008). For HER2, the staining intensity was scored based on the CAP/ASCO guidelines (Wolff et al., 2007); cases with 3+ were considered positive for this current study.

Spearman correlation was used for the analysis of continuous variables which were not normally distributed. A p value of <0.05 was considered to be statistically significant. Kaplan-Meier analysis was used to model the overall survival and the Wilcoxon Log-rank test was used to assess differences between groups. Cox proportional hazard method was used for univariate and multivariate analysis. Statistical analysis was performed using the SPSS statistical software version 15 (Chicago, IL).

2.3. RESULTS

2.3.1. Demographic and pathologic data

The demographic and pathologic data of these 72 cases of breast cancer is summarized in Table 2.1. The median age of patients was 53.5 years (range, 37-87 years). The median time to death or last follow-up was 66 months (range, 1-362 months). The pathologic features of these 72 tumors are as follows: 59 (81.9%) invasive ductal carcinomas of no special type, 9 (12.5%) invasive lobular carcinomas, 3 (4.2%) carcinomas of mixed ductal and lobular carcinomas, and one (1.3%) tubular carcinoma. Approximately half (32 of 72, 44.4%) of the tumors had a histologic grade of 3. Axillary lymph node involvement was found in 33 of 60 (55.0%) patients who had the lymph nodes examined. Using immunohistochemistry, 50 (69.5%) and 42 (58.3%) of the 72 tumors were positive for the estrogen receptor (ER) and the progesterone receptor (PR), respectively. Results regarding the HER2 status were available in 64 cases, only 5 tumors were positive (i.e. 3+).

Table 2.1. Patients' demographics and clinical parameters

	All patients Total cases=72 n (%)	**ER positive patients Total cases=50 n (%)**
Age (mean)	53.5 years	56.5 years
Grade		
1	13 (18.1%)	13 (26.0%)
2	27 (37.5%)	23 (46.0%)
3	32 (44.4%)	14 (28.0%)
Stage		
I	22 (30.6%)	17 (34.0%)
II	30 (41.7%)	20 (40.0%)
III	5 (6.9%)	3 (6.0%)
IV	2 (2.8%)	1 (2.0%)
Data not available	13 (18.1%)	9 (18.0%)
Tumor size		
T1	23 (31.9%)	25 (50.0%)
T2	30 (41.7%)	10 (20.0%)
T3	3 (4.2%)	3 (6.0%)
Data not available	16 (22.2%)	12 (24.0%)
Axillary Lymph nodes		
Positive	33 (45.8%)	24 (48.0%)
Negative	27 (37.5%)	17 (34.0%)
Data not available	12 (16.7%)	9 (18.0%)
pGSK-3β score		
Positive (≥ 1.7)	34 (47.2%)	25 (50.0%)
Negative (< 1.7)	38 (52.8%)	25 (50.0%)
pAkt score		
Positive (>1)	35 (48.6%)	27 (54.0%)
Negative (≤ 1)	37 (51.4%)	23(46.0%)
β-catenin		
Positive	30(41.6%)	24(48.0%)
Negative	42 (58.4%)	26 (52.0%)
Cyclin D1 score		
Positive (≥ 1.3)	28(38.9%)	27(54.0%)
Negative (< 1.3)	44 (61.1%)	23 (46.0%)
Ki67 score		
Positive (≥ 24%)	30(41.6%)	11(22.0%)
Negative (< 24%)	42 (58.4%)	39 (78.0%)

2.3.2. pGSK-3β is expressed in a subset of breast cancer

We assessed the expression of pGSK-3β in these 72 cases by immunohistochemistry, and the results are summarized in Table 2.1 and illustrated in figure 2.1a-c. The immunostaining was heterogeneous between cases, and sometimes within the same case. Thus, our scoring method took into account of both the staining intensity and the percentage of the tumor showing different staining intensity (detailed in Methods and Materials). Fibroblasts, macrophages and benign lymphocytes were non-reactive. Overall, pGSK-3β was scored positive, as defined by ≥ the cut off point of 1.7, in 34 (47.2%) cases. The staining was largely restricted to the cytoplasm, although a very small number of cases had some degree of nuclear staining (which was not included in the scoring). As shown in Table 2.1, the pGSK-3β positivity in the ER-positive group was 50.0% (25 of 50), and that in the ER-negative group was 40.0% (8 of 22). Normal adjacent ductal epithelial cells were negative or faintly positive for pGSK-3β.

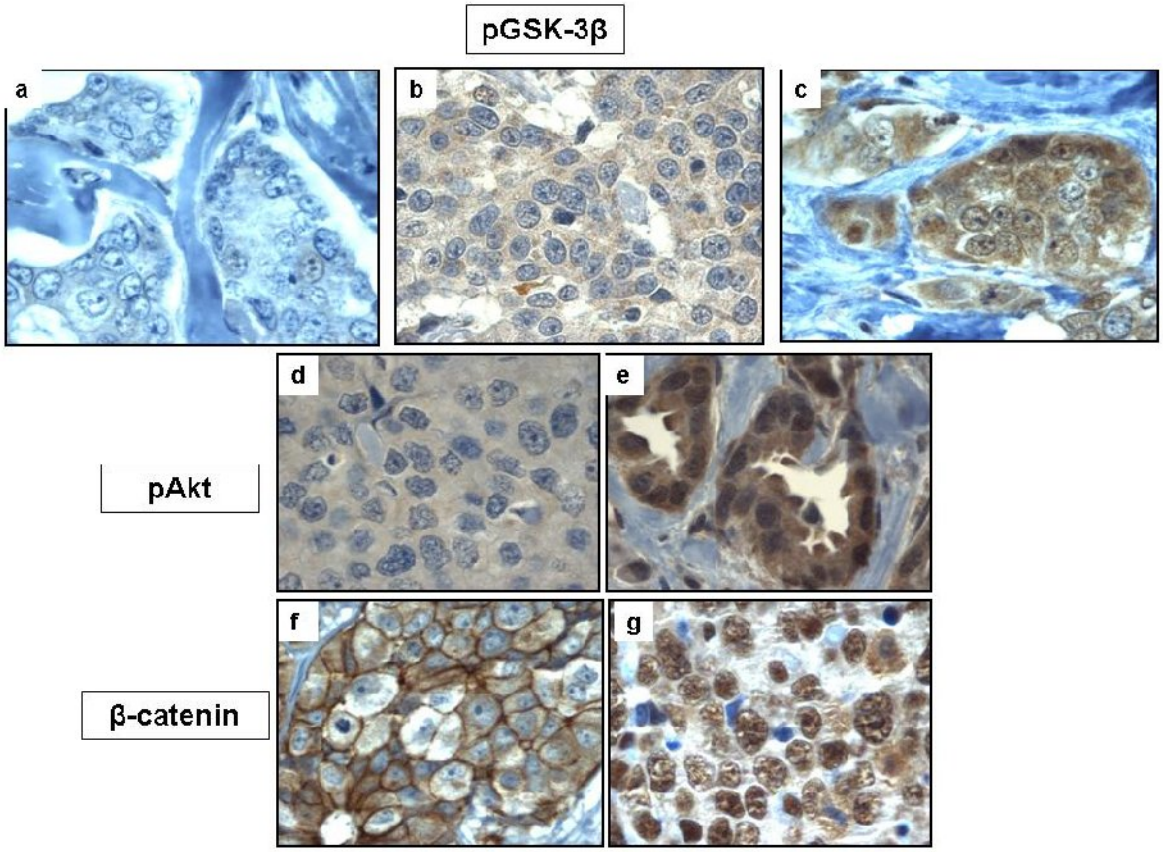

Figure 2.1. Immunohistochemistry for pGSK-3β, pAkt and β-catenin in breast cancer. Figure 2.1a, b, c: Immunohistochemical staining in breast cancer showing a pGSK-3β negative case (a), pGSK-3β weakly positive case (b) and a pGSK-3β strongly positive case (c). The pGSK-3β staining was cytoplasmic in most cases. Figure 2.1 d, e: Immunohistochemical staining in breast cancer showing a pAkt negative case (d) and a pAkt positive case (e). The pAkt staining was mostly cytoplasmic. Figure 2.1 f, g: Immunohistochemical staining in breast cancer showing a β-catenin negative case with only membraneous staining (f) and a β-catenin positive case with prominent nuclear staining (g).

Table 2.2. Correlation between pGSK-3β and pAkt with other markers

Markers	pGSK-3β p-value (Spearman)		pAkt p-value (Spearman)	
	Overall group	ER positive group	Overall group	ER positive group
pAkt	0.01*	0.003*		
pGSK-3β			0.01*	0.003*
β-catenin	0.26	0.29	< 0.0001*	0.001*
Cyclin D1	0.91	0.68	0.02*	0.28
Ki67	0.48	0.10	0.96	0.08

* Statistically significant

2.3.3. pGSK-3β correlates with pAkt expression

We then assessed if pGSK-3β correlates with pAkt. Similar to pGSK-3β, the immunostaining was heterogeneous between cases, and sometime, within the same case. The pAkt immunostaining, as illustrated in figure 2.1d and 1e, was largely restricted to the cytoplasm, although a small number of cases had some degree of nuclear staining (not included in the scoring). pAkt was found positive, as defined by >1.0, in 31 (43.1%) cases. Normal adjacent ductal epithelial cells were either negative or only faintly positive. There was significant correlation between pAkt and pGSK-3β in the entire group (p=0.01, Spearman correlation) (Table 2.2). In addition, in the ER -positive group (n=50), the correlation was found to be highly significant (p=0.003, Spearman) (Table 2.2). The frequency of pAkt positivity in ER-positive group was similar to the overall group (54.0% as opposed to 48.6%). In ER negative group (n=22), the pAkt positivity was 36.4% (8 of 22). However, no significant correlation was found between pAkt and pGSK-3β in this group.

2.3.4. Nuclear localization of β-catenin significantly correlates with pAkt but not pGSK-3β

We next assessed the expression of β-catenin, as it has been described to be negatively regulated by GSK-3β (Hagen et al., 2002). Since β-catenin is a transcriptional factor, we used the nuclear localization of β-catenin as a surrogate marker for its transcriptionally active status. The β-catenin staining is illustrated in Figure 2.1f and 1g. Overall, 30 (41.6%) of cases were positive. No significant correlation was found between nuclear β-catenin and pGSK-3β (p=0.26, Spearman) (Table 2.2). Similar results were obtained in ER-positive group (n=50) (p=0.29, Spearman). Nevertheless, we identified a statistically significant positive correlation between the expression of β-catenin and pAkt in the overall group (p< 0.0001, Spearman), as well as in the ER-positive group (p=0.001, Spearman).

2.3.5. pAkt but not pGSK-3β correlates with cyclin D1

Since cyclin D1 is one of the known downstream targets of GSK-3β and β-catenin, we sought possible correlations among these markers. Cyclin D1 was assessed positive, as defined by ≥ 1.3, in 28 (38.9%) cases. No significant correlation was found between cyclin D1 and pGSK-3β (Spearman correlation p=0.91) (Table 2.2). Similar results were obtained in the ER-positive group (p=0.68, Spearman). Interestingly, we found a significant correlation between pAkt expression and cyclin D1 in the entire group (p=0.02, Spearman), but no significant correlation was found in the ER-positive group (p=0.2, Spearman). We found no significant correlation between cyclin D1 and β-catenin in the whole group or in the ER positive group (p=0.1 and 0.25, Spearman, respectively). Lastly, there was a significant correlation between cyclin D1 positivity and a positive ER status (p< 0.0001, Spearman).

2.3.6. pGSK-3β does not correlate with Ki67

To assess if pGSK-3β has any impact on cell proliferation, we correlated high Ki67 staining with that of pGSK-3β. Ki67 was positive, as defined by ≥ 24%, in 30 (41.6%) cases. No significant correlation was found between Ki67 positivity and pGSK-3β positivity in the entire group (p=0.4, Spearman), or in the ER-positive group (p=0.1 Spearman).

2.3.7. pGSK-3β significantly correlates with poor survival

pGSK-3β significantly correlated with the overall survival (p=0.004, Log rank) (figure 2.2). However, in the ER positive cases, no significant correlation was found between survival and pGSK-3β. In addition, we did not find any significant correlation between the pGSK-3β status and the tumor size, lymph node status or clinical stage (p=1.0, 0.4 and 1.0 respectively). The expression of pAkt, cyclin D1 and β-catenin did not significantly correlate with the overall survival. Cox regression analysis

was performed to determine if the clinical/pathologic features correlated with the overall survival. The clinical/pathological features included axillary lymph node involvement, tumor stage, tumor grade, the ER status, and biomarkers examined in this study (pGSK-3β, pAkt, cyclin D1, β-catenin and Ki67). Univariate analysis revealed that pGSK-3β and the clinical stage significantly correlated with the overall survival. When these variables were included in the multivariate analysis, only pGSK-3β significantly correlated with survival (p=0.001; hazard ratio=7.9). Patients with a high clinical stage (stage II, III or IV) showed a trend for a shorter survival (p=0.053, hazard ratio=3.3) (Table 2.3).

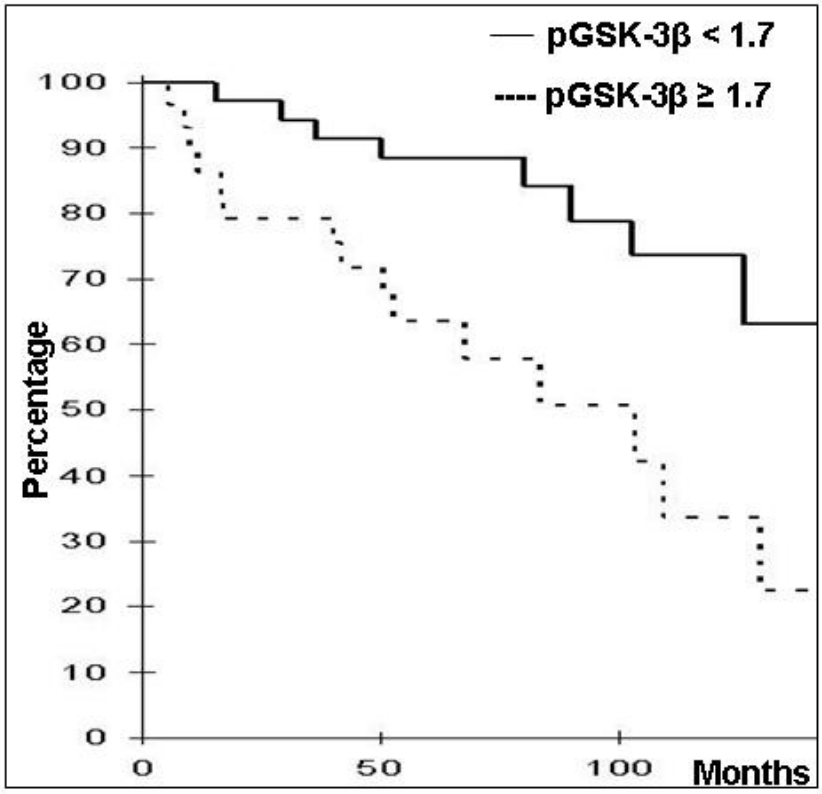

Figure 2.2. pGSK-3β expression significantly correlates with a worse clinical outcome (p=0.004, log-rank). Cut point analysis for overall survival was used to dichotomize continuous pGSK-3β. The optimal cut-off point for pGSK-3β was ≥ 1.7.

Table 2.3. Results of univariate and multivariate analysis using Cox proportional hazards to determine the prognostic value of different variables in relation to the overall survival

Variable	Univariate p-value	Multivariate p-value
Axillary lymph node involvement	NS	NS
Tumor stage	0.126*	0.053*
Tumor grade	NS	NS
pGSK-3β	0.016*	0.001*
pAkt	NS	NS
β-catenin	NS	NS
Cyclin D1	NS	NS
Estrogen receptor status	NS	NS

NS=not statistically significant

* Statistically significant

2.4. DISCUSSION

In view of the known biological importance of GSK-3β in cancer, we used immunohistochemistry to assess the abundance and localization of the inactivated/phosphorylated form of pGSK-3β in a series of 72 primary breast cancers. We determined that 47.2% of breast cancers in our cohort were positive for pGSK-3β. This frequency of pGSK-3β positivity is quite similar to that reported by *Ding et. al*., who reported 60% in their cohort (Ding et al., 2007).

One of our key questions was to test the hypothesis that Akt activation contributes to GSK-3β inactivation in breast tumor samples, as it has been reported to be the case in various *in-vitro* models (Cohen et al., 1997; Pap and Cooper, 1998). Our finding that pGSK-3β significantly correlates with pAkt supports this hypothesis. Given that this correlation was highly significant in the ER-positive subgroup (p=0.003, Spearman), we believe that GSK-3β can be inactivated via other mechanisms (e.g. the WCP), especially in ER-negative tumors. Other mechanisms that are known to inactivate GSK-3β, such as cyclic AMP-dependent protein kinase (PKA) and p90Rsk**,** may also play a role (Fang et al., 2000; Stambolic and Woodgett, 1994). These alternative mechanisms may result in the increased discordance between pGSK-3β and pAkt in breast cancer.

Our study showed that β-catenin is positive in 41.6% of breast cancer. This finding is slightly lower than those found in 3 other studies (i.e. in the range of 60-70%) (Chung et al., 2004; Lin et al., 2000; Ryo et al., 2001). We believe that this discrepancy is most likely due to the fact that the other three studies scored the staining regardless of its localization (i.e. cytoplasmic versus nuclear). We scored only the nuclear β-catenin staining since we believe that the nuclear β-catenin is more representative of its transcriptional activity status. We found a significant correlation between β-catenin and pAkt both in the overall cases and in the ER-

positive subgroup ($p < 0.0001$ and 0.001 respectively). However, there was no significant correlation between β-catenin and pGSK-3β. These results suggest that β-catenin may be predominantly regulated by pAkt by mechanisms not involving pGSK-3β. This is in keeping with a study showing that pAkt can directly enhance the nuclear accumulation and the transcriptional activity of β-catenin by phosphorylating β-catenin at the cell-cell junctions, thereby enhancing its release into the cytosol/nuclei (Fang et al., 2007).

Our study yielded a cyclin D1 positivity of 38.9%, which is similar to what has been reported in previous studies. In addition, we found a significant correlation between cyclin D1 overexpression and the positive ER status ($p < 0.0001$), and this finding is similar to that previously reported (Elsheikh et al., 2008). We found no significant correlation between pGSK-3β and cyclin D1. This result is not overly surprising to us; while previous *in-vitro* studies have shown that GSK-3β regulates the protein level, nuclear localization and phosphorylation of cyclin D1 (Diehl et al., 1998), a more recent report showed that inhibition of the GSK-3 activity has no influence on the cyclin D1 level (Yang et al., 2006). In the same study, the authors proposed that the cyclin D1 levels are regulated in accordance with various stages of the cell cycle rather than by signaling activity (Yang et al., 2006).

The biological significance of GSK-3β inactivation appears to be cell-type specific. In cancers of colon, ovary and pancreas, active GSK-3β is believed to promote cell survival and proliferation (Ougolkov and Billadeau, 2006). However, *Ding et. al.* reported that active GSK-3β sensitizes breast cancer cells to chemotherapeutic agents by down regulating Mcl-1, an anti-apoptotic protein (Ding et al., 2007). Thus, it is conceivable that GSK-3β inactivation may promote tumor cell survival and perhaps, confer a worse clinical outcome by virtue of its anti-apoptotic

effects. In support of this hypothesis, we found that expression of pGSK-3β significantly correlated with shorter overall survival. To our knowledge, our study is the first correlating the expression of pGSK-3β and the survival of cancer patients, and our findings suggest that immunohistochemical detection of pGSK-3β may provide prognostically useful information for breast cancer patients. Due to the relatively small sample size in this study, larger studies with the full representation of various molecular subtypes of breast cancer are required to confirm the prognostic significance of pGSK-3β in breast cancer. Of note, we had a relatively a low number of HER2-positive cases which may be due to several factors: 1) we did not do *in situ* hybridization for equivocal cases (n=6); 2) Only 64 cases had adequate tissues; thus, 8 cases were not evaluated for HER2 and this may have lowered the number of HER2-positive cases; 3) 12.5% of cases in our cohort were invasive lobular carcinomas, which are generally HER2-negative.

The prognostic value of pAkt in breast cancer is controversial in the literature; while studies found no statistical prognostic value of this marker, other studies suggested that pAkt is prognostically useful (Panigrahi et al., 2004; Schmitz et al., 2004). In our study, we found that pAkt is not prognostically significant. While β-catenin has previously been suggested to be prognostic in one study (Lin et al., 2000), their method for scoring β-catenin was different from ours, as described above. Additional studies may be needed to verify the prognostic value of this marker in breast cancer.

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❖ Chapter 3

BIOLOGICAL AND CLINICAL SIGNIFICANCE OF GSK-3β IN MANTLE CELL LYMPHOMA – AN IMMUNOHISTOCHEMICAL STUDY

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*Chung R and Peters A are first co-authors
Armanious H, is second author and contributed in the immunohistochemical studies and writing the manuscript

3.1. INTRODUCTION:

GSK-3β is an important signalling protein that has been shown to regulate a wide range of cellular functions including apoptosis and cell proliferation. Dysregulation of GSK-3β has been implicated in a wide range of human diseases including diabetes, Alzheimer's, schizophrenia, and cancer (Jope and Johnson, 2004; Jope et al., 2007; Karim et al., 2004; Kim and Kimmel, 2000). One of the major biological functions of GSK-3β is to inhibit β-catenin by sequestration and promotion of its proteasome degradation. GSK-3β itself is known to be regulated by the PI3K/Akt and the Wnt canonical pathways (Failor et al., 2007; Joshi et al., 2007; Manoukian and Woodgett, 2002). GSK-3β inactivation is mediated by the phosphorylation of its serine 9 residue. Upon inactivation, its inhibitory effect on β-catenin is released, and β-catenin is allowed to accumulate and translocate to the nucleus, where it upregulates the transcription of multiple genes including *cyclin D1*. GSK-3β also has been shown to directly regulate cyclin D1 via modifying its rate of proteolysis and nuclear export (Diehl et al., 1998; Takahashi-Yanaga and Sasaguri, 2008).

Mantle cell lymphoma (MCL) is a specific subtype of aggressive B-cell lymphoma recognized by the World Health Organization Classification Scheme (WHO) (Jaffe et al., 2004). The genetic hallmark of this disease is the recurrent chromosomal abnormality, t(11;14)(q13;q32), which brings the *cyclin D1(CCND1)* gene under the influence of the enhancer of the immunoglobulin heavy chain (*IGH*) gene, leading to cyclin D1 overexpression. Although it has been shown that cyclin D1 overexpression is not sufficient for the induction of lymphoma in animal models, this abnormality is considered to be the primary oncogenic event in MCL (Diehl, 2002; Jares et al., 2007). In view of the roles of GSK-3β in regulating the expression and subcellular localization of cyclin D1, as discussed above, we hypothesized that the activation status of GSK-3β is likely to be relevant to the pathogenesis of MCL. In support of this hypothesis, we have recently shown that GSK-3β is inactivated and phosphorylated in all MCL cell lines and a subset of MCL tumors examined (Gelebart et al., 2008). In another

study, using a constitutive active GSK-3β construct, Dal Col et. al. (Dal Col and Dolcetti, 2008) recently provided the first direct evidence that GSK-3β phosphorylates cyclin D1 at Thr^{286}, thereby promoting its nuclear export in MCL. Nevertheless, the biological and clinical significance of GSK-3β in MCL has not been comprehensively examined. Specifically, whether the activation status of GSK-3β correlates with the nuclear expression of β-catenin, the cyclin D1 expression level and/or other clinicopathologic parameters is not known.

In this study, using immunohistochemistry, we assessed the expression of phosphorylated/inactive form of GSK-3β (pGSK-3β) in 83 formalin-fixed, paraffin-embedded MCL tumors. We then correlated the expression of pGSK-3β with the nuclear expression of β-catenin, the expression level of cyclin D1 and Ki67 labelling in these tumors. We also evaluated whether pGSK-3β correlates with various clinical parameters including the overall survival.

3.2. MATERIALS AND METHODS

3.2.1. MCL tumors

All cases of MCL were diagnosed at the Cross Cancer Institute (Edmonton, Alberta, Canada) between 1994 and 2007, and the diagnostic criteria were based on those described in the World Health Organization Classification Scheme (Jaffe et al., 2004). All cases were confirmed to express cyclin D1 by immunohistochemistry. Six of these 83 MCL tumors used in this study were blastoid variant. The use of these tissues has been approved by our Institutional Ethics Committee.

3.2.2. Immunohistochemistry

To detect pGSK-3β, β-catenin and Ki67, antigen retrieval was performed using microwave-treated citrate buffer (pH 6.0) for 20 minutes. To detect cyclin D1, antigen retrieval was performed using EDTA (pH=8.0). After antigen retrieval, tissue sections were incubated with 3% hydrogen peroxide (H_2O_2) for 10 minutes to block endogenous peroxidase activity. Subsequently, sections were incubated

overnight at 4˚C with a rabbit polyclonal antibody reactive with pGSK-3β (Ser 9)(1:50, Cell Signaling Technology, Danvers, MA), a rabbit polyclonal anti-β-catenin (1:1200 dilution, Sigma, clone C2206, Oakville, Ontario, Canada), anti-cyclin D1 (1:75 dilution, clone SP4, Medicorp, Montreal, Quebec, Canada) or anti-Ki67 (1:60 dilution, clone M7240, Dako Canada Inc., Mississauga, Ontario, Canada). Immunostaining was visualized with a labelled streptavidin-biotin (LSAB) method using DAB as a chromogen (Dako Canada).

3.2.3. Scoring of the markers and statistical analysis

The cytoplasmic expression of pGSK-3β was assessed as 3+ (strong staining), 2+ (moderate staining), 1+ (weak staining), 0 (no staining). For β-catenin, cases showing definitive nuclear immunostaining in >20% of the tumor cells were considered positive, and the β-catenin nuclear staining was scored as 3+ (strong staining), 2+ (moderate staining) and 1+ (weak staining). Score for pGSK-3β was calculated as follows: 3 points were given for 3+ staining, 2 points for 2+ staining, 1 point for 1+ staining and 0 for no staining. Cut point analysis for overall survival was used to dichotomize continuous pGSK-3β with the minimum p-value method applied to estimate the optimum pGSK-3β division. The analysis found a pGSK-3β score of 0.6 is the optimal cut point. For nuclear β-catenin, <20% of the tumor cells showing staining or a score of 1 was considered negative, and a score of 2+ or 3+ was considered positive. The scoring used to assess the expression of cyclin D1 and Ki67 have been previously detailed (Hui et al., 2005). All of the staining was reviewed by two observers (RC and RL) independently and discrepant cases were reviewed under a double-headed microscope to reach a consensus.

The correlations between pGSK-3β and the other biological markers were assessed using Fisher's exact test for tables and Spearman rank correlation for continuous variables. Univariate and multivariate Cox regression analysis was used to determine the influence of pGSK-3β and various biological and clinical parameters on overall survival. Overall survival plots of the factor groups were

calculated using the Kaplan-Meier method. Statistical tests are two-tailed with a P value <0.05 considered to be statistically significant. The SAS computer program SAS (r) 9.2 (TS1M0) was used to perform the analysis

3.3. RESULTS

3.3.1. Clinical characteristics of MCL patients

The clinicopathologic characteristics of the 83 MCL patients included in this study are summarized in Table 3.1. There were 69 men and 14 women, and the median age at diagnosis was 64 years (range, 41-88 years). Fifty patients died during the follow-up and the median time from the initial diagnosis to either the last follow-up or death was 29 months. Treatment for each MCL patient was determined during our weekly lymphoma conference based on our provincial lymphoma treatment protocol. For first-line treatment, the majority of these patients received CHOP-based (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy, and small subsets of patients received chlorambucil-based chemotherapy or more novel treatments such as proteasome inhibitor PS341 and flavopirodol.

Table 3.1. Patients' Demographics and Clinical Parameters

	pGSK3-β		
	(negative = <0.6) Total cases = 27 n (%)	**(postive = ≥ 0.6) total cases = 56 n (%)**	**Total cases = 83**
Age (mean)	63.3	65.5	83
Gender			
Female	5 (18.5)	9 (16.1)	14
Male	22 (81.5)	47 (83.9)	69
Stage			
I-II	4 (14.8)	4 (7.1)	8
III	6 (22.2)	14 (25.0)	20
IV	15 (55.6)	37 (56.1)	52
Missing	2 (7.4)	1 (1.8)	3
B Symptoms			
Yes	8 (29.6)	15 (26.8)	23
No	17 (63.0)	39 (69.6)	56
Missing	2 (7.4)	2 (3.6)	4
GI Involvement			
Yes	6 (22.2)	6 (10.7)	12
No	19 (70.4)	48 (85.7)	67
Missing	2 (7.4)	2 (3.6)	4
IPI			
0 - 2	15 (55.6)	36 (64.3)	51
≥ 3	4 (14.8)	12 (21.4)	16
missing	8 (29.6)	8 (14.3)	16
β-catenin			
0-1	23 (85.2)	27 (48.2)	50
2-3	3 (70.4)	23 (41.1)	26
Missing	1 (3.7)	6 (10.7)	7
Cyclin D1			
1-2	18 (66.7)	23 (41.1)	41
3	3 (11.1)	26 (46.4)	29
Missing	6 (22.2)	7 (12.5)	13
Ki67			
0-3	21 (77.8)	37 (66.1)	58
4	4 (14.8)	18 (32.1)	22
Missing	2 (7.4)	1 (1.8)	3

3.3.2. pGSK-3β significantly correlates with β-catenin positivity

Of the 83 MCL tumors, 56 (67.5%) were considered pGSK-3β positive, based on a minimum p-value cut-off of ≥ 0.6. The expression of pGSK-3β was found to be largely localized to the cytoplasm of the MCL cells (Figure 3.1 a, b). Of the 76 MCL tumors that were also evaluated for the expression of β-catenin, 26 (32.5%) were assessed positive. In contrast with pGSK-3β, β-catenin was largely expressed in the nuclei of MCL cells (Figure 3.1 c, d). pGSK-3β positivity significantly correlated with the expression of β-catenin ($p = 0.0025$, Fisher's exact) (Table 3.2).

3.3.3. pGSK-3β significantly correlates with cyclin D1 expression

As illustrated in Figure 3.1 e and f, the cyclin D1 immunostaining was largely restricted to the nuclei of MCL cells in all cases. Although all cases were positive for cyclin D1 (as per definition of MCL), the proportion of strongly positive cells was highly variable among tumors. To ensure that the assessment of the cyclin D1 expression status was uniform, we excluded 13 cases for which a different anti-cyclin D1 antibody was used and insufficient tissues were available to repeat the staining. Of the remaining 70 cases, 29 (41.4%) were considered high cyclin D1-expressing. Statistical analysis revealed that the high-expressing cyclin D1 status correlates significantly with positive pGSK-3β ($p = 0.0032$) (Table 3.3).

Since the gene expression of cyclin D1 has been shown to be up-regulated by β-catenin in various in-vitro models, we addressed whether there is a significant correlation between the expression of cyclin D1 and β-catenin positivity. Data for both markers was available in 63 cases. In the β-catenin positive group (n=23), 13 (56.5%) were high cyclin D1-expressing. In the β-catenin negative group (n=40), only 12 (30%) were high cyclin D1-expressing; the two markers had a correlation that was not quite significant ($p = 0.06$) (Table 3.4).

3.3.4. pGSK-3β does not correlate with the Ki67

The percentage of tumor cells with Ki67 expression was assessed in all MCL tumors and categorized into 2 groups: <50% or ≥ 50% (Figure 3.1 g, h). Three of 83 cases did not have sufficient Ki67 data. Of the remaining 80 cases, 22 had a ≥50% Ki67 labelling. Only 4 (16%) of the 25 pGSK-3β negative cases had a ≥50% Ki67 labelling, as compared to 18 of 55 (49%) pGSK-3β positive cases.
This correlation is not statistically significant ($p = 0.177$, Fisher's exact). Ki67 also did not significantly correlate with β-catenin or cyclin D1.

Table 3.2. Correlation between pGSK-3β and β-catenin

	β-catenin			
	Negative (0 or 1)	**Positive (2 or 3)**	**Total**	**P**
pGSK3β				
negative (< 0.6)	23	3	26	0.0025
positive (≥ 0.6)	27	23	50	
Total	50	26	76	

Table 3.3. Correlation between pGSK-3β and cyclin D1

	Cyclin D1			
	Low (1 or 2)	**High (3)**	**Total**	**P**
pGSK-3β				
negative (< 0.6)	18	3	21	0.0032
positive (≥ 0.6)	23	26	49	
Total	41	29	70	

Table 3.4. Correlation between cyclin D1 and β-catenin

	β-catenin			
	Negative (0 or 1)	**Positive (2 or 3)**	**Total**	**P**
Cyclin D1				
Low (1 or 2)	28	10	38	0.0606
High (3)	12	13	25	
Total	40	23	63	

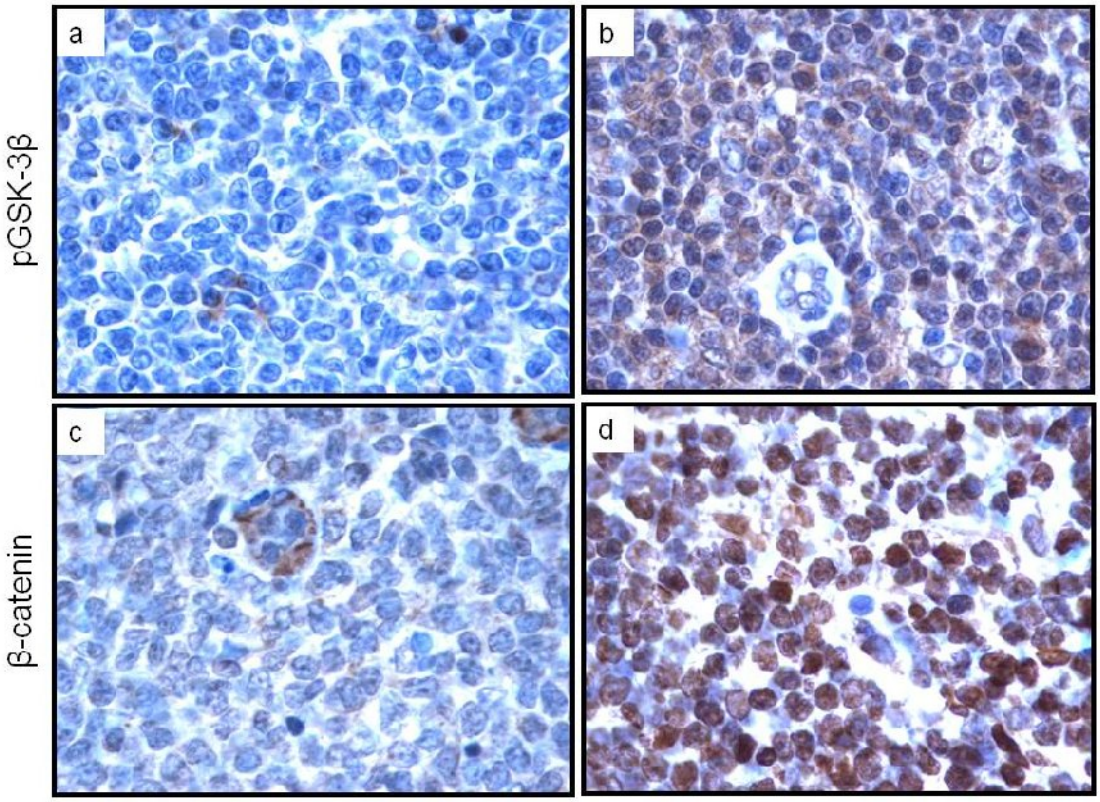

Figure 3.1(a-d). Immunohistochemistry of pGSK-3β and β-catenin in MCL. Figure 3.1a, b: Immunohistochemical staining of pGSK-3β in MCL tumors revealed a subset of negative cases and a subset of positive case (a and b respectively). The staining was predominantly cytoplasmic. Figure 3.1c, d: Immunohistochemical staining of β-catenin in MCL tumors revealed a subset of negative cases and a subset of positive case (illustrated in c and d respectively). Of note, the staining in the tumor cells was predominantly nuclear; in contrast, the staining was mostly found in the cytoplasm of the endothelial cells.

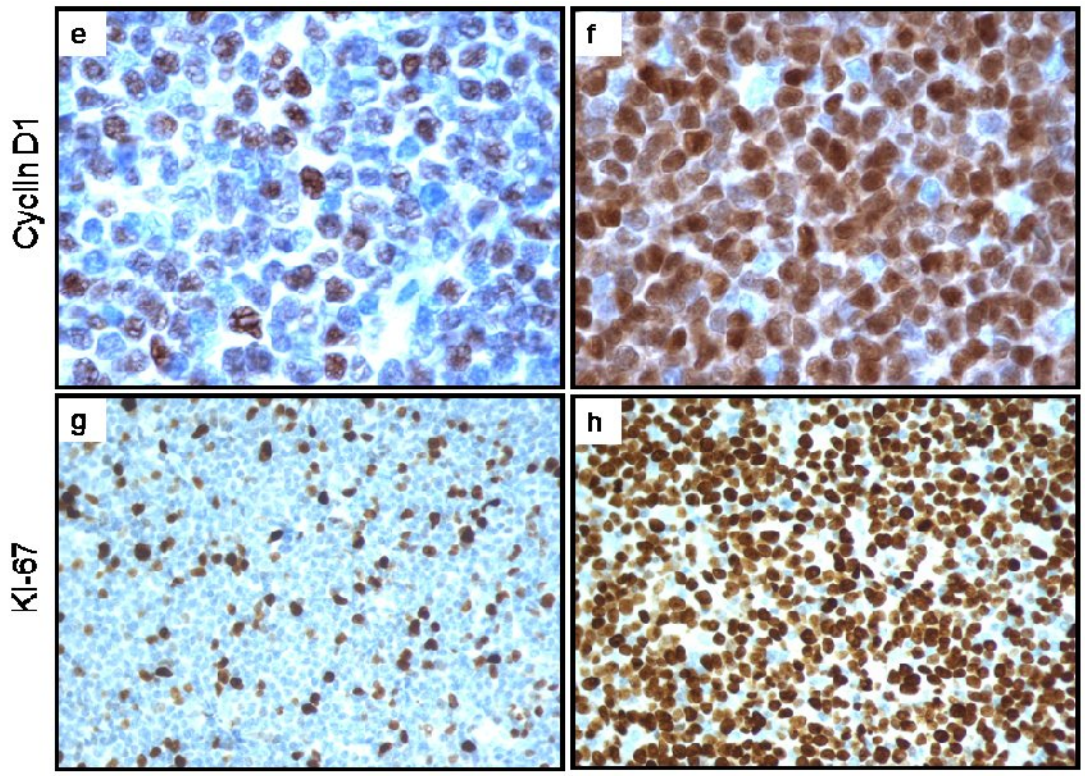

Figure 3.1(e-h). Immunohistochemistry of cyclin D1 and Ki67 in MCL. Figure 3.1e, f: Immunohistochemical staining of cyclin D1 in MCL tumors was heterogeneous with regard to the proportion of intensely positive cells. The case in 1e was assessed cyclin D1-low, as strongly positive cells were <50% of the neoplastic cell population. The case in figure 3.1f was assessed cyclin D1-high, as strongly positive cells were ≥ 50% of the neoplastic cell population. Figure 3.1 g, h. Immunohistochemical staining of Ki67 in MCL tumors also revealed a high degree of heterogeneity in the proportion of positive cells, showing a Ki67-negative case and a Ki67 positive case (g and h respectively).

3.3.5. pGSK-3β significantly correlates with overall survival and absolute lymphocytosis

Using a pGSK-3β cut point of ≥ 0.6 univariate Cox survival analysis we found a significant correlation between pGSK-3β and overall survival ($p = 0.045$, HR = 1.89), with a pGSK-3β negative status associated with a better outcome (Table 3.5). The Kaplan-Meier survival curves are shown in Figure 3.2. In keeping with our previously published data cases with ≥50% cells positive for Ki67 significantly correlated with a shorter survival ($p<0.0001$) (Hui et al., 2005). Multivariate analysis found the prognostic value of pGSK-3β ($p = 0.030$, HR = 2.10) was independent of Ki67 ($p<0.0001$, HR = 4.40). There was no significant interaction effect between pGSK-3β and Ki67 ($p = 0.71$). Neither cyclin D1 nor β-catenin shows a significant correlation with the overall survival (Table 3.5).

We also assessed if there is a correlation between the expression of pGSK-3β and various clinical parameters, including clinical stage, the international prognostic index, absolute lymphocytosis, elevation of lactate dehydrogenase, patient age at diagnosis and extra-nodal involvement; only peripheral blood lymphocytosis (using a cut-off of $>6 \times 10^9$/L) significantly correlates with pGSK-3β status ($p = 0.0011$, Spearman).

Table 3.5. Cox regression analysis of overall survival

		Unitivariate		Multivariate	
	Total cases assessed	**HR (95% CI)**	**P**	**HR (95% CI)**	**P**
pGSK-3β < 0.6 ≥ 0.6	83	1.00 1.89 (1.01-3.53)	0.045	1.00 2.10 (1.075-4.09)	0.030
Ki67 0-3 4	80	1.00 4.45 (2.29 - 8.67)	<0.0001	1.00 4.40 (2.24-8.64)	<0.0001
Cyclin D1 1 or 2 3	70	1.00 1.17 (0.79-1.73)	0.442		
β-catenin 0 or 1 2 or 3	76	1.00 1.06 (0.78-1.44)	0.732		
IPI 0-2 3-5	81	1.00 2.08 (1.08-4.03)	0.030		

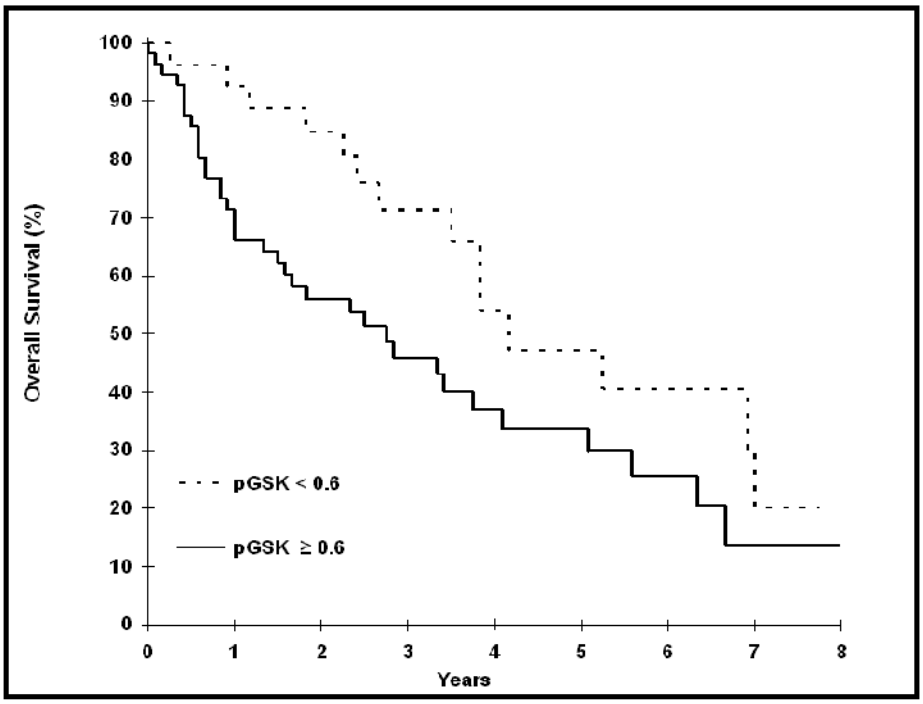

Figure 3.2. pGSK-3β expression significantly correlates with overall survival (p=0.045, Log rank).

3.4. DISCUSSION

GSK-3β is a biologically important signalling protein. In the Wnt canonical pathway (WCP), it plays a central role in sequestrating β-catenin and promoting the proteasome destruction of β-catenin (Staal and Clevers, 2005). In this study, using pGSK-3β as a surrogate marker detectable by an immunohistochemical method, we comprehensively examined the GSK-3β inactivation status in 83 paraffin-embedded MCL tumors. We found evidence that GSK-3β is inactivated in two-thirds in this cohort. We have also provided evidence that the pGSK-3β positive status significantly correlates with the nuclear expression of β-catenin, a relatively high expression level of cyclin D1, absolute lymphocytosis in the peripheral blood, and shorter overall survival in MCL patients. In summary, our data has provided evidence to support that GSK-3β is both biologically and clinically important in MCL.

With regard to the mechanism by which GSK-3β is inactivated, our group has recently published evidence of constitutive activation of the WCP in MCL cell lines and patient tumours, which is likely to be attributed to the autocrine and/or paracrine stimulation of various Wnt ligands including Wnt3 (Gelebart et al., 2008). These findings echo those of two recently published studies. In the first study, the authors showed that RNA-interference to abrogate the expression of Fz2, a receptor for Wnt ligands, decreases cell proliferation in a MCL cell line (Ortega-Paino et al., 2008). In the second study, the authors employed gene expression profiling experiments and identified a significant upregulation of several genes in the WCP in MCL compared to naïve B cells (Rizzatti et al., 2005). In addition to the WCP, the PI3K/Akt signalling pathway may also contribute to GSK-3β inactivation. To this end, it has been previously reported that the PI3K/Akt signalling pathway is constitutively activated in a subset of MCL (Rudelius et al., 2006).

In various in-vitro experimental models, β-catenin has been shown to accumulate and translocate to the nucleus upon inactivation of GSK-3β. This appears to be

the case for MCL, since we observed that the pGSK-3β positive status significantly correlates with the nuclear expression of β-catenin (i.e. p = 0.0025, Fisher's exact). In this regard, β-catenin nuclear accumulation coupled with GSK-3β inactivation is also observed in a number of other types of cancer, including chronic myeloid leukemia in blast crisis, precursor B-cell acute lymphoblastic leukemia and specific types of epithelial cancers (Clevers, 2006; Jamieson et al., 2004; Radich et al., 2006). Nevertheless, in our study, a substantial proportion of pGSK-3β positive cases, namely 27 of 50 (54%) cases, did not have detectable β-catenin. One likely explanation is that additional mechanisms may be required for the nuclear transport of β-catenin, and these mechanisms are not operational in some MCL tumors.

The finding of heterogeneous cyclin D1 immunostaining in paraffin-embedded MCL tissues is commonly observed by diagnostic hematopathologists. It is highly unlikely that this staining heterogeneity is due to variations in fixation or other technical reasons, since strongly cyclin D1-positive cells were seen in almost all cases. Thus, we believe that there is a genuine biological difference in the protein expression level of cyclin D1. Our findings of a significant correlation between a high level of cyclin D1 expression and the pGSK-3β positive status (p = 0.0032, Fisher's exact) support the concept that GSK-3β plays an active role in regulating the protein level of cyclin D1. Variation in the cyclin D1 protein level in MCL is also likely to be related to the variations in the cyclin D1 mRNA levels, as previously reported by multiple groups (Marzec et al., 2006).

Of various clinical parameters analyzed in this study, only absolute lymphocytosis (arbitrarily defined as >6.0 x 10^9/L in this study) and overall survival significantly correlate with pGSK-3β. It is unlikely that the correlation between lymphocytosis and pGSK-3β is related to the tumor burden, since the pGSK-3β status does not significantly correlate with the clinical stage. In view of the fact that GSK-3β has been shown to regulate cell motility and cell-to-cell adhesions (Bianchi et al., 2005; Etienne-Manneville and Hall, 2003; Jope et al.,

2007; Zhou et al., 2004), we have considered the possibility that GSK-3β may directly modulate the cell migratory and adhesion properties of MCL cells, such that inactivation of GSK-3β predisposes to a leukemic phase.

One of the key findings in this study is the observation that GSK-3β inactivation is significantly associated with a worse clinical outcome in MCL patients. Since pGSK-3β did not significantly correlate with Ki67, it is unlikely that its association with a worse clinical outcome is directly linked to increased cell proliferation. In view of the biologic importance of GSK-3β in various normal cellular functions and the pathogenesis of various cancers, we believe that this finding is rather not too surprising. Since the expression of β-catenin does not show a significant correlation with survival, the prognostic value of GSK-3β is likely to be mediated via downstream effectors other than β-catenin.

In contrast with two other previous studies (Rosenwald et al., 2003; Yatabe et al., 2000), we did not identify a significant correlation between the high cyclin D1 expression status and overall survival. A plausible explanation for this discrepancy is related to the difference in the experimental approach employed. While we measured the protein level expression of cyclin D1 using immunohistochemistry, the other two studies assessed the levels of *cyclin D1* mRNA in MCL tumors. Of note, while MCL cells express two cyclin D1 mRNA species, namely cyclin D1a and cyclin D1b, only cyclin D1a is translated into protein in MCL cells (Marzec et al., 2006). Moreover, the anti-cyclin D1 antibody used in this immunohistochemical study recognizes only the cyclin D1a isoform. These two factors may have contributed to this discrepancy of our conclusions.

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❖ Chapter 4

STAT3 UPREGULATES THE PROTEIN EXPRESSION AND TRANSCRIPTIONAL ACTIVITY OF β-CATENIN IN BREAST CANCER

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4.1. INTRODUCTION

β-catenin is known to function as an adhesion molecule that is associated with E-cadherin and actin filaments at the cell membrane (Huber and Weis, 2001). In addition, it has been shown that β-catenin can act as a transcriptional factor involved in a number of cellular signaling pathways such as the Wnt canonical pathway (WCP) (Brennan and Brown, 2004; Polakis, 2000). In the WCP, β-catenin is normally sequestered by the so-called 'destruction complex', which consists of glycogen synthase kinase-3β (GSK3β), the adenomatous polyposis coli, axin and casein kinase 1 (Clevers, 2006; Gordon and Nusse, 2006). Upon ligation of the soluble Wnt proteins to their receptors, the dishevelled proteins (Dvl's) will become phosphorylated, which is believed to result in inactivation and phosphorylation of GSK3β, leading to the dissociation of the destruction complex. Consequently, β-catenin is allowed to evade proteasome degradation, accumulate in the cytoplasm and translocate to the nucleus. Forming heterodimers with T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) in the nucleus, β-catenin has been shown to regulate the expression of a wide range of important genes including *c-myc* and *cyclin D1* (He et al., 1998; Mikels and Nusse, 2006; Miller, 2002; Shtutman et al., 1999).

β-catenin has been implicated in the pathogenesis of a wide range of human cancer (Ewan and Dale, 2008). There are also links between β-catenin and breast cancer. For instance, the expression of a β-catenin mutant with an abnormally high stability has been shown to induce breast adenocarcinomas in a transgenic mouse model (Imbert et al., 2001). By immunohistochemistry, the expression of β-catenin in breast cancer (reported to be up to 60% of the cases) has been reported to significantly correlate with a poor prognosis or relapse in breast cancer patients in previous studies (Asgarian et al., 2009; Lim and Lee, 2002; Lin et al., 2000). A few previous studies have shed light to the mechanisms

underlying the relatively high level of β-catenin expression in a subset of breast cancer. For instance, the WCP, which is known to regulate the expression and activity of β-catenin, is known to be constitutively active in a subset of breast cancer (Benhaj et al., 2006). In another study, it has been shown that manipulation of the WCP can modulate β-catenin in breast cancer cells (Schlange et al., 2007). In addition to the WCP, other mechanisms also may be involved in regulating β-catenin in breast cancer. For instance, Pin1 was found to promote the dissociation of β-catenin from the destruction complex, and thus, increasing its stability (Ryo et al., 2001). Other studies showed that p53 downregulates β-catenin through ubiquitylation (Levina et al., 2004; Liu et al., 2001). Thus, the high level of β-catenin expression in a subset of breast cancer may be multi-factorial.

Signal transducer and activator of transcription-3 (STAT3) belongs to a family of latent transcription factors the STAT family (Turkson, 2004). In breast cancer, STAT3 is constitutively activated in approximately 50-60% of primary breast tumors; downregulation of STAT3 resulted in decrease in the tumorigenecity of breast cancer cells xenografted in nude mice (Dien et al., 2006; Kunigal et al., 2009). Blockade of STAT3 using a dominant negative construct has been recently shown to decrease the nuclear localization and transcriptional activity of β-catenin in colon cancer cell lines (Kawada et al., 2006). Given that both β-catenin and STAT3 are activated in a subset of breast tumors, we hypothesized that STAT3 may represent another mechanism by which β-catenin is regulated in breast cancer cells. In addition, we evaluated the biological and clinical significance of β-catenin in breast cancer.

4.2. MATERIALS AND METHODS

4.2.1. Cell lines and tissue culture

MCF-7 and BT-474 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). They were grown at 37^0C and 5% CO_2 and maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA). The culture media were enriched with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA). MCF-7 cells permanently transfected with the tetracycline-controlled transactivator and TRE-STAT3C plasmids (labeled $STAT3C^{tet\text{-}off}$ MCF-7) have been described previously (Dien et al., 2006), this stable cell line was maintained by the addition of 800 µg/ml geneticin (Life Technologies, Inc.) to the culture media.

4.2.2. Subcellular protein fractionation, Western blot analysis and antibodies

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA, USA) and followed the manufacturer's instructions. Preparation of cell lysates for Western blots was done as follows: cells were washed twice with cold phosphate-buffered saline (PBS, pH=7.0), and scraped in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 50mM Tris pH 8.0) supplemented with 40.0 µg/mL leupeptin, 1 µM pepstatin, 0.1 mM phenylmethylsulfonyl-fluoride and sodium orthovandate. Cell lysates were incubated on ice for 30 minutes and centrifuged for 15 minutes at 15000*g* at 4^0C. Proteins in the supernatant were then extracted and quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL). Subsequently, cell lysates were then loaded with 4x loading dye (Tris-HCl pH 7.4, 1%SDS, glycerol, dithiothreitol, and bromophenol blue), electrophoresed on 8% or 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA). After the membranes were blocked with 5% milk in Tris buffered saline (TBS) with Tween, they were incubated

with primary antibodies. After washings with TBS supplemented with 0.001% Tween-20 for 30 minutes between steps, secondary antibody conjugated with the horseradish peroxidase (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was added to the membrane. Proteins were detected using enhanced chemoluminescence detection kit (Pierce, Rockford, IL). Antibodies employed in this study included anti-β-catenin (1:4000, BD Biosciences Pharmingen, San Diego, CA, USA), anti-STAT3 and anti-pSTAT3 (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FLAG, anti-HDAC, anti-α-tubulin and anti-β-actin (1:3000, Sigma-Aldrich).

4.2.3. β-catenin transcriptional activity assessed by TOPFlash/FOPFlash

To assess the transcriptional activity of β-catenin in breast cancer cell lines, we employed the TOPFlash/FOPFlash luciferase system. This method has been previously described in details (Staal et al., 2002). MCF-7 and BT-474 cells were transiently transfected with β-catenin responsive firefly luciferase reporter plasmids, TOPFlash (Millipore, Billerica, MA, USA) or the negative control, FOPFlash (Millipore). After 24 hours, cells were harvested and cell extracts were prepared using a lysis buffer purchased from Promega (Madison, WI, USA). The luciferase activity was assessed using 20 µL of cell lysate and 100 µL of luciferase assay reagent (Promega). The luciferase activity measured was normalized against the *β-galactosidase* activity, which was measured by incubating 20 µL of cell lysates in a 96 well plate with 20 µL of o-nitrophenyl-β-D galactopyranoside solution (0.8 mg/mL) and 80 µL H_2O, absorbance was measured at 415 nm at 37ºC. Data are reported as means ± standard deviations of three independent experiments, each of which was performed in triplicates.

4.2.4. Gene transfection

Transient gene transfection of cell lines with various expression vectors were performed using Lipofectamine 2000 transfection reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacture's protocol. Briefly, cells were grown in 60 mm culture plates until they are ~90% confluence, culture medium was replaced with serum-free Opti-MEM (Life Technologies) and cells were transfected with the DNA-lipofectamine complex. For all *in-vitro* experiments, STAT3C$^{tet\text{-}off}$ MCF-7 cells were transiently transfected with 3 µg TOPFlash or FOPFlash and 4 µg of *β-galactosidase* plasmid. To manipulate the expression level of STAT3C in these cells, various concentrations of tetracycline (Invitrogen) were added to the cell culture. For MCF-7 and BT-474, 2 µg of TOPFlash or FOPFlash, 3 µg of *β-galactosidase* plasmid and 2 µg of STAT3C (or an empty vector) were transfected.

4.2.5. Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed using a commercially available kit according to the manufacturer's protocol (Upstate, Charlottesville, VA, USA). Briefly, DNA-protein was cross-linked using 1% formaldehyde for 10 minutes at 37^{0}C. Cells were lysed using the SDS buffer, followed by sonication. Immunoprecipitation was done using protein A/G agarose beads conjugated with either a rabbit anti-human STAT3 antibody or a rabbit IgG antibody overnight at 4^{0}C. The DNA-protein-antibody complex was separated and eluted. DNA was extracted using Phenol/Chloroform/ethanol. Primer pairs were designed by Primer 3 Input 0.4 to detect the *β-catenin* gene promoter region containing putative STAT3 binding sites. The primer sequences are as follows: primer 1 forward: 5'-CCGAGCGGTACTCGAAGG-3' and reverse 5'-GTATCCTCCCCTGTCCCAAG-3'; primer 2 forward: 5'-CCAAAGAAAAATCCCCACAA-3' and reverse 5'-TCCTTAGGAGTACCTACTGTGAACAA-3'; and primer 3 forward 5'-

AATTGGAGGCTGCTTAATCG-3' and reverse 5'-TTCCATTTTTATCTGGTTCCAC-3'.

4.2.6. Short interfering RNA (siRNA)

siRNA for β-catenin were purchased from Sigma-Aldrich. siRNA for STAT3 were purchased from Qiagen Science (Mississauga, ON, Canada) and used as described before (Sekikawa et al., 2008). Scrambled siRNA was purchased from Dharmacon (Lafayette, CO, USA). siRNA transfections were carried out using an electro square electroporator, BTX ECM 800 (225V, 8.5ms, 3 pulses)(Holliston, MA, USA) according to the manufacturer's protocol, the dose of siRNA used was 100 picomole/1x10^6 cells. Cells were harvested at 24 hours after transfection. The β-catenin or STAT3 protein levels were assessed by Western blot analysis to evaluate the efficiency of inhibition.

4.2.7. MTS assay

MCF-7 cells transfected with either β-catenin siRNA or scrambled siRNA were seeded at 3,000 cells/well in 96-well plates. MTS assay was conducted following the manufacturer's instructions (Promega). The measurements were obtained at a wavelength of 450 nM using a Biorad Micro plate Reader (Bio-Rad Life Science Research Group, Hercules, CA, USA). The absorbance values were normalized to the wells with media only using the microplate Manager 5.2.1 software (Biorad). All experiments were performed in triplicates.

4.2.8. Immunohistochemistry and breast cancer specimens

A cohort of 129 consecutive, primary breast carcinoma specimens was retrieved from the centralized hormone receptor laboratory at the Cross Cancer Institute in Edmonton, Alberta, Canada. Morphologic features, including the histologic grade and the presence/absence of lymphatic invasion, were reviewed. The hormone receptor expression status was

determined by immunohistochemistry at the time of initial diagnosis. The use of these human tissue samples has been reviewed and approval by our institutional ethics board. Immunohistochemistry was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μM thickness were deparafinized and hydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH=6) and a microwave histoprocessor (RHS, Milestone, Bergamo, Italy). After antigen retrieval, tissue sections were incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Tissue sections were then incubated with anti-β-catenin (1:50) and anti-pSTAT3 (1:50) overnight in a humidified chamber at 4°C. All of these primary antibodies were the same as those used for Western blots. Immunostaining was visualized with a labeled streptavidin-biotin (LSAB) method using 3,3'-diaminobenzidine as a chromogen (Dako Canada Inc., Mississauga, Ontario, Canada) and counter-stained with hematoxylin. For pSTAT3, the absence of nuclear staining or the presence of definitive nuclear staining in <10% of tumor cells was assessed negative; the presence of nuclear staining in ≥10% of tumor cells was assessed positive. ALK-positive anaplastic large cell lymphomas served as the positive control, whereas the lymphoid cells in benign tonsils served as the negative control. For β-catenin, only nuclear staining was scored. Moderate to strong nuclear β-catenin staining was assessed positive whereas the absence or weak (i.e. not definitive) nuclear staining was scored negative. Epithelial cells in benign tonsils served as the positive control whereas lymphoid cells in tonsils served as negative controls.

4.2.9. Statistical analysis

Data are expressed as mean +/- standard derivation. Unless stated otherwise, statistical significance was determined using Student's t-test and statistical significance was achieved when the p value is <0.05.

4.3. RESULTS

4.3.1. STAT3 binds to *β-catenin* gene promoter

DNA sequence analysis of the -1000 bases of the *β-catenin* gene promoter region revealed 7 consensus sequences for the STAT family, characterized by TTN $_{(4-6)}$ AA (Seidel et al., 1995). Three of these 7 sequences contained the specific STAT3 binding sequence, namely TTMXXXDAA (D: A,G, or T;M:A or C)(summarized in Table 4.1) (Ehret et al., 2001). These putative STAT3 binding sites are located at positions -314, -856 and -938, upstream of the ATG transcription initiation site. To provide direct evidence that STAT3 binds to these three sites, we performed chromatin immunoprecipitation using MCF-7 cells. As shown in figure 4.1, both primer 2 (to detect STAT3 binding to the -856 site) and primer 3 (to detect STAT3 binding to the -938 site) showed amplifiable products. In contrast, no detectable amplification was observed for primer 1 (to detect STAT3 binding to the -314 site). The input lanes were included as a control for the PCR effectiveness. PCR without the addition of DNA templates was used as a negative control. The *SALL4* primer served as the positive control, as published previously (Bard et al., 2009).

Table 4.1. Putative STAT binding sites on human *β-catenin* gene promoter:

Site number	Location relative to ATG	Consensus sites TTN $_{(4-6)}$AA
1	-254	TTCCCCAA
2	-314	TTCGGGAAA*
3	-782	TTGTTGAA
4	-856	TTAACCTAA*
5	-938	TTCTCCAAA*
6	-970	TTTCACAAA
7	-1000	TTCTCTATAA

*Specific STAT3 binding site

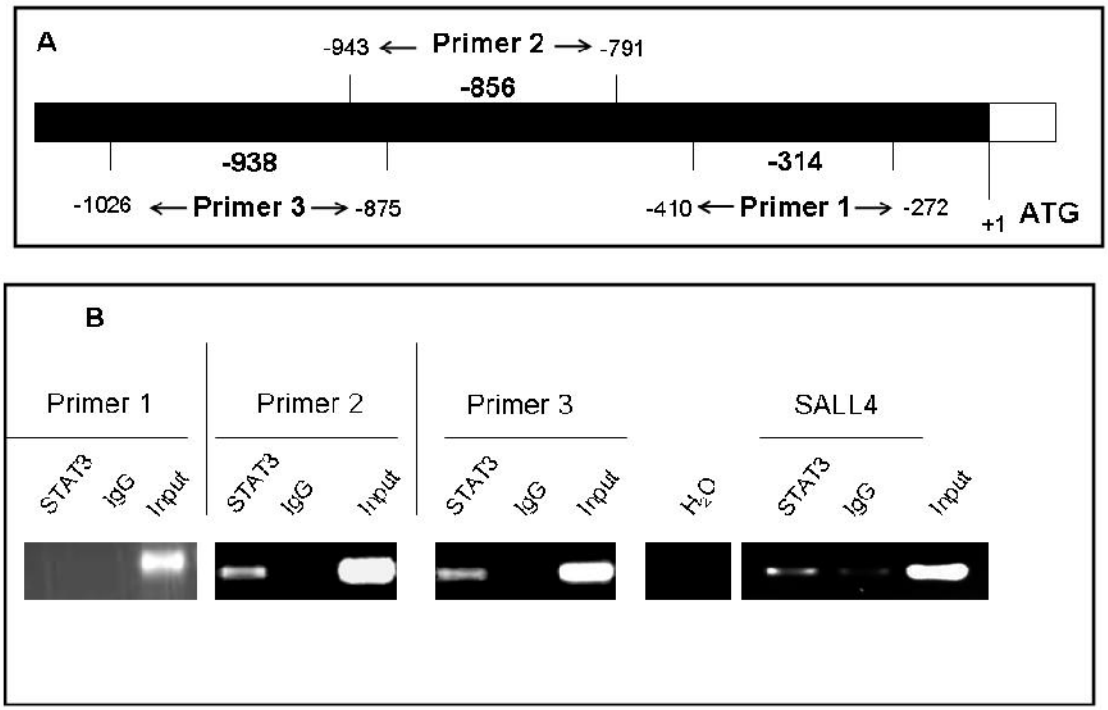

Figure 4.1. STAT3 binds to *β-catenin* gene promoter (A) Schematic representation of the three primers sets specific for three putative STAT3 binding sites in the *β-catenin* gene promoter region. (B) Chromatin immunoprecipitation was performed using MCF-7 cells. A rabbit polyclonal antibody against STAT3 was used. Normal rabbit IgG antibody instead of anti-STAT3 served as a negative control. PCR with both primer 2 and primer 3 revealed amplicons. In contrast, no amplicons were detected with primer 1 and in the negative control. *SALL4* gene promoter primer served as the positive control.

4.3.2. STAT3 regulates the transcriptional activity and protein levels of β-catenin

To determine if the expression of STAT3 affects the transcriptional activity and/or protein level of β-catenin, we subjected two breast cancer cell lines (MCF-7 and BT-474) to STAT3 knock-down using siRNA. As shown in figure 4.2A, transfection of STAT3 siRNA resulted in a substantial downregulation in the STAT3 protein levels in both cell lines. In the same blots, the protein levels of β-catenin were also decreased. We also found evidence that STAT3 regulates the transcriptional activity of β-catenin. As shown in Figure 4.2B, downregulating STAT3 using siRNA in MCF-7 cells resulted in a significant downregulation of the β-catenin transcriptional activity, as assessed by the TOPFlash/FOPFlash system ($p=0.0006$)(Figure 4.2B). Furthermore, transient transfection of *STAT3C* (i.e. constitutively active STAT3) in MCF-7 and BT-474 cells led to a significant increase in the transcriptional activity of β-catenin, as compared to transfection of an empty vector ($p=0.003$ for both cell lines) (Figure 4.3A). Also, we performed subcellular fractionation after STAT3C transfection on MCF-7 but we did not see any change in β-catenin nuclear translocation (Figure 4.3B). Lastly, to further support that STAT3 regulates β-catenin, we employed our generated MCF-7 cell clone that has been stably transfected with an inducible (tetracycline-off) STAT3C expression vector (labeled as $STAT3C^{tet\text{-}off}$ MCF-7), as previously described (Dien et al., 2006). As shown in figure 4.4A, increasing levels of tetracycline added to these cells resulted in a gradual downregulation of the total STAT3 level as well as the FLAG tag. Using the TOPFlash/FOPFlash system, the luciferase level from cells treated with 20 μg/ml and 60 μg/ml tetracycline were significantly lower than that of negative controls ($p=0.04$ and 0.03 respectively) (Figure 4.4B). These results are derived from triplicate experiments. Also, we performed subcellular fractionation after downregulation of STAT3C levels using tetracycline; however we did not see any change in β-catenin nuclear translocation (data not shown).

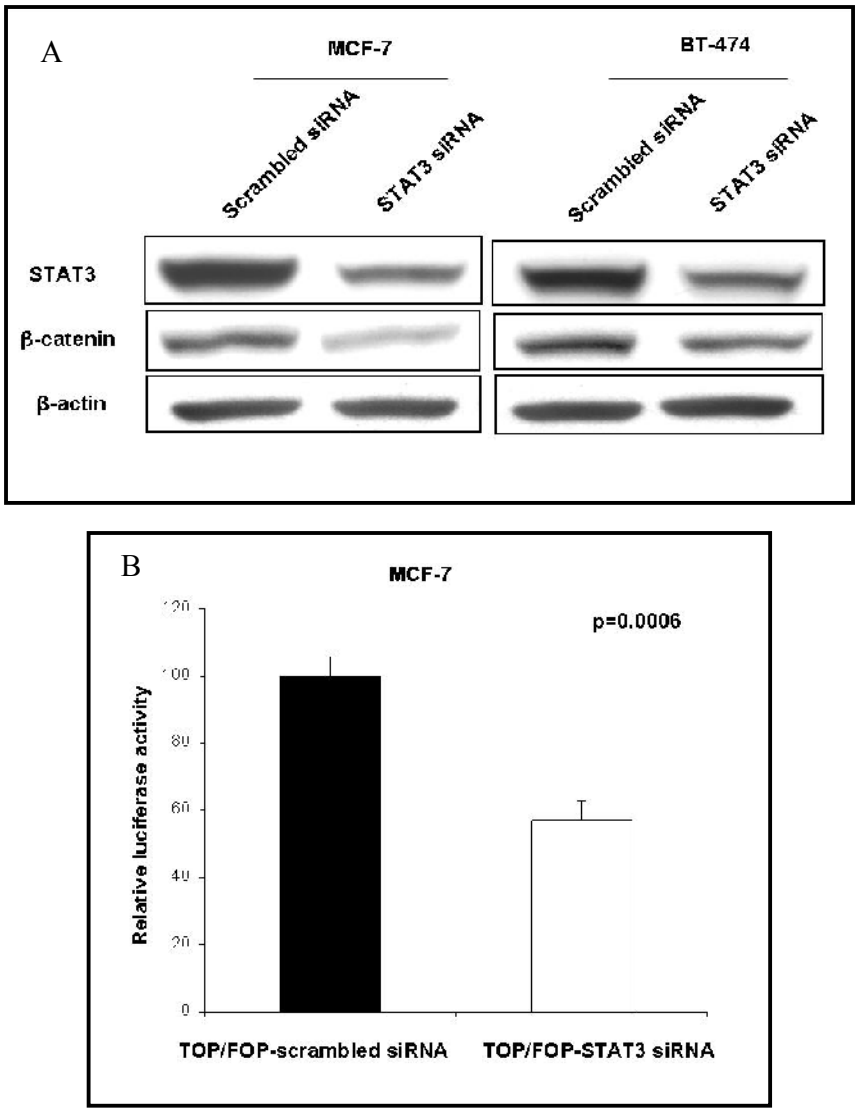

Figure 4.2. STAT3 regulated the protein levels and transcriptional activity of β-catenin. (A) Significant downregulation of the STAT3 protein level using siRNA in MCF-7 and BT-474 cells led to a substantial downregulation of β-catenin. Results are representative of four independent experiments. β-actin served as the loading control. (B) In MCF-7 cell line, in comparison to cells treated with scrambled siRNA, cells treated with STAT3 siRNA showed a significant decrease in the β-catenin transcriptional activity, as assessed by the TOPFlash/FOPFlash luciferase assay (p=0.0006).

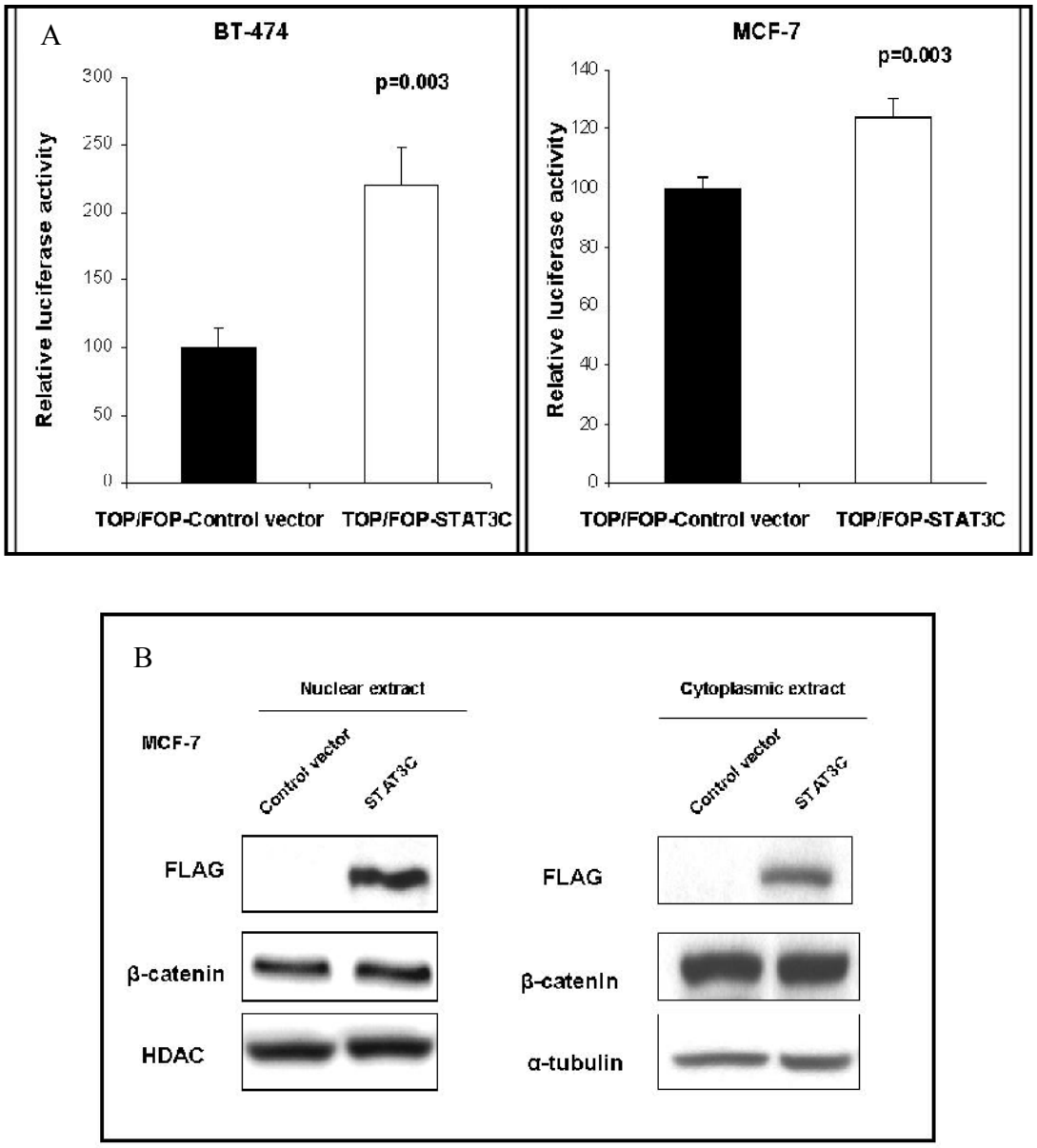

Figure 4.3. Increase in transcriptional activity of β-catenin after STAT3C transfection (A) Transient transfection of STAT3C in MCF-7 and BT-474 led to a significant upregulation of the transcriptional activity of β-catenin, as assessed by the TOPFlash/FOPFlash luciferase assay (p=0.003 for both cell lines). (B) Subcellular fractionation after STAT3C transfection in MCF-7 showing no change in nuclear translocation of β-catenin.

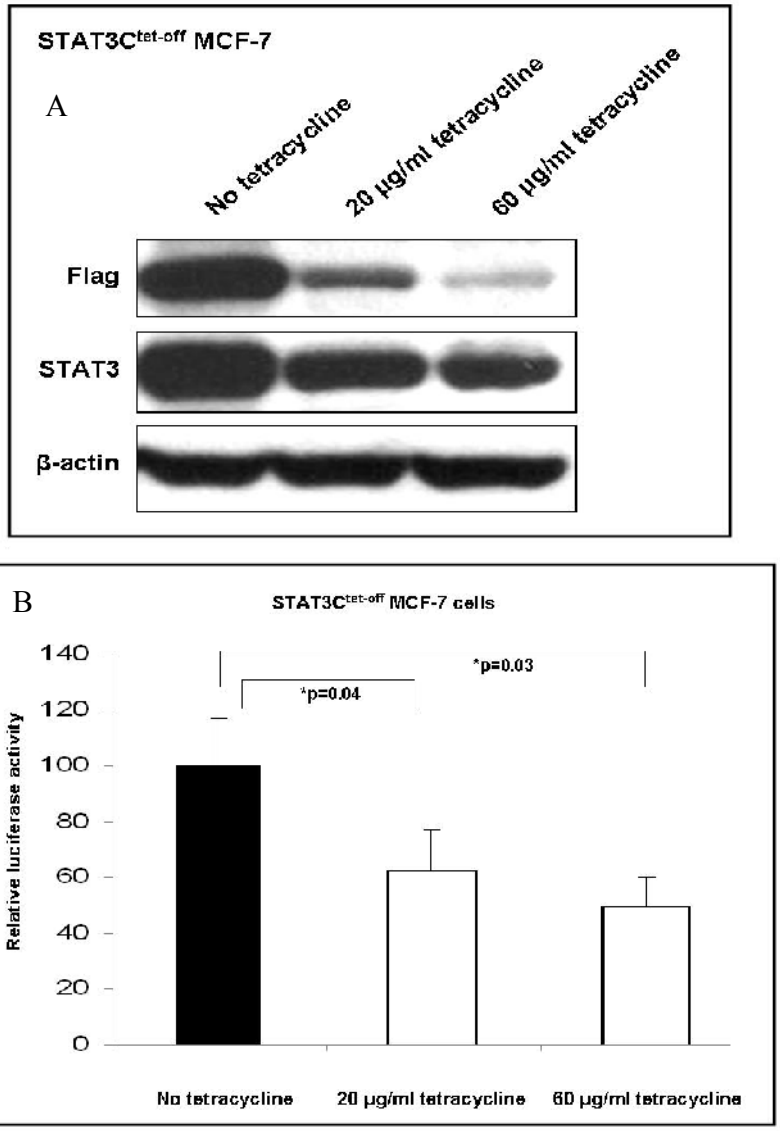

Figure 4.4. Decrease in transcriptional activity of β-catenin in STAT3C$^{tet\text{-}off}$ MCF-7 (A) Downregulation of exogenous STAT3C using tetracycline in STAT3C$^{tet\text{-}off}$ MCF-7 cells was revealed by western blot analysis, as the expression levels of the FLAG tag and total STAT3 were gradually decreased with increasing concentrations of tetracycline. Cell lysates were prepared 24 hours after tetracycline was added to the cell culture. (B) STAT3C$^{tet\text{-}off}$ MCF-7 cells treated with 20 µg/ml and 60 µg/ml tetracycline had a significant decrease in the transcriptional activity of β-catenin, as compared to cells without tetracycline (p=0.04 and 0.03 respectively). Results were derived from four independent experiments, each performed in triplicate.

4.3.3. Nuclear expression of β-catenin significantly correlates with pSTAT3 expression in breast cancer samples

Using an anti-β-catenin antibody and immunohistochemistry, we surveyed the expression of nuclear β-catenin in a cohort of formalin-fixed, paraffin-embedded breast cancer samples (n=129). Nuclear β-catenin was detected in 24 (19%) cases. Similarly, we surveyed the expression of pSTAT3 using a monoclonal antibody and immunohistochemistry. pSTAT3 was detectable in 61 (47%) cases (Table 4.2). Importantly, the expression of these two markers significantly correlated with each other (p=0.003, Fisher exact test). However, the expression of these two markers did not significantly correlate with the overall survival. The staining results for pSTAT3 are illustrated in Figure 4.5A and 4.5B. The staining results for β-catenin are illustrated in Figure 4.5C-F. Cases scored negative for β-catenin showed no detectable nuclear staining, but some cases had staining on the cell membrane (Figure 4.5C) whereas other cases showed both membraneous and cytoplasmic staining (Figure 4.5D). Cases scored positive for β-catenin showed definitive nuclear staining, with some cases also showing staining in the cytoplasm (Figure 4.5E) whereas other cases showing only nuclear staining (Figure 4.5F).

Table 4.2. Immunohistochemistry of pSTAT3 and β-catenin in breast cancer patients' samples (p=0.003)

	β-catenin positive	β-catenin negative	Total
pSTAT3 positive	18	43	61
pSTAT3 negative	6	62	68
Total	24	105	129

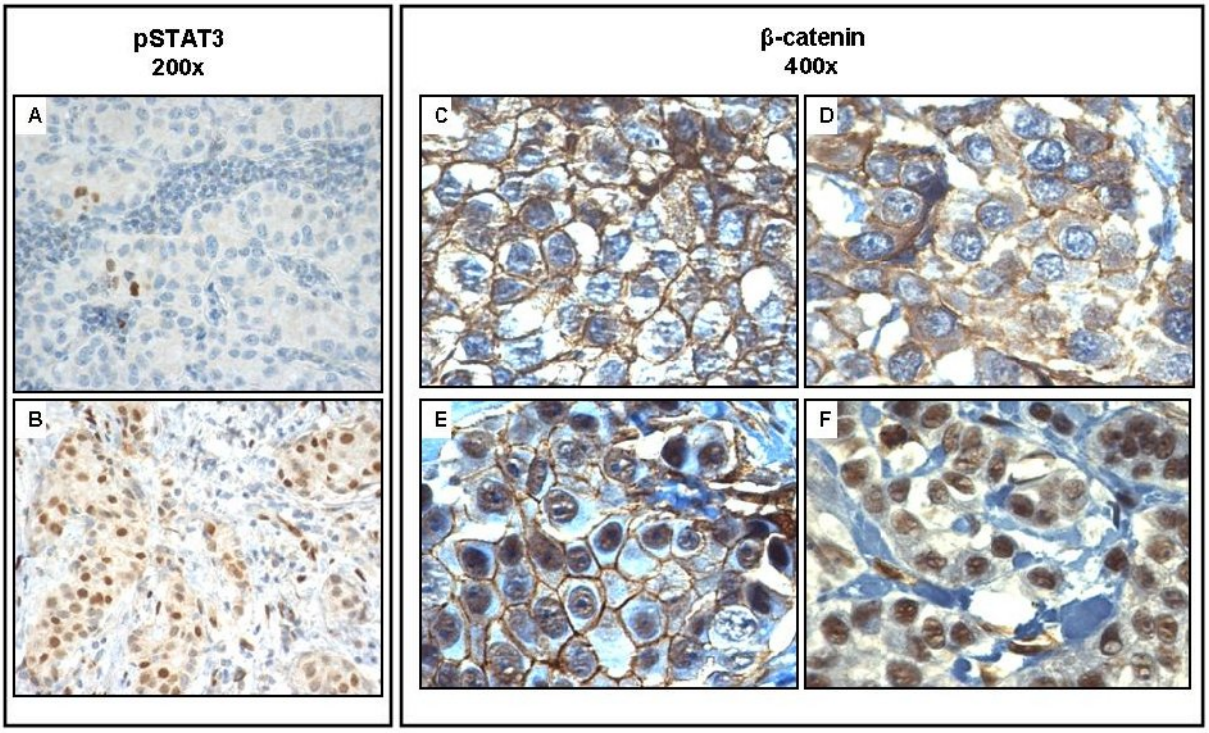

Figure 4.5. Immunohistochemistry for pSTAT3 and β-catenin in breast tumors. A pSTAT3-negative case containing only rare positive cells (i.e. <10%) is shown in A, whereas a pSTAT3-positive case containing ≥10% stained cells is shown in B. pSTAT3 staining was largely nuclear. Staining for β-catenin is illustrated in Figure 4.5C-F. Figure 4.5C and 4.5D show two negative cases in which no definitive nuclear staining was detectable. The case shown in 4.5C had membranous staining whereas the case in Figure 4.5D had both membranous and cytoplasmic staining. Figure 4.5E and 4.5F show two positive cases, in which definitive nuclear staining was detectable. The case shown in Figure 4.5E also showed cytoplasmic staining, whereas the case in Figure 4.5F showed nuclear staining only.

4.3.4. β-catenin promotes cell growth in breast cancer

To investigate the biological importance of β-catenin on breast cancer, we downregulated β-catenin levels in MCF-7 using siRNA, and a significant downregulation of β-catenin was shown on Western blot (Figure 4.6A). An MTS assay was performed on day 1, 2, and 3 after β-catenin was downregulated. As shown in Figure 4.6B, there was a significant decrease in cell growth in cells treated with β-catenin siRNA, as compared to those treated with scrambled siRNA. Apoptosis, as detected by PARP and caspase 3 cleavages, was not detectable (not shown).

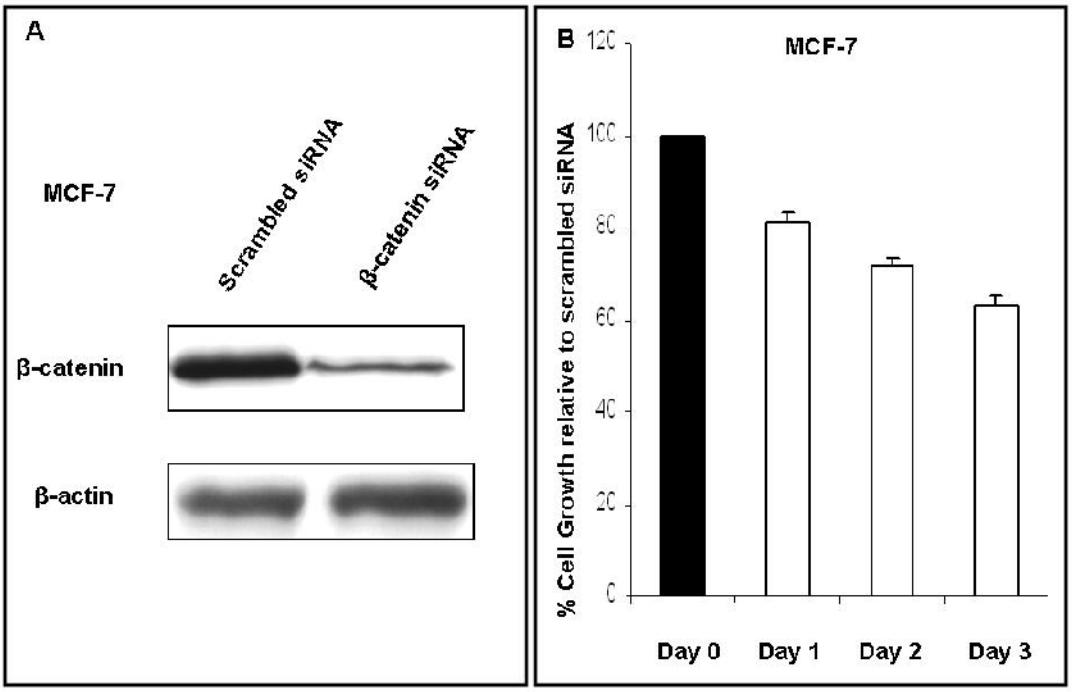

Figure 4.6. β-catenin promotes cell growth in breast cancer. A, B) Downregulation of β-catenin using siRNA led to a significant decrease in cell growth compared to scrambled siRNA, as assessed by the MTS assay.

4.4. DISCUSSION

Our presented data support the concept that STAT3 is a regulator of β-catenin in breast cancer. Specifically, we found that the gene promoter of *β-catenin* carries multiple STAT3 consensus sequences and our chromatin precipitation experiments provided direct evidence of STAT3 binding at two specific sites (-856 and -938) in the *β-catenin* gene promoter region. Furthermore, in two different breast cancer cell lines, we found evidence that the protein level and transcriptional activity of β-catenin can be modulated in response to a change in the expression and/or activity of STAT3, in both transient and stable transfection conditions. In further support that STAT3 regulates β-catenin in breast cancer, our immunohistochemical studies using a large cohort of breast tumors revealed a significant correlation between the expression of pSTAT3 and β-catenin. Our conclusion regarding β-catenin being a downstream target of STAT3 echoes the findings described in a recent study of colon cancer, which showed that STAT3 activity
regulates the transcriptional activity of β-catenin in colon cancer cells (Kawada et al., 2006). In contrast with the same study, we did not detect any change in the nuclear translocation of β-catenin following STAT3 knockdown using nuclear/cytoplasmic fractionation. Based on our overall study results, the regulation of the transcriptional activity of β-catenin by STAT3 in breast cancer appears to be related to the observations that STAT3 controls the total protein level of β-catenin.

In the present study, we have presented evidence that STAT3 can directly regulate the gene transcription of *β-catenin* and its protein expression level. We are also aware of other mechanisms by which STAT3 can potentially regulate β-catenin through modulating the upstream of the WCP. Specifically, the gene promoter of *Wnt3a* has been shown to carry the consensus binding sequence for STAT3 (Li et al., 2008). STAT3 also has been shown to induce the expression of Wnt5a in rat cardiac

myocytes (Fujio et al., 2004; Miyagi et al., 2004). Taken together, it is possible that STAT3 regulates β-catenin via multiple mechanisms: 1) direct modulation of the gene transcription of *β-catenin*, and 2) modulate the secretion of different Wnt's, thereby regulating β-catenin via the WCP.

While we did not observe a prognostic significance for β-catenin in our cohort of breast cancer patients, we are aware of the results of a previously published study which showed that β-catenin is prognostically important in breast cancer (Lin et al., 2000). In contrast with the study by Lin *et al*, who scored the β-catenin regardless of the staining pattern (Lin et al., 2000), we considered β-catenin positivity only when the staining was definitively nuclear. We also would like to point out that, one of the authors (JM) in a paper recently published that β-catenin is useful in predicting relapse in breast cancer patients (Asgarian et al., 2009); however, no significant correlation between β-catenin nuclear staining and the overall survival was found (personal communication).

Although the focus of this manuscript is to document the functional interaction of STAT3 and β-catenin, we also examined whether β-catenin is biologically important in breast cancer cell lines. As shown in this study, downregulation of β-catenin using siRNA inhibited cell growth in MCF-7. Growth inhibition induced by a downregulation of β-catenin has been observed for esophageal cancer, colon cancer and glioma (Huang et al., 2007; Pu et al., 2009; Wang et al., 2009). β-catenin was found to promote cell migration in a breast cell line (Simpson et al., 2008), but we are not aware of any previous study in which the growth-promoting effect of β-catenin was examined in breast cancer. Thus, to our knowledge, these findings represent the first evidence that β-catenin promotes cell growth in breast cancer.

Both β-catenin and STAT3 have been shown to be oncogenic in various types of human cancer including breast cancer (Imbert et al., 2001; Kunigal et al., 2009). Our findings described in this manuscript raise the possibility that the oncogenic effects of STAT3 may synergize with that of β-catenin. Of interest, it have been reported that β-catenin regulates the STAT3 transcriptional activity in esophageal cancer cell lines, thus potentially creating a positive a feedback loop between these two signaling proteins (Yan et al., 2008). Since specific agents inhibiting STAT3 and β-catenin are available (Takemaru et al., 2008; Yue and Turkson, 2009), our data provide the rationale for combining these inhibitors in treating specific forms of cancer, such as in a subset of breast cancer in which both STAT3 and β-catenin are activated. *In-vitro* studies evaluating the potential synergistic effects of combining these agents will be of great interest.

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❖ Chapter 5

CONSTITUTIVE ACTIVATION OF METALLOPROTEINASE ADAM10 IN MANTLE CELL LYMPHOMA PROMOTES CELL GROWTH AND ACTIVATES THE TNFα/NFκB PATHWAY

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Armanious H, Gelebart P, Anand M, Belch A, Lai R. Constitutive activation of metalloproteinase ADAM10 in mantle cell lymphoma promotes cell growth and activates the TNFα/NFκB pathway. Blood. 2011;117:6237-6246

5.1. INTRODUCTION

A disintegrin and metalloproteinase 10 (ADAM10), a member of the ADAM family of metalloproteinases, was discovered in the protein extract of brain myelin membranes and subsequently found to be a homologue of the Drosophila *kuzbanian (kuz)* gene (Chantry and Glynn, 1990; Chantry et al., 1989; Yavari et al., 1998). ADAM10 is secreted as a precursor protein, and it consists of multiple functional domains including a prodomain, catalytic domain, cysteine-rich domain, transmembraneous domain and cytoplasmic domain (Edwards et al., 2008). To become the active/mature form, the precursor ADAM10 protein needs to be cleaved by proprotein convertase 7 and furin, both of which remove the prodomain of ADAM10 (Anders et al., 2001). ADAM10 is biologically important, since *ADAM10* knockout mice die on day 9 of embryogenesis due to multiple abnormalities in the nervous and cardiovascular systems (Hartmann et al., 2002). The key biological function of ADAM10 appears to be attributed to its enzymatic activity as a metalloproteinase. Specifically, ADAM10 is involved in the intra-membrane proteolysis process, whereby it mediates ectodomain shedding of various membrane bound receptors, adhesion molecules, growth factors and cytokines (Endres and Fahrenholz; Mochizuki and Okada, 2007; Seals and Courtneidge, 2003). For instance, ADAM10 is involved in the regulation of the shedding of Notch, HER2, CD44, IL-6 receptor, amyloid precursor protein and cadherins (Duffy et al., 2009). Directly relevant to our study, ADAM10 was recently found to be one of the enzymes responsible for cleaving tumor necrosis factor alpha (TNFα) and releasing its active form (Hikita et al., 2009; Lunn et al., 1997; Mezyk-Kopec et al., 2009). Furthermore, it has been reported that ADAM10 is important for the development of the marginal zone B-cells (Gibb et al.). By regulating the bioavailability of ligands to various cellular signaling receptors, ADAM10 modulates the activation status of various cellular signaling pathways that have an impact on various cellular responses such as proliferation and migration (Seals and Courtneidge,

2003). ADAM10 has been shown to be constitutively active in a number of solid tumors, and this biochemical defect is implicated in the pathogenesis of these tumors. For instance, xenografting of colorectal cancer cells with enforced expression of ADAM10 in nude mice induced the formation of liver metastasis compared to the negative control cells, and this effect can be attributed to ADAM10-mediated cleavage and release of L1-CAM, a cell adhesion molecule (Gavert et al., 2007). In another study, ADAM10 expression in colorectal cancer patient samples detectable by immunohistochemistry was found to correlate with a higher clinical stage (Knosel et al., 2005). Using immunohistochemistry, it was also found that ADAM10 is overexpressed in squamous cell carcinomas of the oral cavity, as compared to the benign epithelial cells; knockdown of ADAM10 expression using siRNA in the cell lines derived from these tumors was shown to induce a significant decrease in cell growth (Ko et al., 2007). Similar findings were made in pancreatic cancer, where inhibition of ADAM10 expression in pancreatic carcinoma cell lines resulted in a significant decrease in invasiveness and migration (Gaida et al.). Lastly, ADAM10-mediated cleavage of N-cadherin was found to regulate the migratory properties of glioblastoma cells (Kohutek et al., 2009). While the pathogenetic role of ADAM10 has been well documented in solid tumors, its role in hematologic malignancies is largely unknown. To our knowledge, there is only one published study that performed a survey of the expression of various ADAM family members in benign and malignant hematopoietic cells using RT-PCR, and the authors reported the expression of the *ADAM10* mRNA in myeloma, erythroleukemia and a subset of lymphoma cell lines (Wu et al., 1997). Since only RT-PCR was used in this particular study, whether ADAM10 is in its active form could not be assessed; the biological significance of ADAM10 in these malignancies also was not examined (Wu et al., 1997).

Mantle cell lymphoma (MCL) is a specific subtype of aggressive B-cell lymphoma recognized by the World Health Organization Classification Scheme (Jaffe et al., 2004). The genetic hallmark of this disease is the recurrent chromosomal abnormality, *t(11;14)(q13;q32)*, which brings the *cyclin D1(CCND1)* gene under the influence of the enhancer of the immunoglobulin heavy chain (*IgH*) gene, leading to cyclin D1 overexpression (Campo et al., 1999). Although it has been shown that cyclin D1 overexpression is not sufficient for the induction of lymphoma in animal models, this abnormality is considered to be the primary oncogenic event in MCL (Diehl, 2002; Jares et al., 2007). Large-scale cDNA microarray studies using frozen MCL tumors have revealed a relatively large number of biochemical abnormalities in MCL, with these defects are frequently implicated in the regulation of apoptosis, survival and DNA damage (Hofmann et al., 2001; Martinez et al., 2003; Rosenwald et al., 2003; Schaffner et al., 2000). An increasing number of cellular signaling pathways also have been found to be abnormal. For instance, NFκB has been reported to be constitutively active in MCL, and this biochemical defect is biologically important to the pathogenesis of MCL (Pham et al., 2003). The activation of NFκB in MCL can be partly attributed to TNFα (Gelebart et al., 2009; Shishodia et al., 2005), the bioavailability of which has been shown to be regulated by ADAM10, as mentioned above (Hikita et al., 2009; Lunn et al., 1997; Mezyk-Kopec et al., 2009).

In this report, we described for the first time that ADAM10 is constitutively activated in MCL cell lines and tumors. In view of the importance of TNFα in the biology of MCL, and the previous report that ADAM10 can increase the bioavailability of TNFα (Hikita et al., 2009; Lunn et al., 1997; Mezyk-Kopec et al., 2009) , we hypothesize that ADAM10 may be important in the pathogenesis of MCL.

5.2. MATERIALS AND METHODS

5.2.1. Cell lines and tissue culture

The characteristics of the three MCL cell lines, Jeko-1, Mino, and SP53, have been previously described (Amin et al., 2003). All 3 cell lines are negative for the Epstein-Barr virus nuclear antigen. They were grown at 37^0C and 5% CO_2 and maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO). The culture media were enriched with 10% fetal bovine serum (Life Technologies, Carlsbad, CA). Ficoll-Paque (GE Health care, Quebec, Canada) was used to isolate peripheral blood mononuclear cells (PBMC) from healthy donors (n=5) and leukemic MCL patients (n=3). OCT-embedded frozen tumors from classical MCL (n=9), chronic lymphocytic leukemias (CLL, n=5), follicular lymphomas (FL, n=4), diffuse large B-cell lymphomas (DLBCL, n=4) and marginal zone lymphomas (MZL, n=4) were also included for comparison. The use of these patient samples has been reviewed and approved by our Institutional Ethics Committee.

5.2.2. Subcellular protein fractionation, Western blots and antibodies

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA) and followed the manufacturer's instructions. Preparation of cell lysates for Western blots was done as previously described (Armanious et al.). Antibodies employed in this study included those reactive with ADAM10, phospho-NFκBp65 (Chemicon, Temecula, CA), NFκBp65, cyclin D1, α-tubulin, HDAC and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), $p21^{Waf1,}$ $p27^{Kip1}$, p15, p16, cleaved PARP and cleaved caspases 3, 7 and 9 (Cell Signaling Technology, Danvers, MA).

5.2.3. Short interfering RNA (siRNA)

Two siRNA species for ADAM10 from 2 different commercial sources were used in this study. siRNA #1 was a pool of 4 siRNA species from Dharmacon (Lafayette, CO) and siRNA #2 was from Invitrogen

(Burlington, Ontario, Canada). Scrambled siRNA was purchased from Dharmacon. Transfection of siRNA was carried out using an electrosquare electroporator, BTX ECM 800 (225V, 8.5ms, 3 pulses) (Holliston, MA). The concentration of siRNA used was 200 pmol/1x10^6 cells, and cells were harvested at 48 hours after transfection. The ADAM10 protein levels were assessed using Western blot to evaluate the efficiency of inhibition.

5.2.4. Cell viability

A total of 100,000 cells suspended in 1 mL of culture medium were plated in triplicate. To assess cell viability, trypan blue exclusion assay (Sigma-Aldrich) was performed every 24 hours for up to 3 days following transfection of ADAM10 siRNA or scrambled siRNA. MTS assay was also performed during this time frame and the manufacturer's instructions were followed (Promega, Madison, WI). The measurements were obtained at a wavelength of 450 nM using a Bio-Rad Micro plate Reader (Bio-Rad, Hercules, CA). The absorbance values were normalized to the wells with media only using the microplate Manager 5.2.1 software (Bio-Rad). All experiments were performed in triplicates.

5.2.5. Recombinant ADAM10, TNFα assay, bortezomib and MG132

Human recombinant ADAM10 was purchased from BD biosciences (Pharmingen, San Diego, CA) and it corresponds to the segment spanning from Thr214 to Glu672, which represents the active/mature form of ADAM10. It was added at a concentration of 100 ng/ml to 1x10^6 cells; after 24 hours, cell lysates were prepared and cell count was done. TNFα secretion was monitored using a commercially available ELISA kit (R&D Systems Inc., Minneapolis, MN). Aliquots of the culture medium from MCL cell lines collected 48 hours after transfection with either scrambled siRNA or ADAM10 siRNA were centrifuged at 15,000 g, and the supernatant was assayed for the TNFα levels as per manufacturer's protocol. Bortezomib (LC laboratories, Woburn, MA), a proteasome inhibitor was added at a

concentration of 5 nM to MCL cells 24 hours after siRNA transfection, and cell count using trypan blue exclusion assay was performed 48 hours after the initiation of the experiment. Another proteasome inhibitor MG132 (Calbiochem, EMD Biosciences, Darmstadt, Germany) was added at a concentration of 1 μM to MCL cells 24 hours after siRNA transfection, and cell count using trypan blue exclusion assay was performed 48 hours after the initiation of the experiment.

5.2.6. NFκB transcriptional activity

To assess the transcriptional activity of NFκB in MCL cell lines, we employed the NFκB responsive firefly luciferase reporter plasmid and renilla reporter plasmid (Promega). After 48 hours of transfection of the reporter plasmid (with the renilla luciferase plasmid as an internal control) together with either scrambled siRNA or ADAM10 siRNA, MCL cells were harvested and cell extracts were prepared using a lysis buffer purchased from Promega. The firefly luciferase activity and renilla luciferase activity were assessed using the dual luciferase reagent (Promega).

5.2.7. Cell cycle analysis by flow cytometry

Cells transfected with either scrambled siRNA or ADAM10 siRNA were fixed with ice-cold 70% ethanol 24 hours after gene transfection. These cells were then subjected to RNase treatment and propidium iodide (PI) staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated to exclude cell doublets and the cell-cycle phase distribution was determined using the CellQuest program (20,000 events were counted).

5.2.8. Assessment of cyclin D1 expression using quantitative RT-PCR

The expression of *cyclin D1* in MCL cells treated with ADAM10 siRNA was assessed using quantitative RT-PCR (qRT-PCR). The assay was performed using the Applied Biosystems 7900 HT, and the SYBR®

GreenER™ qPCR SuperMix from Invitrogen. The primer sequence for *ADAM10* was as follows: forward 5'-AGCAACATCTGGGGACAAAC -3'; reverse 5'-CTTCCCTCTGGTTGATTTGC-3'. The primer sequence for *cyclin D1* was as follows: forward 5'-CAAATGGAGCTGCTCCTGGTG-3'; reverse 5'-TGGCACCAGCCTCGGCATTTC-3' (Gelebart et al., 2008). Triplicate experiments were performed and the statistical significance of the differences was assessed using Student *t*-test.

5.2.9. Immunohistochemistry and archival MCL tumors

Formalin-fixed, paraffin-embedded tumors from a cohort of 23 patients with classical MCL were retrieved from the files at the Cross Cancer Institute (Edmonton, Alberta, Canada). All MCL primary tumors were diagnosed at the Cross Cancer Institute and the diagnostic criteria were based on those described in the World Health Organization Classification Scheme (Jaffe et al., 2004). All cases were confirmed to express cyclin D1 by immunohistochemistry. The use of these tissues has been approved by our Institutional Ethics Committee. Immunohistochemistry was performed using standard techniques. Briefly, formalin-fixed, paraffin-embedded tissue sections of 4 μM thickness were deparaffinized and hydrated. Heat-induced epitope retrieval was performed in a pressure cooker using citrate buffer (pH=6) in a microwave. After antigen retrieval, tissue sections were incubated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Tissue sections were then incubated with anti-ADAM10 antibody (1:200) (same antibody used in western blots) overnight in a humidified chamber at 4°C. Immunostaining was visualized with a labeled streptavidin-biotin (LSAB) method using 3,3'-diaminobenzidine as a chromogen (Dako Canada Inc., Mississauga, Ontario, Canada) and counter-stained with hematoxylin. Colon carcinoma case served as the positive control, whereas the lymphoid cells of mantle zone in benign tonsils served as the negative control.

5.2.10. Statistical analysis

Data are expressed as mean +/- standard derivation. Unless stated otherwise, statistical significance was determined using two-tailed Student's *t*-test and statistical significance was achieved when the p value is <0.05. In experiments where we needed to determine the statistical significance between more than two groups analysis of variance (ANOVA) test was applied.

5.3. RESULTS

5.3.1. The active/mature form of ADAM10 is expressed in MCL cells and other B-cell lineage malignancies

The expression of ADAM10 in 3 MCL cell lines was assessed using western blots, and the results are illustrated in figure 5.1A. While the precursor form of ADAM10 at 98 kDa was highly expressed in all 3 cell lines examined, the expression levels of the active/mature form of ADAM10 at 72 kDa were variable among these 3 cell lines. Specifically, although the active/mature form of ADAM10 was readily detectable in Jeko-1 and Mino cells, it was barely detectable in SP53 cells. MCF-7, a breast cancer cell line previously reported to have a high level of active/mature form of ADAM10 (Dittmer et al., 2009), served as the positive control. In contrast to MCL cells, PBMC from five healthy donors expressed no detectable active/mature form of ADAM10 at 72 kDa, although the precursor form was readily detectable in all cases. The results from 3 of these 5 healthy individuals are illustrated in figure 5.1A and 5.1B.

Western blot analysis was performed using 9 frozen MCL tumor samples (patient #1-9) and 3 fresh leukemic MCL blood samples (patient #10-12). The active/mature form of ADAM10 was readily detectable in all patient samples, although the level was relatively low in patient #7 (figure 5.1B). Again, PBMC from healthy donor #2 and #3 had no convincing band at 72

kDa. Of note, in contrast with the MCL cell lines (figure 5.1A); most MCL patient samples expressed the active/mature form of ADAM10 at a higher level than its precursor form. We also analyzed the expression of both the precursor and active/mature forms of ADAM10 in frozen tissues derived from other B-cell lineage malignancies (5 CLL cases, 4 DLBCL cases, 4 FL cases and 4 MZL cases). As shown in supplementary figure 5.1, the expression of ADAM10 was readily detectable in all of these B-cell neoplasms, with a pattern similar to that seen in MCL patient samples.

We then performed immunohistochemistry applied to 23 MCL formalin-fixed/paraffin-embedded tumor samples and 5 cases of benign reactive tonsils. We found that the mantle zones in benign reactive tonsils were negative for ADAM10, whereas the germinal centers showed only faint cytoplasmic staining (figure 5.1C). The ADAM10 immunostaining in MCL was generally homogenous within the same tumor, and we considered a MCL tumor to be positive when the majority of the tumor cells showed a staining intensity higher than that of germinal center cells. With this criterion, ADAM10 was assessed positive in 20 of 23 (87%) cases. On higher magnification, the immunostaining was predominantly cytoplasmic in all cases, while a small subset of cases also showed some degree of nuclear staining (figure 5.1C). Of note, 5 of these MCL cases used for immunohistochemical studies were also included in the experiment illustrated in figure 5.1B (western blot studies); all these 5 cases were positive by immunohistochemistry and carried the active form of ADAM10 by western blots.

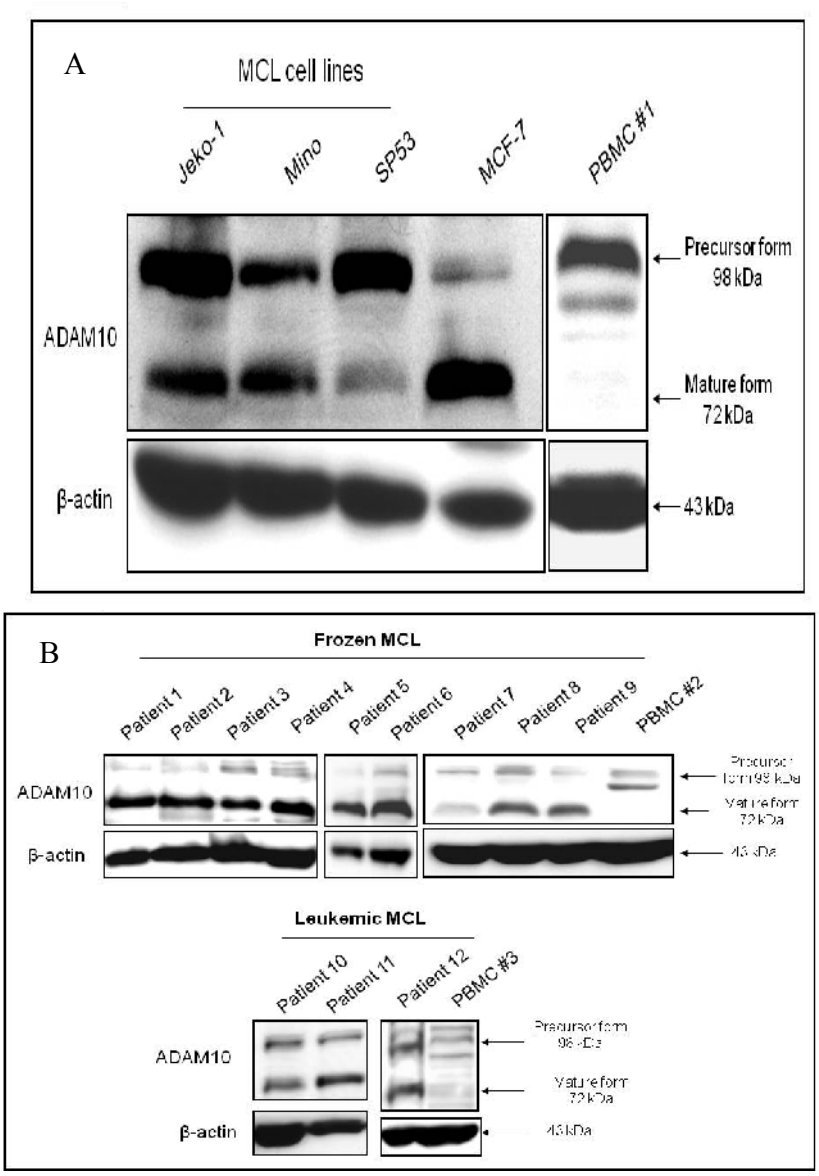

Figure 5.1. ADAM10 expression in MCL cell lines and patient samples (continued). A) Western blots showed the expression of the precursor and active/mature form of ADAM10 in three MCL cell lines. While the precursor was highly expressed, the active/mature form was readily detectable only in Jeko-1 and Mino. A faint band at 72 kDa, representing the active/mature form of ADAM10, was also detectable in SP53. MCF-7 cells were used as the positive control. Peripheral blood mononuclear cells (PBMC) from a healthy individual (#1) showed abundant precursor ADAM10 protein but no detectable active/mature ADAM10. B) Western blots showed the presence of the precursor and active/mature form of ADAM10 in all MCL patients' samples (both frozen #1-9 and leukemic #10-12); PBMC from two healthy individuals (#2 and #3) showed no detectable active/mature ADAM10.

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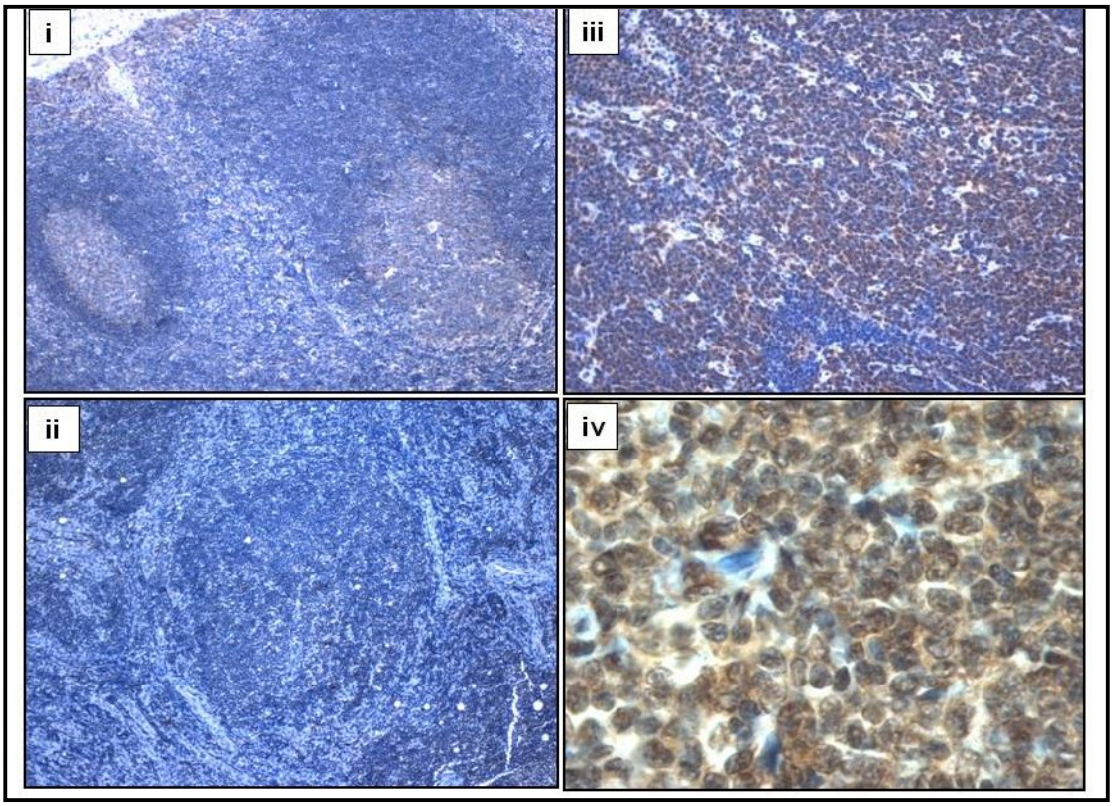
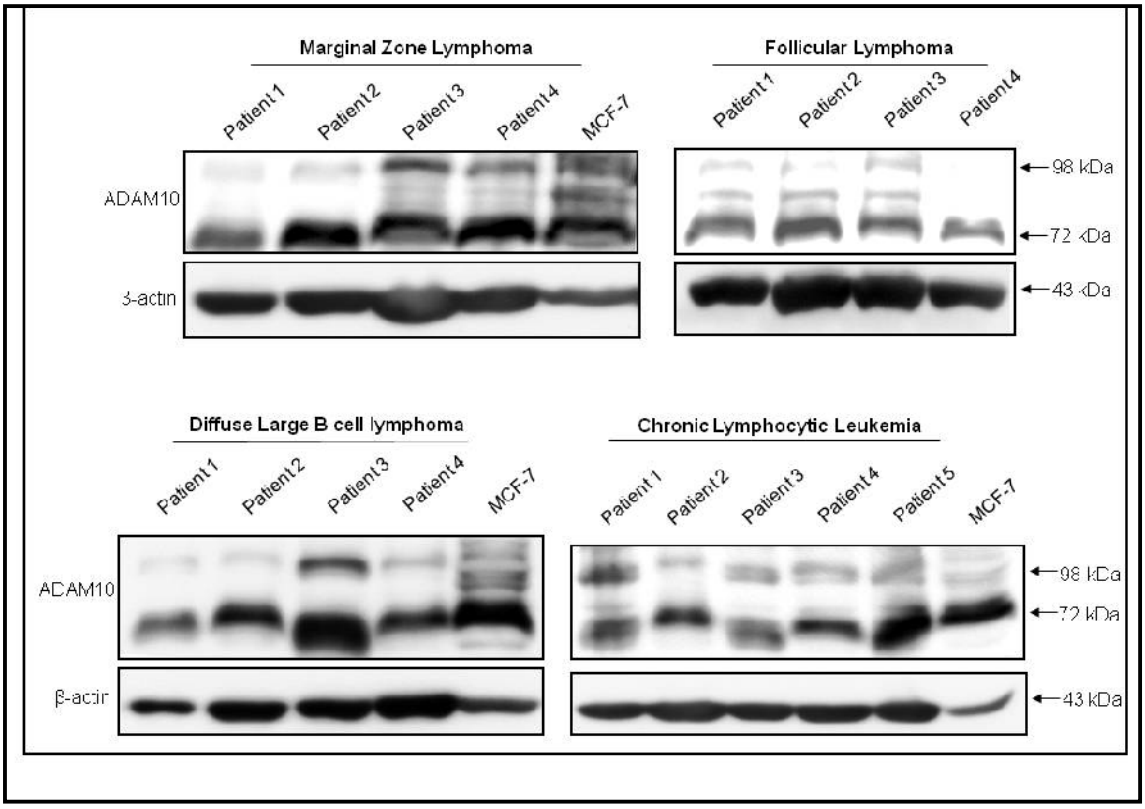


Figure 5.1. ADAM10 expression in MCL cell lines and patient samples. C) Immunohistochemistry showed no detectable signal of ADAM10 in the mantle zone of a reactive tonsil; the germinal centers showed faint staining, 100X (i). A case of MCL tumor showed no detectable ADAM10, 100X (ii). A MCL tumor showed a relatively high level of ADAM10 expression, 100X (iii). On high magnification, the ADAM10 immunostaining pattern was detectable in both of the nucleus and cytoplasm, 1000X (iv).



Supplementary figure 5.1. ADAM10 expression in B-cell non-Hodgkin lymphomas other than MCL. Western blots showed the presence of the precursor and active/mature form of ADAM10 (with a predominance of the active form) in marginal zone lymphoma, follicular lymphoma, diffuse large B cell lymphoma and chronic lymphocytic leukemia, MCF-7 was used as positive control.

5.3.2. ADAM10 promotes cell growth in MCL cells

To assess the functional importance of ADAM10 in MCL cells, we evaluated if siRNA knockdown of ADAM10 has any impact on the growth of MCL cells. As shown in figure 5.2A, transfection of siRNA into Jeko-1 and Mino resulted in a reduction of both the precursor and the active/mature form of ADAM10. With the knockdown of ADAM10, the cell growth (assessed by the trypan blue exclusion assay) was significantly decreased, as compared to cells transfected with scrambled siRNA ($p=0.01$ and 0.007 for Mino and Jeko-1, respectively) three days after transfection (figure 5.2B). The decrease in the viable cell count was not associated with any substantial increase in trypan blue-positive dead cells. Similar experiments were performed using the MTS assay, and we found comparable results ($p<0.0001$ and $p=0.01$ for Mino and Jeko-1, respectively) (figure 5.2C). To further strengthen the conclusion that ADAM10 promotes cell growth in MCL cells, we tested how MCL cells may respond to recombinant ADAM10. SP53 and Mino cells were used for this experiment, since both cell lines had a lower level of active ADAM10 than Jeko-1 cells, and thus, they were expected to show more dramatic response to recombinant ADAM10. As shown in figure 5.2D, the addition of recombinant ADAM10 induced a significant increase in cell growth ($p=0.008$ and 0.01, respectively).

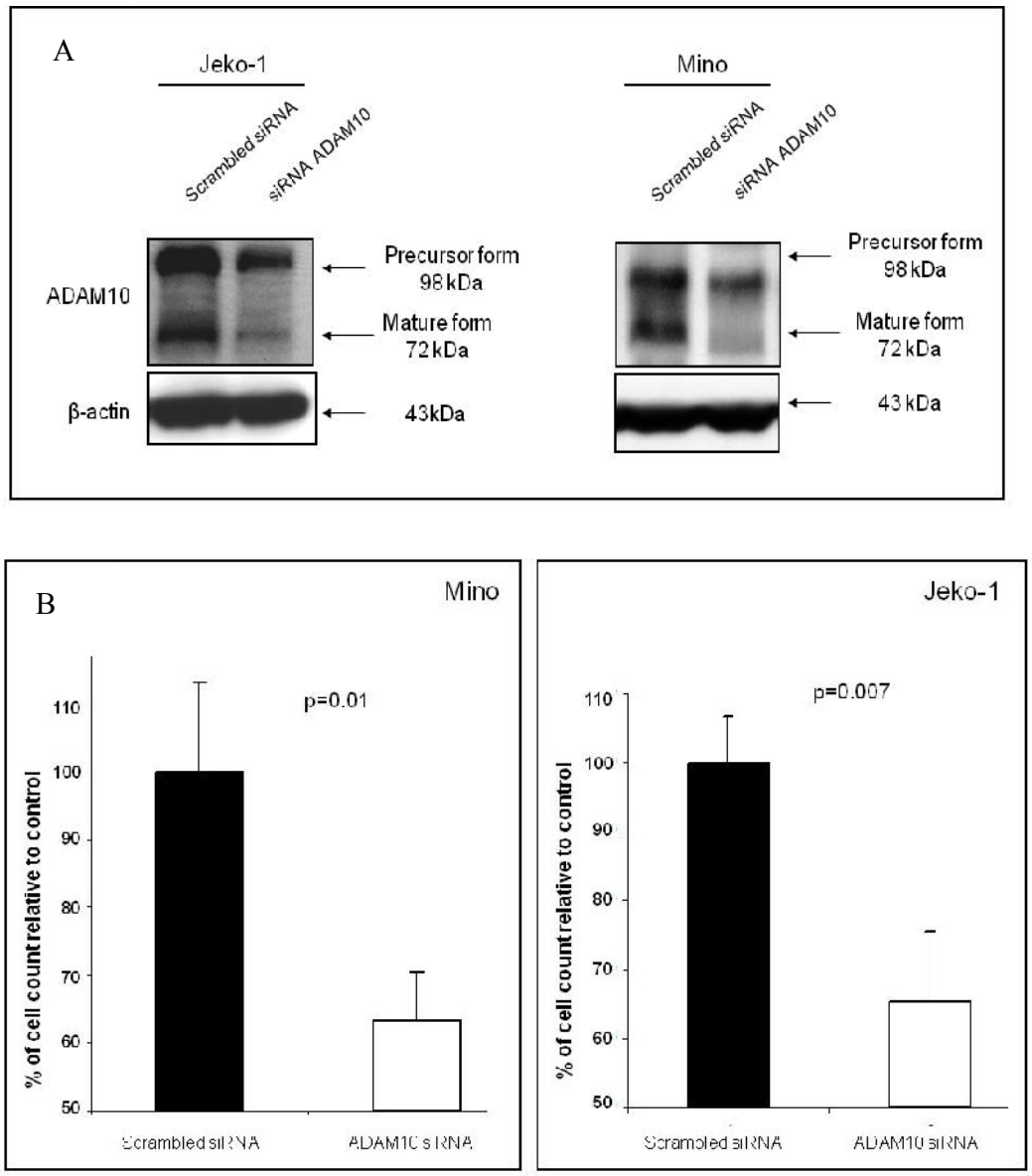


Figure 5.2. ADAM10 promoted cell growth in MCL (continued)
A) Western blots showed downregulation of the precursor and active/mature form of ADAM10 in Jeko-1 and Mino cells with the use of siRNA. B) ADAM10 knockdown induced significant inhibition of cell growth in Mino and Jeko-1 cells three days after transfection, as assessed by trypan blue exclusion assay (p=0.01 and 0.007, respectively). Triplicate experiments were performed.

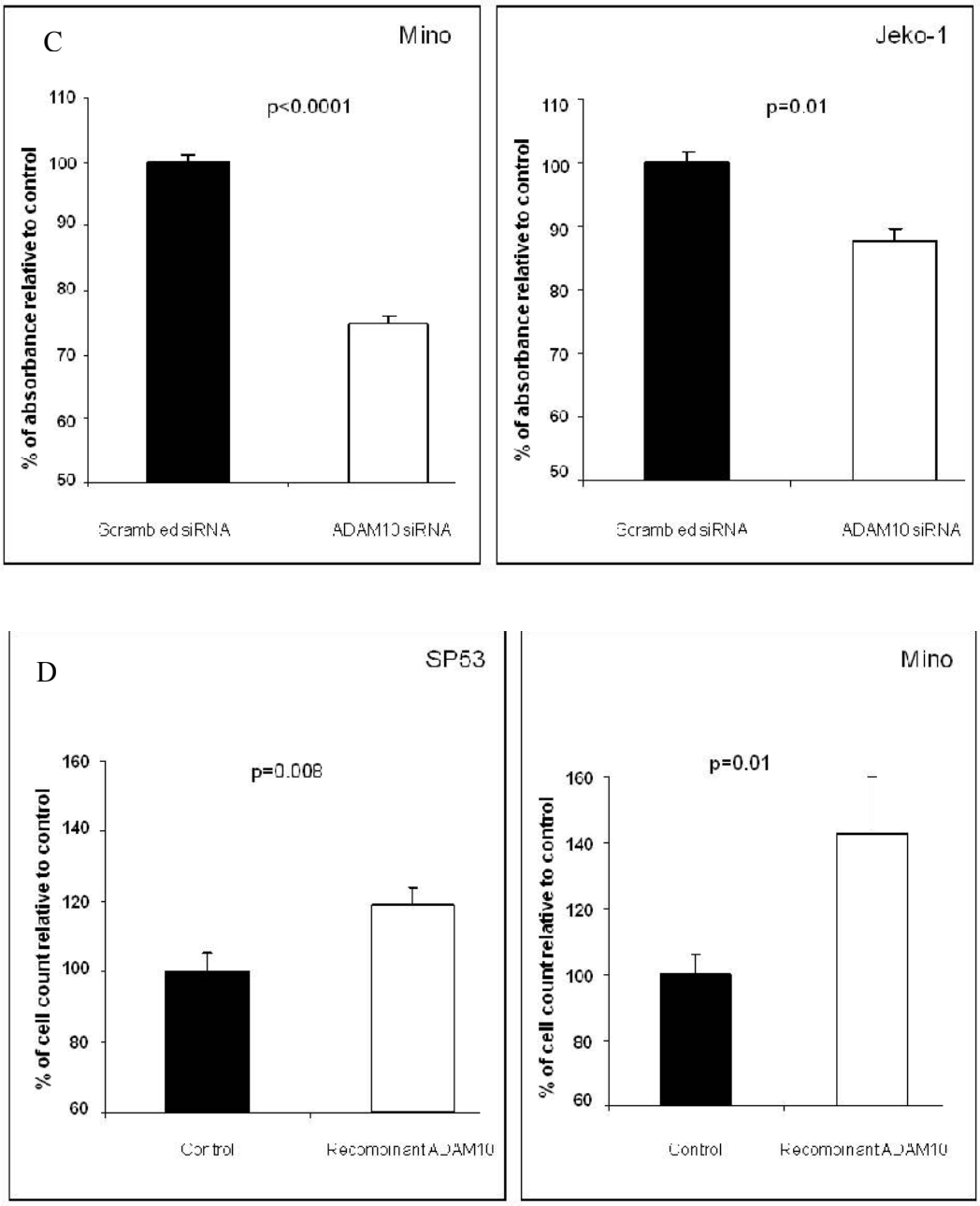
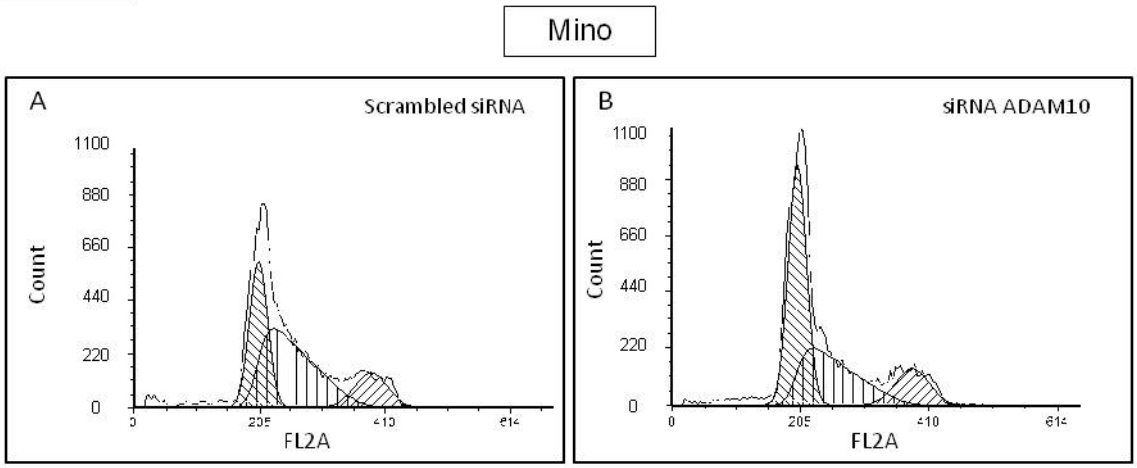


Figure 5.2. ADAM10 promoted cell growth in MCL. C) ADAM10 knockdown induced significant inhibition of cell growth in Mino and Jeko-1 cells three days after transfection, as assessed by MTS assay ($p<0.0001$ and $p=0.01$, respectively). Triplicate experiments were performed. D) Addition of human recombinant ADAM10 (100 ng/ml) to SP53 and Mino cells induced a significant increase in their growth at 24 hours ($p=0.008$ and 0.01, respectively).

5.3.3. Downregulation of ADAM10 induces cell cycle arrest but not apoptosis

To characterize the mechanism by which ADAM10 promotes cell growth in MCL, we performed cell cycle analysis. Transfection of ADAM10 siRNA into Mino and Jeko-1 cells induced a significant $G_{0/1}$ arrest and reduced the proportion of cells in the S phase, as compared to cells transfected with scrambled siRNA ($p=0.02$ and 0.05 for Mino and Jeko-1, respectively). Of note, no appreciable increase in the proportion of cells in the sub$G_{0/1}$ phase was found. All experiments were performed in triplicates and representative results are shown in figure 5.3. To further exclude the occurrence of apoptosis, we performed western blots and found no detectable cleavage of caspases 3, 7, 9 and PARP (supplementary figure 5.2). Cells treated with MG132 served as positive controls.



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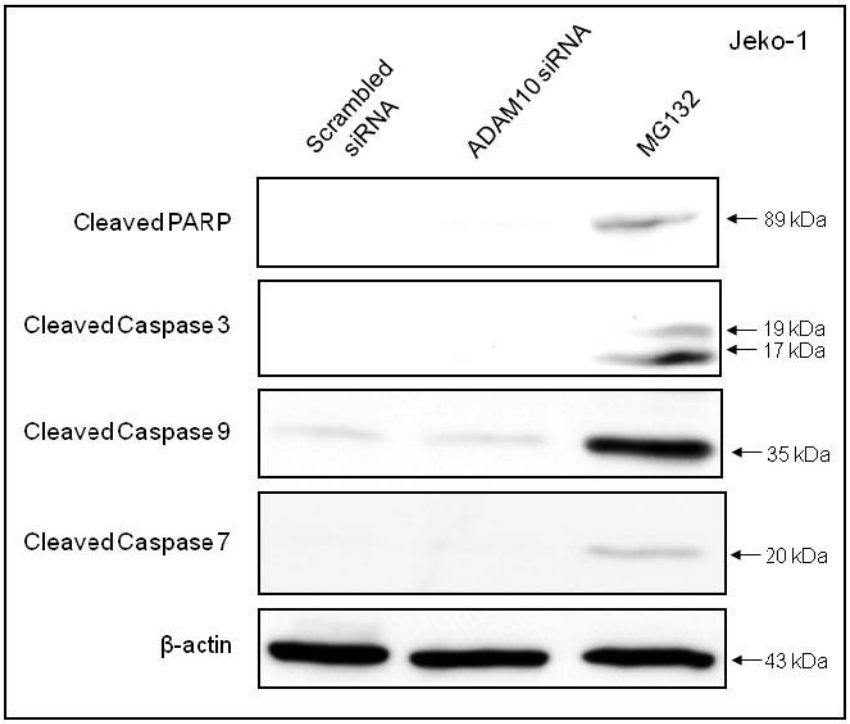
	% G1	%S	%G2	Total
Control	34.43 ± 2.3	54.44 ± 4.2	11.13± 6.4	100
siADAM10	53.6 ± 4.4	35.69 ± 1.1	10.71 ± 5.5	100

Figure 5.3. ADAM10 induced cell cycle arrest. Cell cycle analysis by flow cytometry using propidium iodide showed significant $G_{0/1}$ cell cycle arrest in Mino cells following ADAM10 downregulation using siRNA. Of note, no appreciable increase in the fraction of cells in the sub$G_{0/1}$ was noted.



Supplementary figure 5.2. ADAM10 downregulation did not induce apoptosis. Western blot studies showed that Jeko-1 cells did not undergo apoptosis after ADAM10 downregulation, as there is no evidence of cleavage of PARP, caspase 3, 7 or 9. Jeko-1 cells treated with proteasome inhibitor MG132 were used as positive controls.

5.3.4. ADAM10 regulates the cyclin D1 expression level in MCL cells

Since cyclin D1 overexpression is believed to play an important pathogenetic role in MCL (Campo et al., 1999), we assessed if ADAM10 mediates any effects on the expression of cyclin D1 in MCL cell lines. As shown in figure 5.4A, downregulation of ADAM10 using siRNA appreciably reduced the cyclin D1 protein expression level in Jeko-1 and Mino cells. This decrease in the cyclin D1 protein level correlated well with our observations that the *cyclin D1* mRNA levels were significantly reduced 24 hours after the treatment of ADAM10 siRNA (figure 5.4B). Specifically, the *cyclin D1* mRNA levels detected by quantitative RT-PCR were reduced by 21% and 35% in Jeko-1 and Mino cells, respectively. Opposite effects were observed when recombinant ADAM10 (100 ng/ml) was added to SP53 cells (not shown). Using subcellular fractionation, we found that the increase in the cyclin D1 protein expression level was largely attributed to an increase in cyclin D1 in the nuclear fraction (figure 5.4C). We also examined if downregulation of ADAM10 using siRNA modulates a number of other cell-cycle regulators including $p21^{Waf1,}$ $p27^{Kip1}$, p15 and p16; we found that there was an upregulation of $p21^{Waf1}$, an inhibitor of cyclin-dependent kinase 4 (figure 5.4A); the other three proteins showed no appreciable change (not shown).

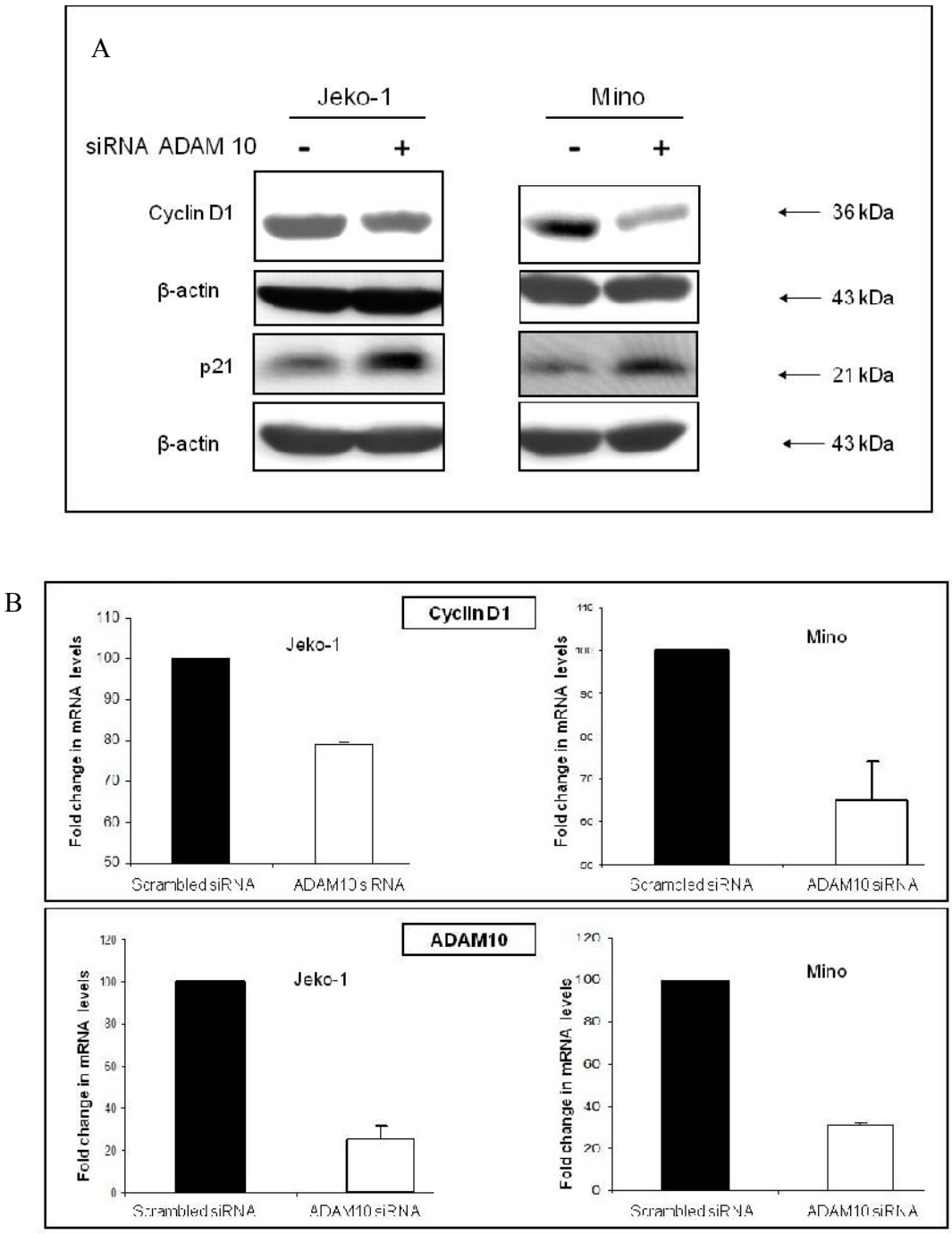


Figure 5.4. ADAM10 regulated cyclin D1 expression in MCL (continued). A) Western blots showed downregulation of cyclin D1 and upregulation of $p21^{Waf1}$ in Jeko-1 and Mino cells following ADAM10 downregulation using siRNA. B) Quantitative RT-PCR showed downregulation of the *cyclin D1* mRNA in Jeko-1 and Mino cells 24 hours after downregulation of ADAM10 using siRNA

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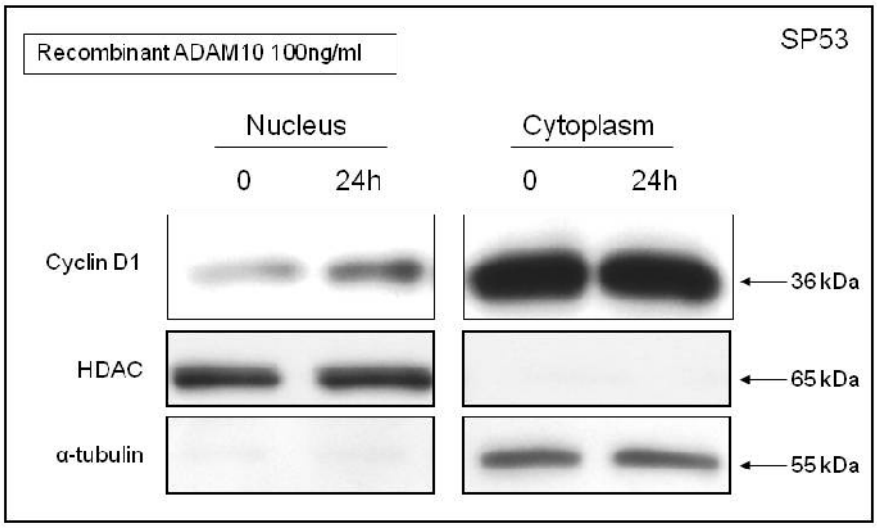
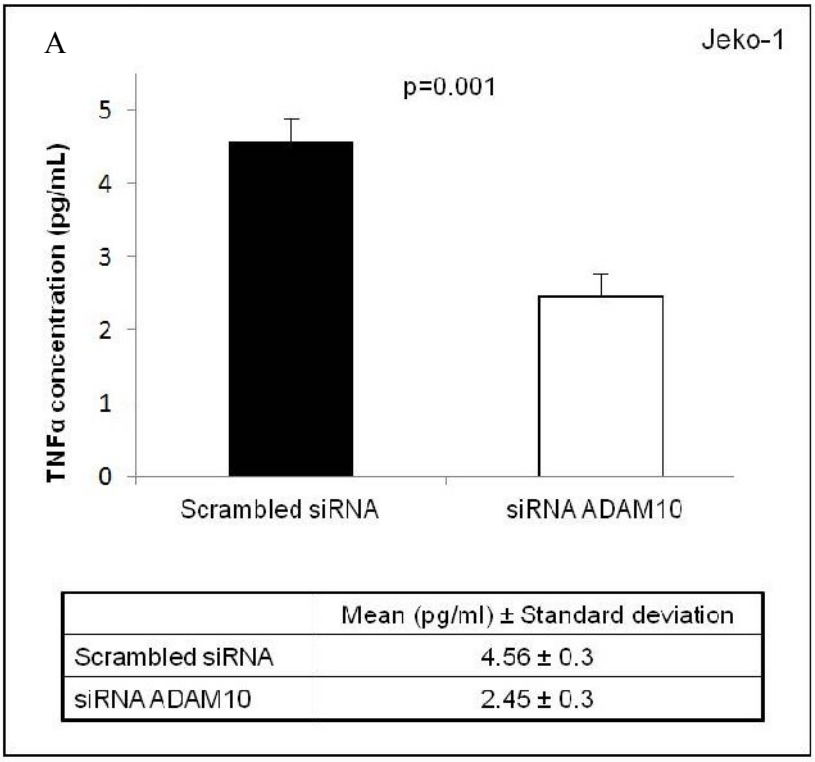


Figure 5.4. ADAM10 regulated cyclin D1 expression in MCL
C) Western blots showed upregulation of the nuclear level of cyclin D1 protein in SP53 cells 24 hours after the addition of human recombinant ADAM10 (100ng/ml); HDAC was used as the loading control for the nuclear extract.

5.3.5. ADAM10 activates the TNFα/NFκB axis

In view of the previous literature that ADAM10 regulates the production of TNFα in murine cell lines and immortalized human cell lines, (Hikita et al., 2009; Mezyk-Kopec et al., 2009) as well as the importance of the TNFα in the biology of MCL (Gelebart et al., 2009; Pham et al., 2003; Shishodia et al., 2005), we hypothesized that ADAM10 may promote the growth of MCL via upregulation of TNFα. In keeping with this concept, we found that downregulation of ADAM10 using siRNA in Jeko-1 cells resulted in a significant decrease in the levels of TNFα present in the culture media ($p=0.001$) (figure 5.5A). Since TNFα is an activator of NFκB (Wullaert et al., 2006), a signaling protein also strongly implicated in the pathogenesis of MCL (Pham et al., 2003), we then assessed the relationship between ADAM10 and this signaling protein. We performed subcellular fractionation experiments in which we found a correlation among the expression levels of the phosphorylated NFκB (active form), total NFκBp65 and ADAM10 in 3 MCL cell lines (supplementary figure 5.3). Specifically, we found that Jeko-1 cells, which have a relatively high level of the active/mature form of ADAM10, carried a higher level of phosphorylated NFκBp65 than SP53 cells, which have a much lower level of the active/mature form of ADAM10. As shown in figure 5.5B, there was a significant downregulation in both the phosphorylated and total NFκBp65 level in Mino and Jeko-1 cells after ADAM10 siRNA treatment. Using subcellular fractionation, we found that the protein level of NFκBp65 in the nuclear fractions was decreased, more so than that in the cytoplasmic fractions (figure 5.5C). Lastly, we assessed the transcriptional activity of NFκB using a commercially available reporter construct. As shown in figure 5.5D, there was significant downregulation of NFκB transcriptional activity following ADAM10 downregulation in Mino cells. In contrast, addition of human recombinant ADAM10 (100 ng/mL) resulted in a significant increase in the NFκB transcriptional activity in these cells (figure 5.5E).



	Mean (pg/ml) ± Standard deviation
Scrambled siRNA	4.56 ± 0.3
siRNA ADAM10	2.45 ± 0.3

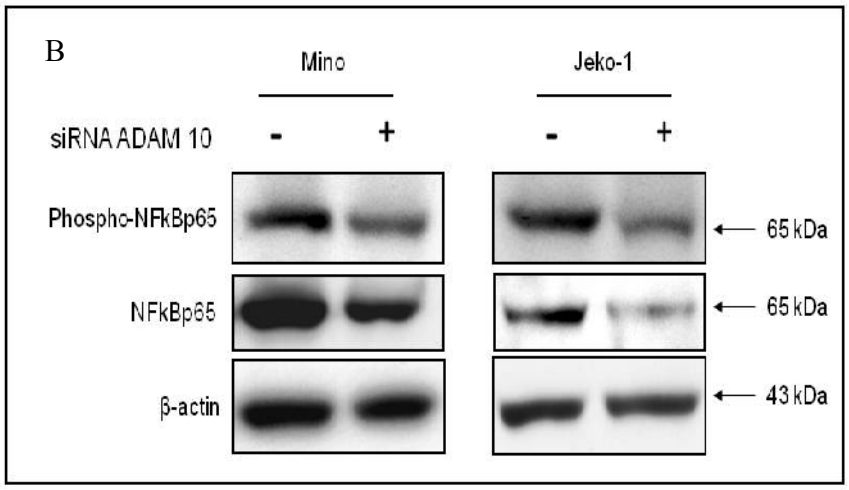


Figure 5.5. ADAM10 activated the TNFα/ NFκB axis (continued). A) Using an ELISA kit, we showed a significant downregulation of TNFα in Jeko-1 cells 48 hours after the transfection of ADAM10 siRNA, as compared to cells transfected with scrambled siRNA. B) Western blots showed a downregulation of phospho-NFκBp65 and total NFκBp65 after ADAM10 downregulation using siRNA, both in the total lysates harvested from Jeko-1 and Mino cells.

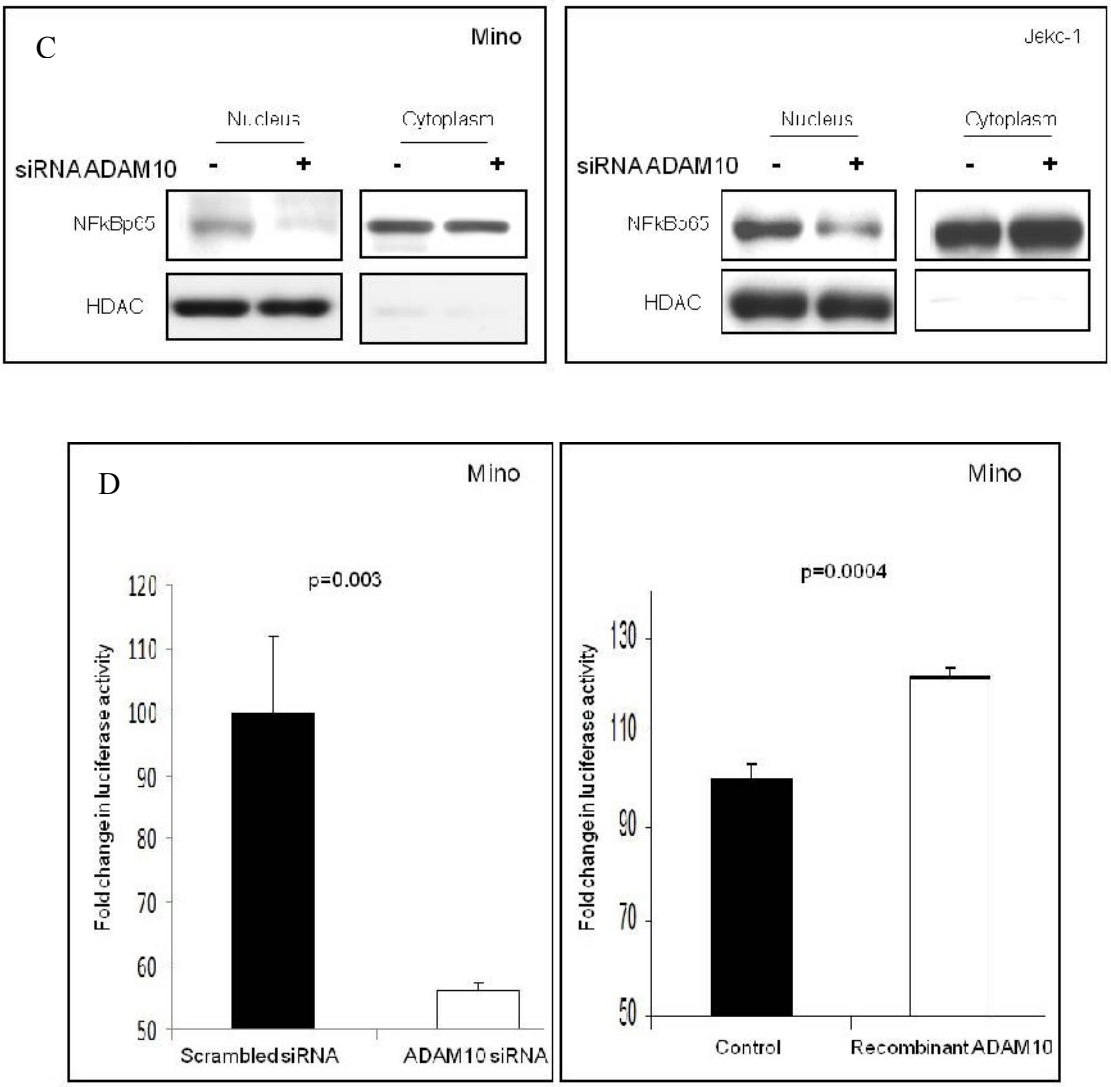
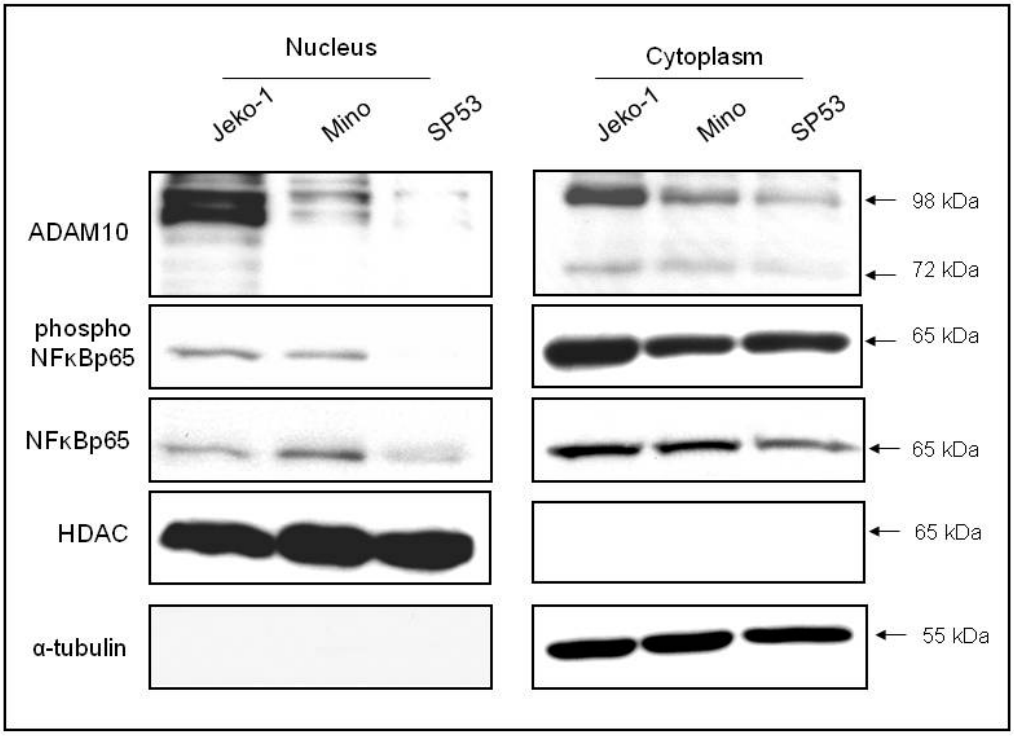


Figure 5.5. ADAM10 activated the TNFα/ NFκB axis. C) Western blots showed a downregulation of total NFκBp65 and in nuclear extract from Jeko-1 and Mino cells. D) Using a NFκB reporter vector, dual luciferase assay, we showed a significant downregulation of the NFκB transcriptional activity in Mino cells after downregulation of ADAM10 using siRNA. E) Using NFκB reporter vector, dual luciferase assay, we showed a significant upregulation of the NFκB transcriptional activity after the addition of human recombinant ADAM10 (100 ng/ml) in Mino cells.



Supplementary figure 5.3. Correlation between ADAM10 and NFkBp65 expression. Western blots of nuclear/cytplasmic fractionation performed in Jeko-1, Mino and SP53 showed a general correlation between the expression level of ADAM10 and those of phosphorylated NFkBp65 and total NFkBp65. Specifically, Jeko-1 cells, which had a higher level of ADAM10 than SP53, carried a higher level of phospho-NFkBp65 than SP53. Of note the precursor form of ADAM10 was found in nuclear and cytoplasmic extracts but the active form was restricted to the cytoplasm.

5.3.6. ADAM10 inhibition enhanced the growth suppressing effect of the proteasome inhibitors MG132 and bortezomib

Proteasome inhibitors have been shown to induce apoptosis and cell-cycle arrest in MCL cells, and these effects are believed to be mediated via an inhibition of the NFκB signaling pathway (McConkey and Zhu, 2008). Since we have shown that ADAM10 mediates its effects via the NFκB pathway, we hypothesized that ADAM10 inhibition may enhance the growth-suppressing effects of proteasome inhibitors such as MG132 in MCL cells. In our initial experiment, we determined that the inhibitory concentration at 50% (i.e. IC50) for MG132 was in the range of 1 μM for Jeko-1 cells (data not shown). Using this concentration of MG132, we found that Jeko-1 cells transfected with ADAM10 siRNA had a significantly more reduction in the number of viable cells, as compared to cells transfected with the scrambled siRNA ($p < 0.0001$, ANOVA) (figure 5.6A). We also performed similar experiments using bortezomib, a proteasome inhibitor currently used in various clinical trials for the treatment of MCL (Strauss et al., 2006). We used 5 nM for this experiment, since cell growth inhibition was approximately 15% at this drug concentration. As shown in figure 5.6B, the combination of bortezomib and ADAM10 siRNA resulted in a significant reduction in number of viable cells, as compared to the use of bortezomib or ADAM10 siRNA alone ($p < 0.0001$, ANOVA).

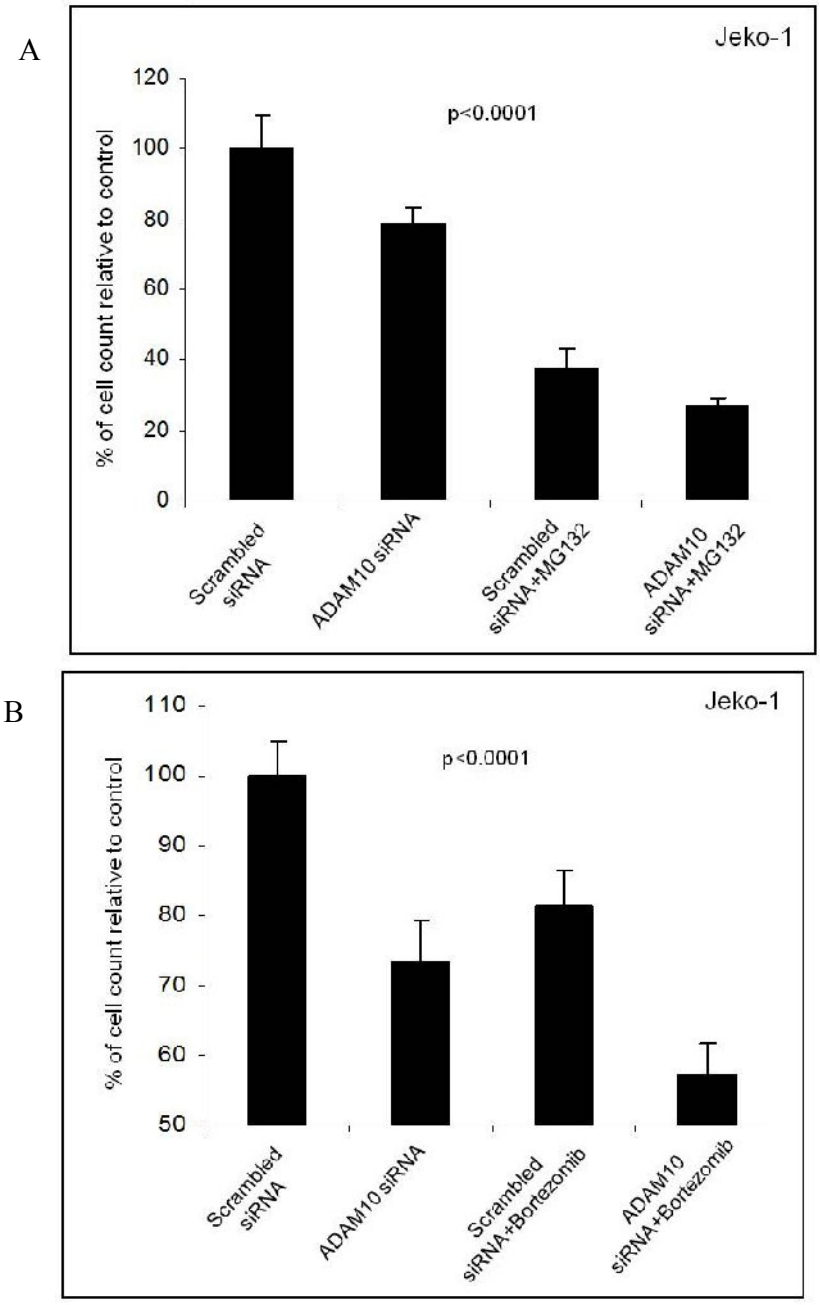


Figure 5.6. ADAM10 inhibition enhanced the growth suppressing effects of proteasome inhibitors bortezomib and MG132 in Jeko-1 cells. A) Jeko-1 cells transfected with either scrambled or ADAM10 siRNA were treated with MG132 (1 μM). At 24 hours after the MG132 treatment, trypan blue exclusion assay was performed and we found that ADAM10 siRNA enhanced the growth suppressing effect of MG132 in Jeko-1 cells ($p < 0.0001$). B) Jeko-1 cells transfected with either scrambled or ADAM10 siRNA were treated with bortezomib (5 nM). At 24 hours after the bortezomib treatment, trypan blue exclusion assay was performed and we found that ADAM10 siRNA enhanced the growth suppressing effect of bortezomib in Jeko-1 cells ($p < 0.0001$, ANOVA).

5.4. DISCUSSION

ADAM10 has recently been implicated in the pathogenesis of several types of malignant solid tumors such as those arising from colon, pancreas, ovary, uterus and the oral cavity (Fogel et al., 2003; Gaida et al.; Gavert et al., 2007; Ko et al., 2007). For the first time, we have demonstrated that constitutive activation of ADAM10 is a highly frequent finding in MCL. Our findings also suggest that ADAM10 is biologically significant in MCL. Specifically, ADAM10 promotes cell-cycle progression and increased cell growth, which are associated with modulations of two important cell-cycle regulators (cyclin D1 and $p21^{waf1}$) and activation of the TNFα/NFκB signaling pathway. While MCL is the focus of this study, we found evidence of constitutive activation of ADAM10 in other types of B-cell non-Hodgkin lymphomas, although the functional significance of ADAM10 in these tumors needs to be further examined.

Regarding the association between ADAM10 and TNFα, we would like to point that, while ADAM10 have been reported previously to be an important sheddase for TNFα, all of these studies were done using murine fibroblasts or other immortalized human cell lines such as the human kidney cell line 293A and human articular chondrocytes (Hikita et al., 2009; Mezyk-Kopec et al., 2009). In this study, inhibition of ADAM10 expression with the use of siRNA led to downregulation of TNFα secretion. To our knowledge, this is the first study directly linking ADAM10 and TNFα in a human cancer model. Furthermore, this is also the first report describing the link between ADAM10 and NFκB. The pathogenetic importance of NFκB in MCL has been previously reported (Pham et al., 2003; Shishodia et al., 2005). Specifically, NFκB appears to confer anti-apoptotic signal in MCL, as blockade of this signaling protein in MCL cells induces downregulation of several anti-apoptotic proteins such as bcl-2 and bcl-$_{xL}$ (Pham et al., 2003). The link between TNFα and NFκB in MCL also has been previously established in one of our previous studies

(Shishodia et al., 2005). Taken together, it appears that ADAM10 upregulates the autocrine production of TNFα, thereby activating the NFκB signaling pathway. Interestingly, although pharmacologic inhibition of NFκB in MCL has been previously shown to result in significant apoptosis in MCL cells (Pham et al., 2003; Shishodia et al., 2005), we did not observe any detectable evidence of apoptosis as a result of ADAM10 knockdown using siRNA. This may be due to the fact that residual NFκB activity is sufficient to prevent the activation of the apoptotic pathway.

In the present study, we have shown that ADAM10 is frequently active in MCL cell lines and tumors, but not in PBMC from healthy donors. Interestingly, in the vast majority of the patient samples (n=12), the active/mature form of ADAM10 was expressed at a higher level than the precursor form of ADAM10. This pattern is in contrast with that of MCL cell lines, in which the precursor form of ADAM10 was expressed at a higher level than the active/mature form of ADAM10. While we cannot provide definitive explanations regarding this discrepancy between cell lines and tumors, we have considered the possibility that the tumor microenvironment may be involved in the regulation of ADAM10.

Our immunohistochemical data revealed that ADAM10 was readily detectable in most MCL tumors examined. This is in contrast with benign mantle zones, which did not show detectable ADAM10 immunoreactivity. These findings suggest that ADAM10 is ‘over-expressed’ in MCL cells, as compared to benign mantle zone cells. Of note, we employed a commercially available, anti-ADAM10 antibody that recognizes this protein regardless of its activation status. The ‘overexpression’ of ADAM10 in MCL tumors *per se* may be biologically important, since one previous report showed that overexpression of ADAM10 in colorectal cancer significantly correlates with a higher clinical stage (Knosel et al., 2005). In this study, we demonstrated that 5 of 5 cases of MCL showing ADAM10

immunoreactivity also expressed the active/mature form of ADAM10. Taken together, it is likely that most MCL tumors overexpress ADAM10 as well as carry constitutive activation of this protein. Regarding our observation that ADAM10 can be localized to the nuclei of MCL cells, we would like to point out that a previous publication suggests that nuclear accumulation of ADAM10 in prostate cancer correlated with a higher Gleason score (Arima et al., 2007). Nevertheless, the biological and/or clinical significance of the nuclear localization of ADAM10 in MCL needs to be further studied.

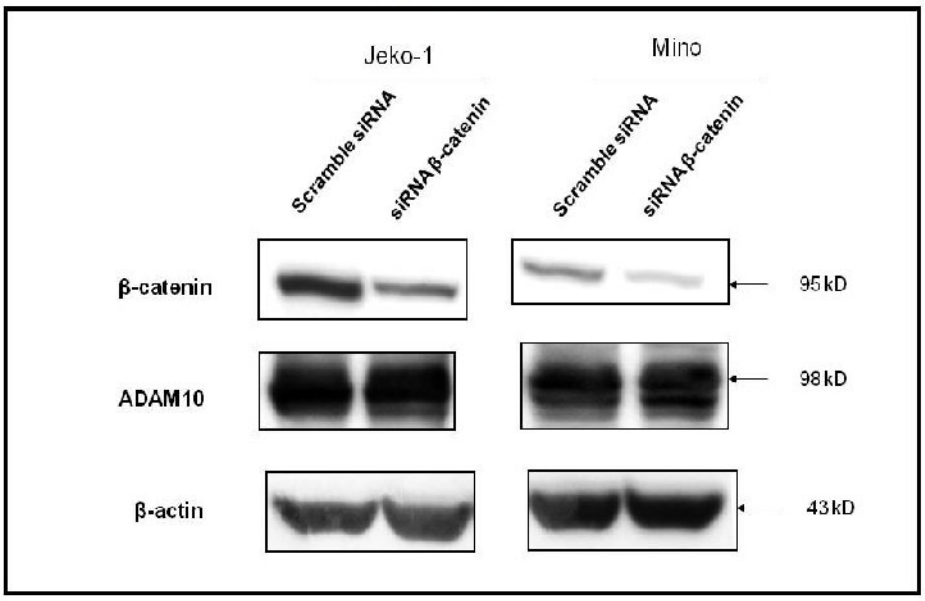
Cyclin D1 overexpression due to t(11;14)(q13;q32), which brings the *cyclin D1(CCND1)* gene under the influence of the enhancer of the *IgH* gene, is considered the hallmark for MCL (Campo et al., 1999). Cyclin D1 has been previously shown to be involved in the regulation of MCL cell proliferation (Klier et al., 2008). In the context of the role of ADAM10 in regulating cell cycle progression in MCL cells, we investigated if ADAM10 regulates the expression of cyclin D1 and other cell cycle-regulatory proteins including $p21^{waf1}$. We observed that ADAM10 downregulation indeed resulted in a substantial downregulation of cyclin D1 and upregulation of $p21^{Waf1}$, which correlated well with cell cycle arrest. Based on our observation that the downregulation of the cyclin D1 transcripts occurred within 24 hours after siRNA treatment, we believe that this change in the cyclin D1 expression is directly due to the downregulation of ADAM10, rather than a consequence to the cell-cycle arrest. In view of the established link between the NFκB pathway and cyclin D1 as well as $p21^{Waf1}$ (Basile et al., 2003; Guttridge et al., 1999), it is possible that ADAM10 modulates the expression of cyclin D1 and $p21^{Waf1}$ via activating the NFκB signaling pathway.

ADAM10 appears to enhance the growth-suppressing effect of bortezomib and MG132, two proteasome inhibitors. This observation is in keeping with

the concept that proteasome inhibitor-induced apoptosis in MCL is at least partly mediated via a suppression of the NFκB signaling pathway (McConkey and Zhu, 2008). Overall, our findings suggest that inhibition of ADAM10 may be a therapeutically useful strategy in treating MCL patients; specifically, inhibition of ADAM10 used in combination with proteasome inhibitors can enhance the overall tumor suppressive effects in MCL. Of note, pharmacologic inhibitors of ADAM10 are available, one of which is being tested in a clinical trial for patients with breast cancer (Duffy et al., 2009).

Different mechanisms have been shown to regulate the ADAM10 protein expression level; for instance, β-catenin has been shown to modulate the expression of ADAM10 in colon cancer (Gavert et al., 2005). Interestingly, β-catenin is known to be activated in a subset of MCL (Gelebart et al., 2008). We tested if blockade of β-catenin using siRNA in MCL cell lines can result in a downregulation of ADAM10; no detectable change was observed (supplementary figure 5.4). Thus, the mechanism by which ADAM10 expression is modulated may well be cell-type specific. Regarding the activation of ADAM10, a number of enzymes are known to activate ADAM10, including proprotein convertase 7 and furin, which remove the prodomain of ADAM10 (Anders et al., 2001). The overexpression and/or aberrant activation of these enzymes in MCL may be involved in the activation of ADAM10. Further studies are needed to investigate the role of these proteins in MCL.

In conclusion, for the first time, our study describes that constitutive activation of ADAM10 is a consistent finding in MCL. We have provided evidence that ADAM10 contributes to the pathogenesis of MCL by activating the TNFα/NFκB signaling pathway. Lastly, we have provided evidence that inhibition of ADAM10 may be a useful approach to enhance the therapeutic effects of other agents (such as proteasome inhibitors) in treating MCL.



Supplementary figure 5.4. No change in ADAM10 protein levels after downregulation of β-catenin. Western blots showed no change in ADAM10 protein levels in Jeko-1 and Mino cells after downregulation of β-catenin using siRNA.

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❖ Chapter 6

CASEIN KINASE 2α PROMOTES CELL CYCLE PROGRESSION AND INCREASES SERINE PHOSPHORYLATION OF NPM-ALK IN ALK-POSITIVE ANAPLASTIC LARGE CELL LYMPHOMA

A version of this chapter will be submitted for publication to *PLoS ONE* Journal:

Armanious H, Gelebart P, Anand M, Lai R. Casein kinase 2α promotes cell cycle progression and increases serine phosphorylation of NPM-ALK in ALK positive anaplastic large cell lymphoma.

6.1. INTRODUCTION

Anaplastic lymphoma kinase positive (ALK^+) anaplastic large cell lymphoma (or ALK^+ALCL) is a specific type non-Hodgkin lymphoma of T/null-cell immunophenotype identified by WHO classification (Jaffe et al., 2004). The expression of NPM-ALK is a result of the reciprocal chromosomal translocation t(2;5)(p23;q35), which leads to the juxtaposition of the nucleophosmin (*NPM*) gene at *5q35* with the *ALK* gene at *2p23* (Morris et al., 1994; Shiota et al., 1994). NPM-ALK has been shown to be oncogenic, and it directly contributes to the pathogenesis of ALK^+ALCL through exerting its constitutively active tyrosine kinase embedded in the ALK portion of the fusion protein (Bischof et al., 1997; Duyster et al., 2001; Fujimoto et al., 1996). NPM-ALK is known to bind and activate a host of cell signaling pathways, including those of JAK/STAT3 (Dien Bard et al., 2009; Zamo et al., 2002), Ras/ERK (Turner et al., 2007) and PI3K/AKT (Bai et al., 2000; Slupianek et al., 2001), all of which are known to regulate important cellular functions such as cell cycle progression and cell survival. Recently, we have reported that β-catenin is transcriptionally active in ALK^+ALCL cells, and this conclusion was supported by its nuclear localization and the results from the TOP/FOP luciferase assay. Also, we found it to be biologically significant in ALK^+ALCL, as down-regulation of β-catenin using siRNA significantly reduced the growth of ALK^+ALCL cells. However, although NPM-ALK downregulation was found to regulate β-catenin transcriptional activity, we found no direct interaction between NPM-ALK and β-catenin. Moreover, we did not detect any downregulation of nuclear protein levels of β-catenin following NPM-ALK downregulation (Anand et al., 2011). Recently, we have newly described that NPM-ALK can be serine phosphorylated and shown for the first time that serine phosphorylation of NPM-ALK was involved in its tumorigenic potential (Wang et al., 2010).

Casein kinase 2 (CK2) is a ubiquitously expressed protein kinase which catalyzes the phosphorylation of serine/threonine residues in proteins (Meggio and Pinna, 2003; Pinna, 2003). CK2 is a tetrameric holoenzyme composed of two catalytic alpha and /or alpha' (α and/or α') subunits and two regulatory beta (β) subunits (Traugh et al., 1990). Both subunits of CK2 enzyme (α and β) have been shown to cause embryonic lethality (Buchou et al., 2003; Lou et al., 2008). CK2α have been shown to be overexpressed in various cancer types as breast, lung and squamous cell carcinoma of the head and neck (Faust et al., 2000; Gapany et al., 1995; Landesman-Bollag et al., 2001). Interestingly, transgenic mice overexpressing CK2 in their lymphocytes develop lymphomas (Seldin and Leder, 1995). Moreover, CK2 has been shown to activate c-myc and promote T cell lymphoma proliferation in mice (Channavajhala and Seldin, 2002). The link between CK2 and WCP was first identified in studies in *Xenopus laevis* embryos as CK2 was shown to modulate dorsal axis formation, a function known to be regulated by Wnt signaling (Dominguez et al., 2004). CK2 was shown to be an important positive regulator of the WCP, as inhibition of CK2 inhibited proliferation of Wnt1 transfected mouse mammary epithelial cells (Seldin et al., 2005; Song et al., 2000). CK2 was also shown to phosphorylate β-catenin at Threonine393, which decreases its affinity for binding to axin (Song et al., 2003; Wu et al., 2009b).

In the present study we investigated the possible cross talk between NPM-ALK the major oncogenic protein in ALK^+ALCL and Wnt pathway and we identified CK2α as a possible link. We also investigated the biological function of CK2α in the biology of ALK^+ALCL.

6.2. MATERIALS AND METHODS

6.2.1. Cell lines and tissue culture

The characteristics of the three ALK^+ALCL cell lines, Karpas 299, SU-DHL-1 and SUPM2, have been previously described (Pulford et al., 1997). All 3 cell lines are negative for the Epstein-Barr virus nuclear antigen. They were grown at 37^0C and 5% CO_2 and maintained in RPMI medium (Sigma-Aldrich, St. Louis, MO). The culture media contain 2 mM L-glutamine and was enriched with 10% fetal bovine serum (Gibco, Carlsbad, CA). Ficoll-Paque (GE Health care, Quebec, Canada) was used to isolate peripheral blood mononuclear cells (PBMC) from healthy donors (n=5). Following PBMC isolation, T-cells were purified using a commercial available kit (Stem Cell Technologies, BC, Canada).

6.2.2. Subcellular protein fractionation, Immunoprecipitation, Western blots and antibodies

For subcellular protein fractionation, we employed a kit purchased from Active Motif (Carlsbad, CA, USA) and followed the manufacturer's instructions. For co-immunoprecipitation, cells were washed in phosphate buffered saline (PBS) and lysed using Cell Lytic Buffer M (Sigma-Aldrich) supplemented with 0.1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), a protease inhibitor mixture (Nacalai Inc., San Diego, CA), and phosphatase inhibitor mixture (Calbiochem, EMD Biosciences, Darmstadt, Germany). After incubating the lysate on ice for 30 min, it was centrifuged at 15,000 X g for 15 min. Two micrograms of the primary antibody was added to 500 µg of protein lysate and rotated overnight at 4 °C. Negative control samples with the primary antibody omitted were included. 50 µl of protein (A/G Plus-agarose) beads (Santa Cruz Biotechnology, Santa Cruz, CA) was added to both the test and control lysates and rocked for 2 h at 4 °C. The beads were then washed 4 times with cold PBS. For co-immunoprecipitation experiments, the final wash was done using cold cell

lysis buffer. For immunoprecipitation experiments, the final wash was done using RIPA buffer. Proteins were then eluted from the beads in 20 µl of SDS protein loading buffer by boiling for 5 min at 100 °C. The complex was then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting. Preparation of cell lysates for Western blots was done as previously described (Gelebart et al., 2008). Antibodies employed in this study included those reactive with CK2α, ALK, phospho tyrosine, tubulin, HDAC and β-actin (Santa Cruz Biotechnology), cyclin D3, cleaved PARP and cleaved caspase 3 (Cell Signaling Technology, Danvers, MA), β-catenin (BD Biosciences Pharmingen, San Diego, CA, USA) and phospho serine (Chemicon, Temecula, CA).

6.2.3. Short interfering RNA (siRNA)

siRNAs for CK2 α/α' and ALK were purchased from (Sigma-Aldrich). Scrambled siRNA was purchased from Dharmacon (Lafayette, CO). Transfection of siRNA was carried out using an electrosquare electroporator, BTX ECM 800 (225V, 8.5ms, 3 pulses) (Holliston, MA, USA). The concentration of siRNA used was 200 pM/1x10^6 cells, and cells were harvested at 48 hours after transfection. Protein levels for either CK2 α/α' and ALK were assessed by Western blot analysis to evaluate the efficiency of inhibition.

6.2.4. Reagents and cell viability

4,5,6,7-tetrabromobenzotriazole (TBB), a pharmacologic inhibitor for CK2 was purchased from Calbiochem. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. A total of 50,000 cells/ ml of culture medium were plated in triplicate and trypan blue (Sigma-Aldrich) exclusion assay was performed every 24 hours for up to 3 days following transfection of CK2 α/α' or scrambled siRNA. In case of TBB or DMSO,

cell count was done after 48 hours of adding the drug. All experiments were performed in triplicates.

6.2.5. Gene expression array analysis of the Wnt pathway

We used the reverse transcription (RT^2) Profiler polymerase chain reaction (PCR) Array Human Wnt Signalling Pathway purchased from SuperArray (Bioscience Frederick, MD). The complete gene list is available on http://www.superarray.com. Total RNA from Karpas 299 was isolated using the TRIZOL Reagent (Invitrogen, Burlington, Ontario, Canada) and measured using the DU1640 Beckman spectrophotometer (Beckman Coulter, Mississauga, ON). First strand cDNA synthesis reaction was performed as follows: 2 µg of extracted RNA was mixed with 10 µL of the SuperArray RT cocktail mix. The products were then incubated at 37°C for 1 hour and heated at 95°C for 5 minutes. Real-time–based SYBR green PCR was performed using an ABI 7900HT instrument (Applied Biosystems, Streetsville, ON) and the following thermal cycling condition was used: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Data analysis and the cycle threshold (CT) values, which were defined as the fractional cycle number at which the fluorescence passes an arbitrarily set threshold, were analyzed using the SDS (version 2.2.2) program (Applied Biosystems). The CT value of each gene was normalized to that of *GAPDH,* which is included in this commercially available kit.

6.2.6. Cell-cycle analysis by flow cytometry

Cells at a concentration of 10^6 cells/ml were prepared 48 hours after TBB treatment. Cells were washed twice with PBS and fixed with 70% cold ethanol for 2 hours. These cells were then subjected to RNase treatment and propidium iodide (PI) staining. DNA content was determined using a FACSCalibur flow cytometer (BD Biosciences). Data acquisition was gated

to exclude cell doublets and the cell-cycle phase distribution was determined using the CellQuest program (20,000 events were counted).

6.2.7. β-catenin transcriptional activity assessed by TOPFlash/FOPFlash

To assess the transcriptional activity of β-catenin, we employed the TOPFlash/FOPFlash luciferase system. This method has been previously described in details (Staal et al., 2002). Karpas 299 was treated for 24 hours with either 50 μM TBB or DMSO then it was transfected with responsive firefly luciferase reporter plasmids, TOPFlash (Millipore, Billerica, MA, USA) or the negative control, FOPFlash (Millipore). After 24 hours of transfection, cells were harvested and cell extracts were prepared using a lysis buffer purchased from Promega (Madison, WI, USA). The firefly luciferase activity and renilla luciferase activity were assessed using the dual luciferase reagent (Promega). Data are reported as means ± standard deviations of three independent experiments, each of which was performed in triplicates.

6.2.8. Statistical analysis

Data are expressed as mean +/- standard derivation. Unless stated otherwise, statistical significance was determined using two-tailed Student's *t*-test and statistical significance was achieved when the p value is <0.05.

6.3. RESULTS

6.3.1. Inhibition of NPM-ALK downregulates CK2α in ALK$^+$ALCL cell lines

To investigate if NPM-ALK is linked to the WCP, we employed WCP-specific oligonucleotide array to evaluate changes in gene expression before and after NPM-ALK was knocked down by the use of siRNA. Karpas 299, an ALK$^+$ALCL cell line was used in this experiment. Triplicate experiments were performed and Student t-test was used for statistical analysis. Of the 83 genes represented on this array, 5 (5.8%) gene showed statistically significant changes. The gene showing one important regulation was CK2α. We confirmed this finding by Western blots. As shown in figure 6.1A, the protein expression of CK2α was readily detectable in all 3 ALK$^+$ALCL cell lines examined, including Karpas 299, SU-DHL-1 and SUPM2. As compared to that of peripheral blood T-cell from healthy donors, the protein levels of CK2α in ALK$^+$ALCL cell lines were generally higher. Of note, SU-DHL-1 expressed both the α form (42 kDa) and α' form (38 kDa) prominently. In contrast, the other two ALK$^+$ALCL cell lines and normal T-cells expressed only low levels of the α' form. To validate the relationship between NPM-ALK and CK2α in ALK$^+$ALCL cells, we found that the protein levels of CK2α were down-regulated in all 3 ALK$^+$ALCL cell lines after NPM-ALK was knockdown by siRNA. While the α form was substantially downregulated in this experiment, no detectable effect was seen on the α' form (figure 6.1B).

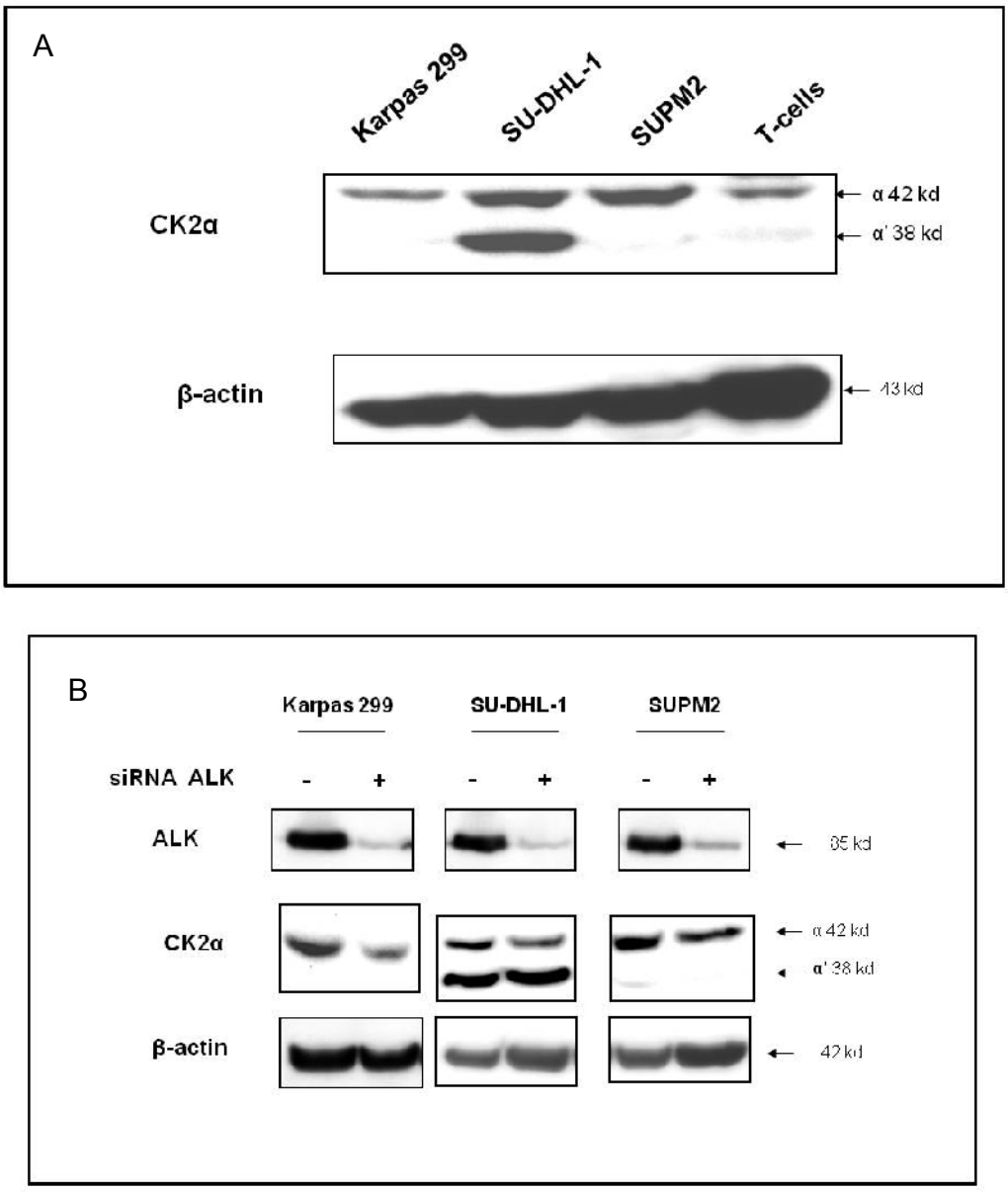


Figure 6.1. Inhibition of NPM-ALK downregulated CK2α in ALK$^+$ALCL cell lines. (A) Western blot revealed the strong expression of CK2α in all 3 ALK$^+$ALCL cell lines compared to T-cells from healthy donors. CK2α showed presence of two forms of catalytic subunit α and α' in SU-DHL-1 cell line. (B) Western blot showed downregulation of CK2α (mainly the α form was downregulated, with no effect on the α' form in SU-DHL-1) in all 3 ALK$^+$ALCL cell lines after ALK downregulation using siRNA.

6.3.2. Inhibition of CK2α results in a significant reduction in cell growth

Next, we sought to examine the biological importance of CK2α in ALK^+ALCL. As shown in figure 6.2A, siRNA pool for CK2α/α' was used in 3 ALK^+ALCL cell lines leading to significant downregulation of protein levels. Three days after the downregulation of CK2α in Karpas 299 and SUPM2, there was a significant decrease in cell growth, as assessed by cell counting after trypan blue staining (p=0.003 and 0.002 for Karpas 299 and SUPM2, respectively) (figure 6.2B). Furthermore, the addition of 4,5,6,7-tetrabromobenzotriazole (TBB), a widely used pharmacological inhibitor of CK2 (Pagano et al., 2008), resulted in a dose-dependent decrease in cell growth as compared to cells treated with DMSO. The inhibitory concentration at 50% (IC 50) was approximately 50 μM for Karpas 299 and SUPM2 (figure 6.2C). To assess the mechanism by which TBB inhibit the growth of ALK^+ALCL *in-vitro*, we performed assays to detect evidence of apoptosis. As shown in figure 6.3A, TBB induced apoptosis, as evidenced by cleaved PARP and caspase 3, at dosages of 100 μM and 150 μM. In contrast, evidence of apoptosis was not detectable with the use of lower dosage (i.e. 25 μM and 50 μM). Similar observations were made with the trypan blue exclusion assay. We then performed cell cycle analysis using flow cytometry on two ALK^+ALCL cell lines Karpas 299 and SUPM2. As shown in figure 6.3B, TBB induced $G_{0/1}$ cell cycle arrest at 50 μM of TBB in keeping with the lack of apoptosis at this dosage (as mentioned above) (p=0.02 for Karpas 299), no substantial sub-$G_{0/1}$ population was detectable. Correlating with TBB-mediated cell cycle arrest, we found that cyclin D3 protein was down-regulated after a treatment of TBB at 50 μM TBB (figure 6.3C).

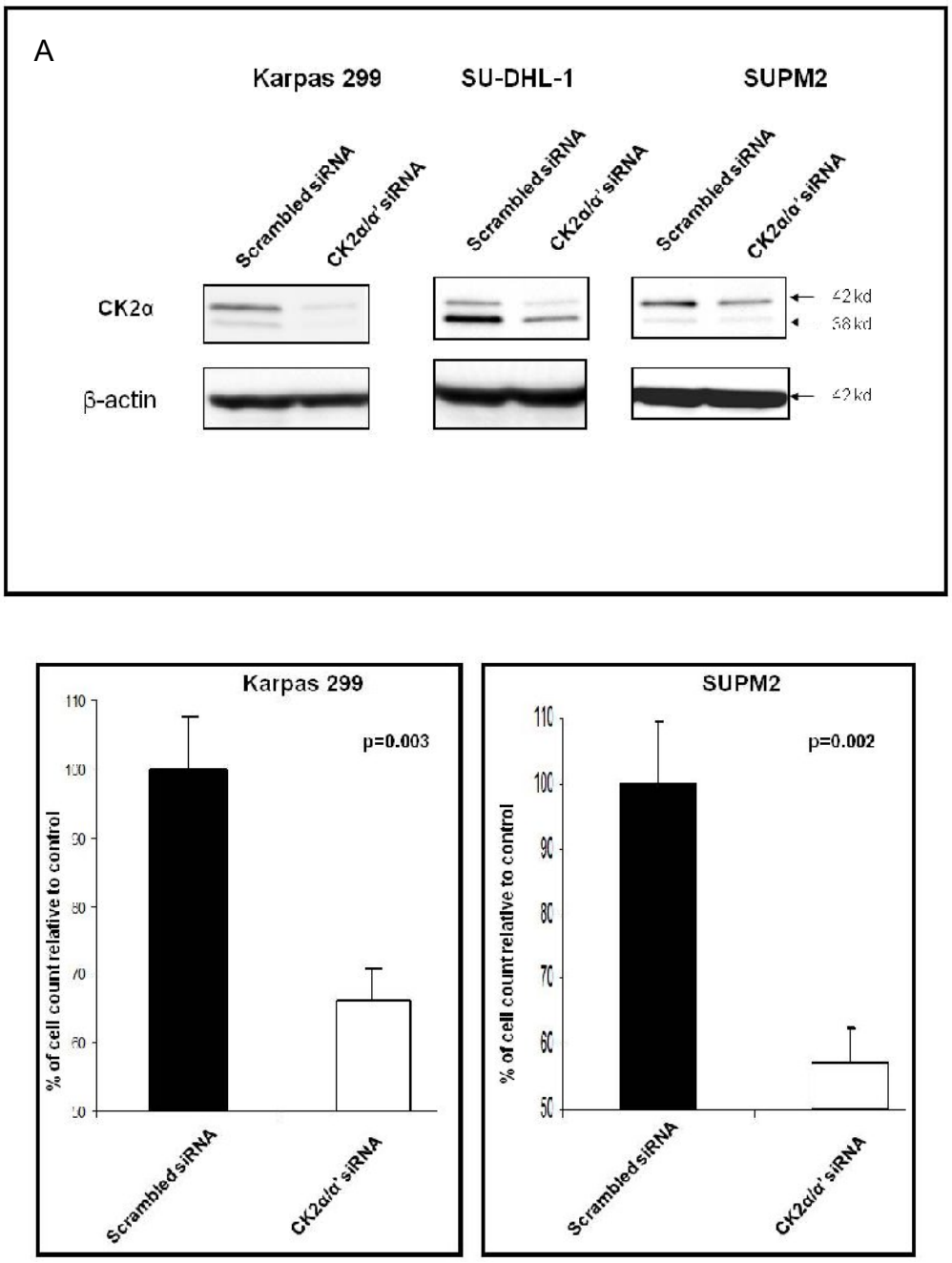


Figure 6.2. Inhibition of CK2α resulted in a significant reduction in cell growth (continued). (A) Western blot showed downregulation of CK2α protein levels in all 3 ALK^+ALCL cell lines after using siRNA CK2α/α'. (B) CK2α knockdown induced significant inhibition of cell growth in Karpas 299 and SUPM2 cells at day 3 after transfection, as assessed by trypan blue exclusion assay (p=0.003 and 0.002, respectively). Three independent experiments were performed.

C

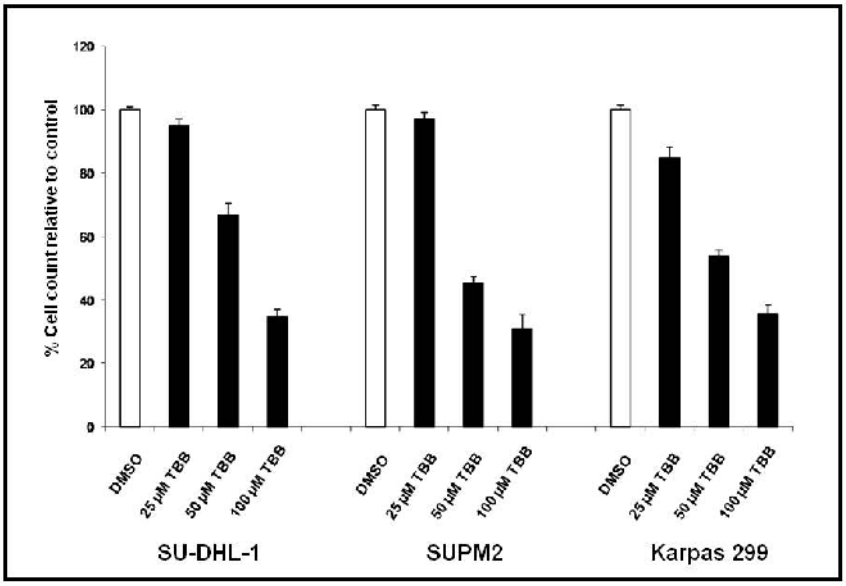
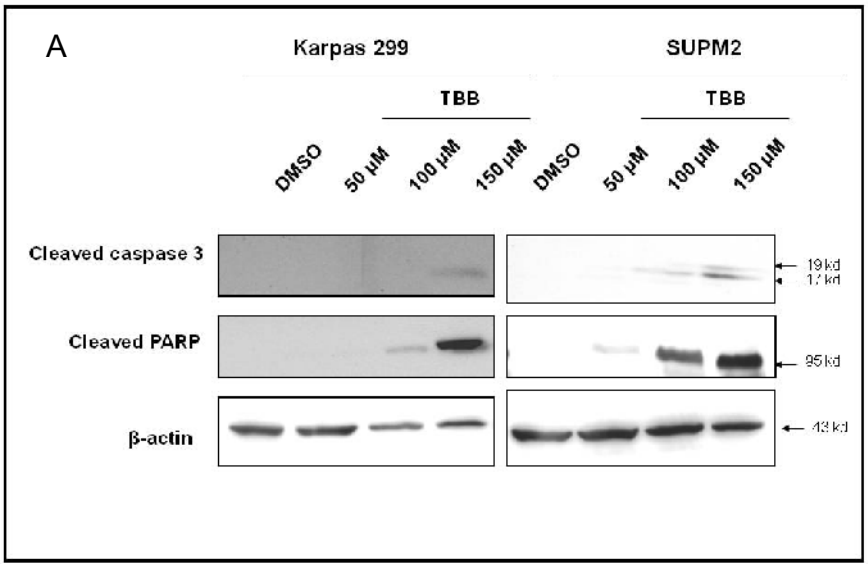


Figure 6.2. Inhibition of CK2α resulted in a significant reduction in cell growth. (C) CK2 inhibition using TBB resulted in dose dependent decrease in cell growth after 48 hours in all 3 ALK^+ALCL cell lines as assessed by trypan blue exclusion assay (IC 50 was around 50 μM in Karpas 299 and SUPM2).



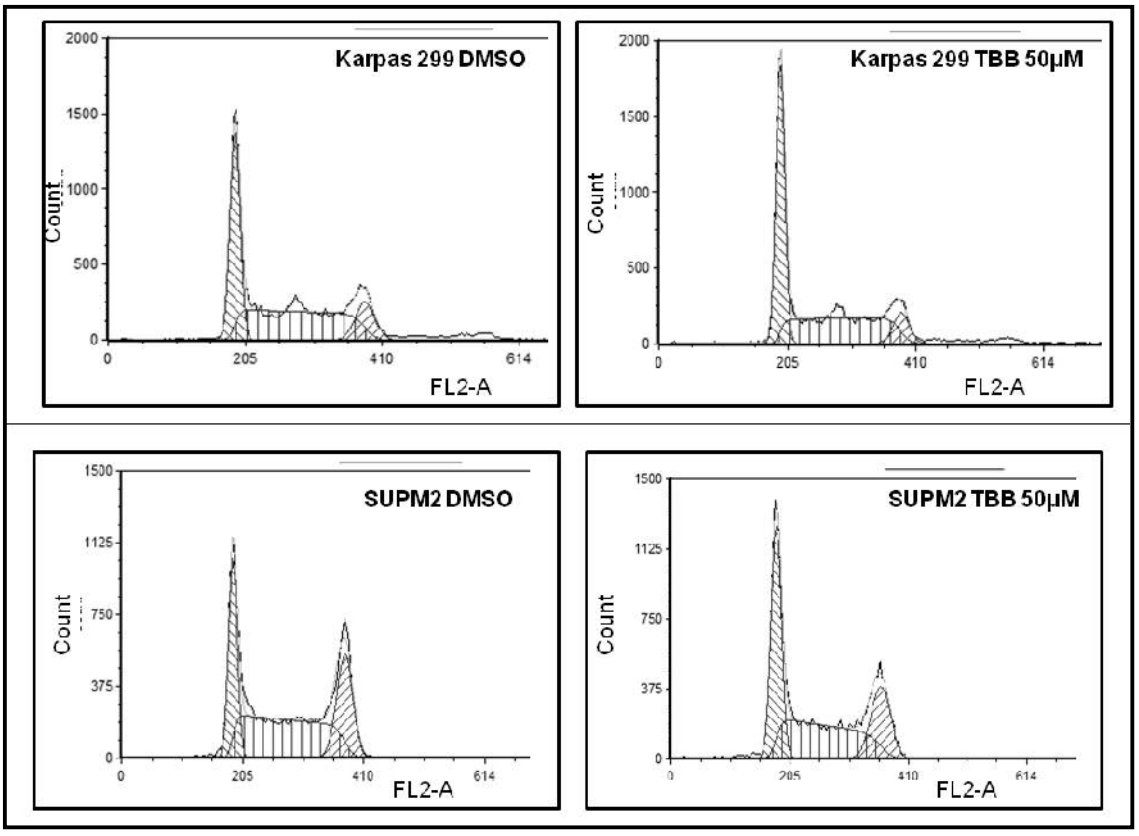


Figure 6.3. TBB resulted in cell cycle arrest (continued). (A) Western blot showed presence of cleaved caspase 3 and PARP only in high doses of TBB (i.e. 100 and 150 μM) not at lower doses i.e. 25 μM and 50 μM in Karpas 299 and SUPM2. (B) Cell cycle analysis by flow cytometry using propidium iodide showed significant $G_{0/1}$ cell cycle arrest in ALK^{+}ALCL cell lines Karpas 299 and SUPM2 following treatment with CK2 inhibitor TBB at a dose of 50 μM, compared to control cells treated with DMSO. Of note, no appreciable increase in the fraction of cells in the sub$G_{0/1}$ was noted.

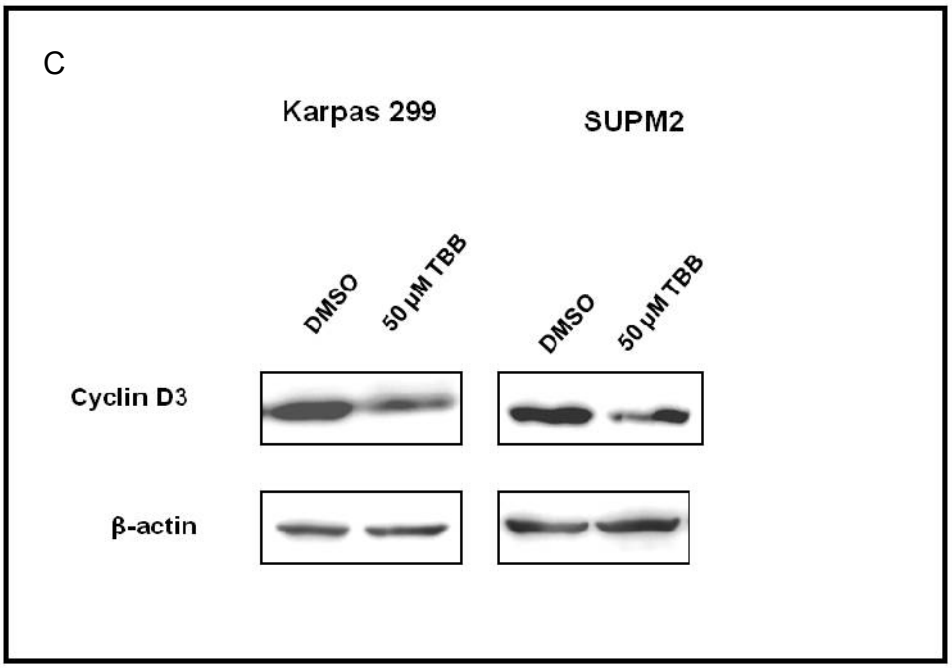


Figure 6.3. TBB resulted in cell cycle arrest. (C) Western blot showed downregulation of cyclin D3 level at dose of 50 μM TBB in Karpas 299 and SUPM2 cells.

6.3.3. CK2 inhibition leads to decreased nuclear localization and transcriptional activity of β-catenin

Addition of TBB at 50 μM for 48 hours resulted in a decrease in the nuclear fraction of β-catenin in Karpas 299 and SUPM2 (figure 6.4A). Using the same experimental approach, we evaluated if TBB induces any change to the transcriptional activity of β-catenin. To achieve it, we employed the TOPFlash/FOPFlash system, as previously described. As shown in figure 6.4B, Karpas 299 cells treated with TBB had a significant downregulation in β-catenin transcriptional activity ($p=0.01$) as compared to cells treated with DMSO.

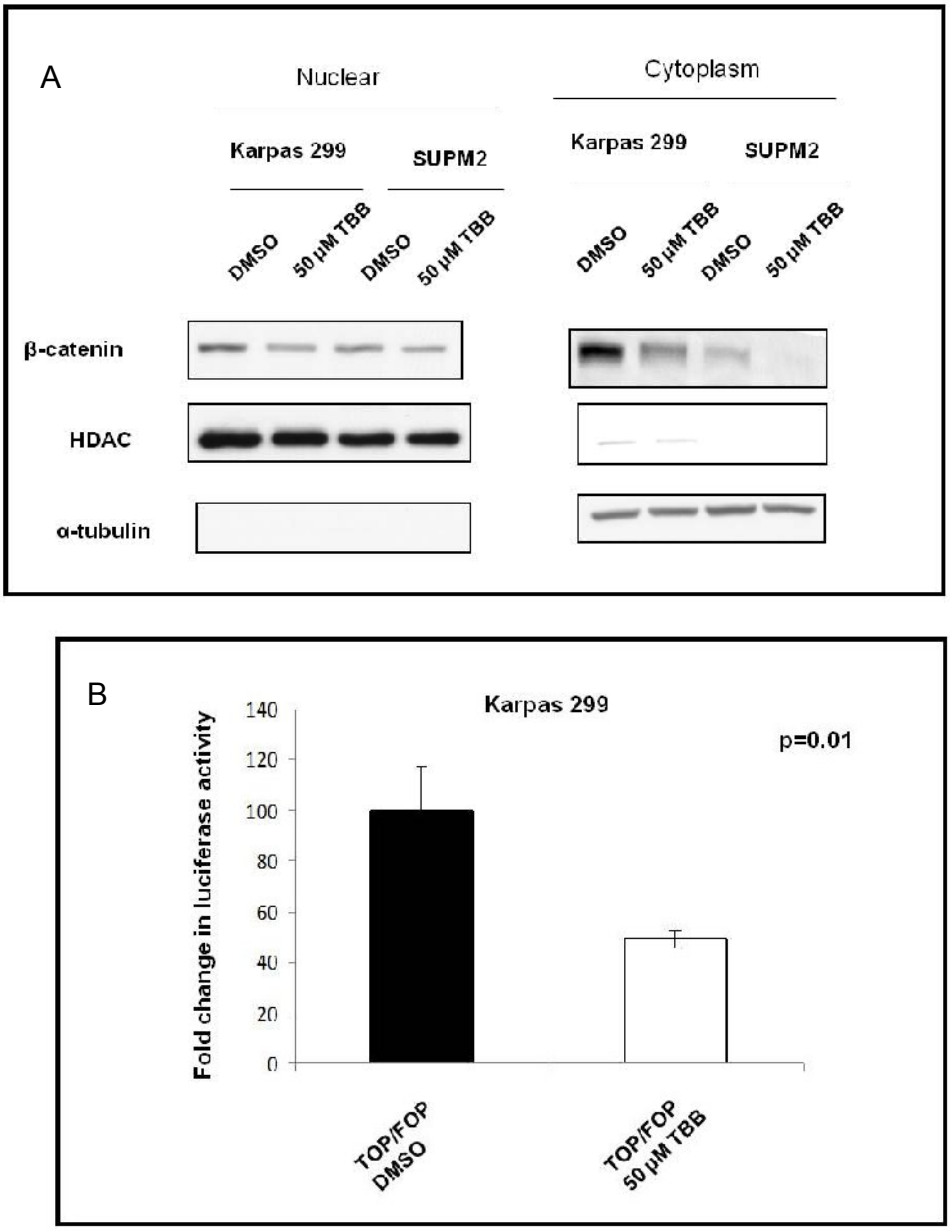


Figure 6.4. CK2 inhibition led to decrease in nuclear levels and transcriptional activity of β-catenin. (A) Western blot revealed decrease in nuclear levels of β-catenin in Karpas 299 and SUPM2 cells after treatment with 50 μM TBB compared to cells treated with DMSO. (B) Using TOPFlash/FOPFlash luciferase system, dual luciferase assay showed a significant downregulation of the β-catenin transcriptional activity (p=0.01) in Karpas 299 cells after treatment with 50 μM TBB compared to cells treated with DMSO.

6.3.4. CK2α inhibition leads to decrease NPM-ALK serine phosphorylation

In view of the importance of NPM-ALK in ALK^+ALCL, we asked if CK2α may modulate the function and/or structure of NPM-ALK. First, we performed co-immunoprecipitation experiment, and we identified evidence of physical interaction between NPM-ALK and CK2α (figure 6.5A). We next sought if CK2α regulates the tyrosine phosphorylation of NPM-ALK as it was shown that CK2 can also mediate tyrosine phosphorylation (Vilk et al., 2008). To achieve this goal, we assessed the level of tyrosine phosphorylation of NPM-ALK using immunoprecipitation and a phospho-tyrosine specific antibody. No detectable difference was found before and after the addition siRNA targeted to CK2α. Since we recently reported that there is serine phosphorylation of NPM-ALK, and serine phosphorylation of NPM-ALK enhances its tumorgenicity (Wang et al., 2011), we investigated if CK2α modulates this property. As shown in figure 6.6B, knockdown of CK2α using resulted in a significant reduction in the level of serine phosphorylation, by 60% and 20% in SU-DHL-1 and SUPM2 cells, respectively.

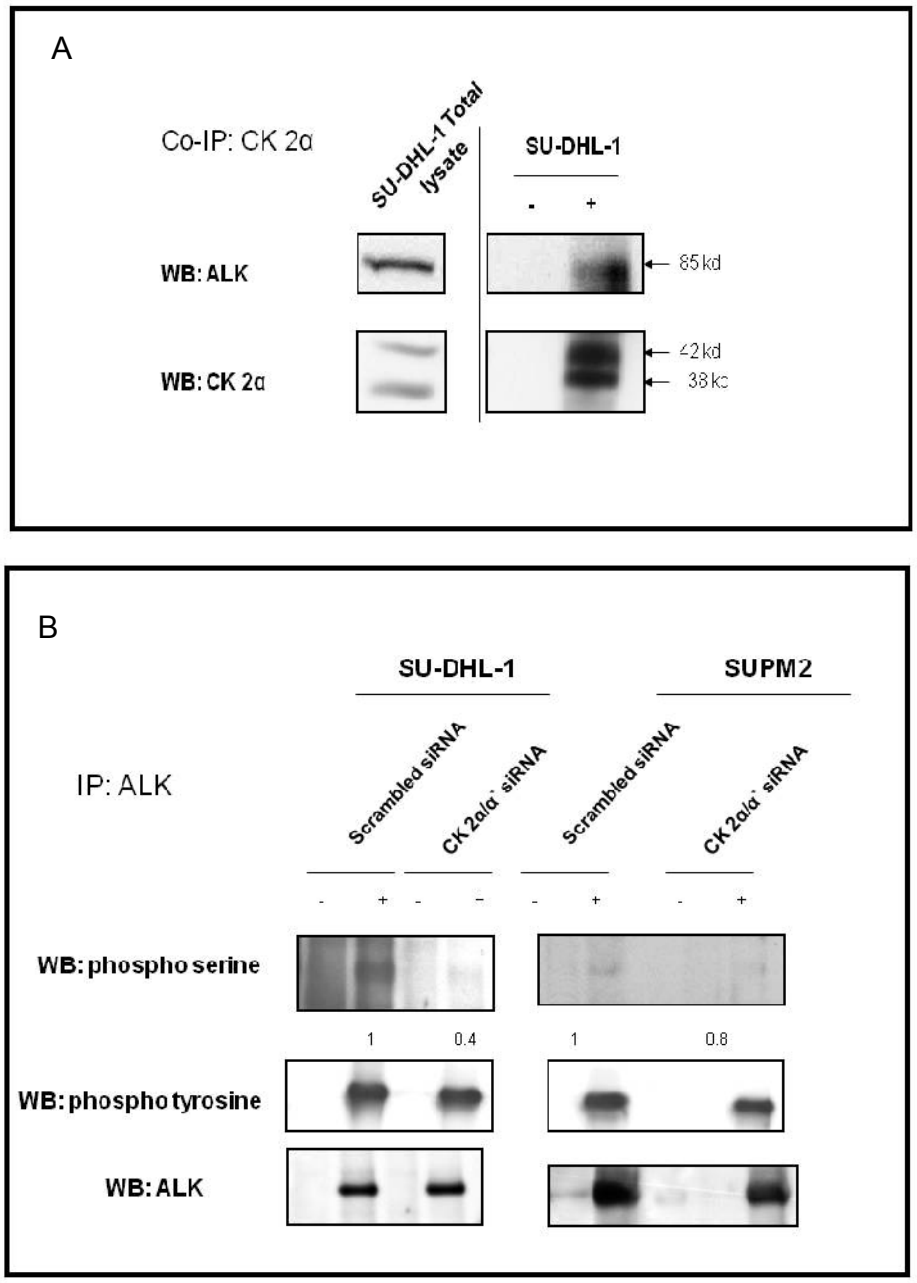


Figure 6.5. CK2α inhibition led to decrease NPM-ALK serine phosphorylation. (A) Western blot showed co-immunoprecipitation using CK2α antibody with NPM-ALK, '+' denotes experiments in which anti CK2α antibody was used for immunoprecipitation and '-' denotes experiments in which the CK2α antibody was omitted. (B) Western blot showed immunoprecipitation using ALK antibody after knockdown of CK2α using siRNA CK2α/α' and blotting with anti phosphoserine antibody showing decrease in serine phosphorylation of NPM- ALK in SU-DHL-1 and SUPM2 cell lines (about 60% and 20% in SU-DHL-1 and SUPM2 respectively). Of note the tyrosine phosphorylation of NPM- ALK did not show any change.

6.4. DISCUSSION

One of our recent studies revealed the constitutive activation of β-catenin in ALK^+ALCL cells (Anand et al., 2011). We found that downregulation of NPM-ALK was able to modulate the transcriptional activity of β-catenin without having an effect on its protein levels, localization and tyrosine phosphorylation status. In order to investigate possible mechanism of regulation of the WCP by the oncoprotein NPM-ALK, we performed knockdown of NPM-ALK, using siRNA, and checked for changes in different Wnt genes using Wnt oligonucleotide array. Specifically, we found that knockdown of NPM-ALK in ALK^+ALCL cells resulted in significant modulations of the expression levels of multiple genes involved in the WCP, of which CK2α was found to be one of the genes affected most dramatically. CK2 is a serine/threonine kinase that has biologic importance as its disruption causes lethality in mouse embryos (Buchou et al., 2003; Lou et al., 2008). Furthermore, CK2 have been identified as a positive regulator of the WCP as it phosphorylates some of its key members like β-catenin (Song et al., 2003; Willert et al., 1997; Wu et al., 2009b).

In addition to the fact that *CK2α* represents the gene which is most affected by the knockdown of NPM-ALK, our choosing of CK2α as the focus of this study is based on its oncogenic importance demonstrated previously in other types of cancer (Ruzzene and Pinna, 2010). Specifically, it has been reported that CK2 is highly expressed in various types of solid tumors, including carcinomas arising from the breast, lungs and the head and neck region (Daya-Makin et al., 1994; Faust et al., 2000; Gapany et al., 1995; Landesman-Bollag et al., 2001). In addition to its role in the WCP (Seldin et al., 2005), CK2 also has been shown to modulate other signaling pathways including those involving Akt/PI3K, c-Myc and NFκB (Channavajhala and Seldin, 2002; Di Maira et al., 2005; McElhinny

et al., 1996). Experiments involving the use of siRNA to knockdown CK2α have provided support that CK2α indeed contributes to the constitutive activation of β-catenin in ALK^+ALCL cells. However, as multiple genes involved in the WCP were implicated, it is highly likely that the cross talk between the WCP and NPM-ALK is established based on multiple interactions at various levels of the WCP.

Interestingly, a study just came out showing that CK2 associate with and phosphorylate JAK tyrosine kinases, this would be of interest as JAK/STAT axis is known to be activated in ALK^+ALCL (Zheng et al., 2011). Of note, CK2 was not previously identified as a NPM-ALK interacting protein in various proteomics studies, including one performed by our research group (Wu et al., 2009a). This discrepancy may be related to the use of different methodologies that carry different sensitivities for various types of protein. Nevertheless, the interaction between CK2α and NPM-ALK correlates with our finding that CK2α appears to alter serine phosphorylation of NPM-ALK. As mentioned, our group recently reported the evidence of phosphorylation of 3 specific serine residues in NPM-ALK; importantly, mutations of these sites that abrogate serine phosphorylation resulted in a significant decrease in the oncogenic potential of NPM-ALK (Wang et al., 2011). However, using colony formation assay after downregulation of CK2α using siRNA in ALK^+ALCL cell lines we did not find significant change in tumorigenecity of cells (data not shown), which could be explained that CK2α is not the only kinase responsible for the serine phosphorylation of NPM-ALK and that other kinases are also implicated.

How NPM-ALK promotes an increase in the protein level of CK2α is unclear. As the STAT3 signaling is probably the most important signaling pathway implicated in the pathogenesis of ALK^+ALCL (Zamo et al., 2002),

we investigated if knockdown of STAT3 can result in a downregulation of CK2α downregulation; however, we did not find any detectable change in CK2α (data not shown). Thus, the exact mechanism by which NPM-ALK affects the CK2α protein level still needs to be further investigated.

In view of the importance of CK2 in cancer, it has been identified as a potential therapeutic target (Bortolato et al., 2008). A recent study showed that pharmacologic inhibition of CK2 (such as TBB) can induce apoptosis in chronic lymphocytic leukemia cells, without significant effect on normal B and T lymphocytes (Martins et al., 2010). The same study emphasizes the relative selectivity of CK2 inhibitors towards neoplastic T-cells as compared to the normal T-cells, and this point carries important therapeutic implications for ALK^+ALCL. Regarding the biologic effect of CK2 inhibition in ALK^+ALCL, to our knowledge, there is only one previous study which used Ellagic acid to inhibit CK2. In the aforementioned study the authors did a virtual screening experiment targeting the ATP binding site of CK2 using different databases, they used a panel of ALCL cell lines both ALK+ and ALK- cell lines to test for the efficacy of identified compounds (Cozza et al., 2006).

In conclusion, our data revealed a novel functional link between CK2 and NPM-ALK, and a positive feedback loop between the WCP and NPM-ALK with CK2α as an important link between the two pathways. Targeting CK2 in ALK^+ALCL may prove to be of clinical importance.

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❖ Chapter 7

Discussion

7.1. Thesis overview

In this thesis, I have extensively evaluated the different mechanisms of cross talk between WCP and various oncogenic networks in different cancer models. I identified novel cross talk between WCP and other signalling pathways implicated in cancer pathogenesis. Previous studies demonstrated the aberrant activation of the WCP in various cancer models including breast cancer and hematopoietic malignancies (Clevers, 2006). However, the dynamic interaction between WCP and various signalling molecules implicated in cancer has not been fully elucidated. I hypothesized the existence of cross talk between various components of the WCP and various oncogenic signalling pathways in lymphoid and solid tumors.

As shown in figure 7.1 the interaction between different WCP members and 4 signalling molecules the PI3K/Akt , STAT3, ADAM10 and NPM-ALK were studied. I found existence of different models of cross talk between WCP members and different oncogenic molecules. Through identifying the cross talk between WCP components and various signalling pathways, we understand the complexity of signalling in cancer and we are warranted to develop combination therapies which will be more beneficial in targeting various signalling pathways.

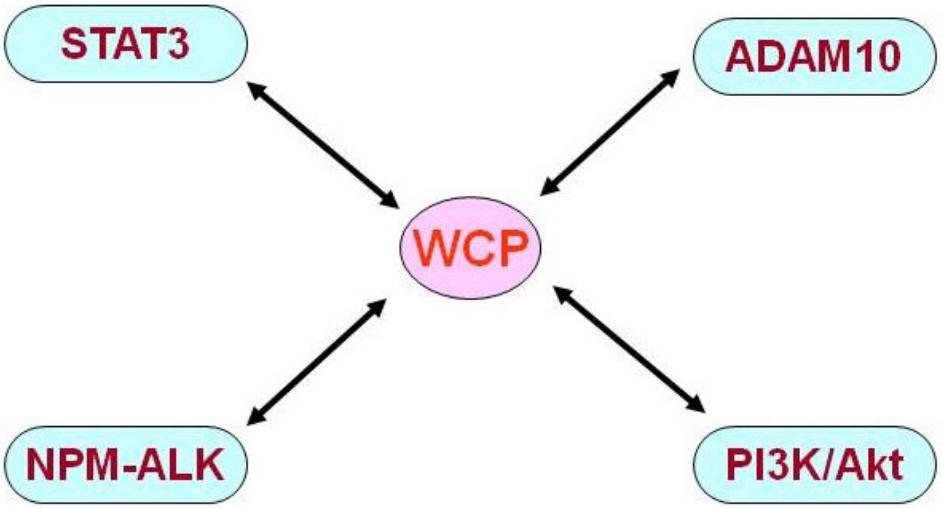

Figure 7.1. WCP cross talking with oncogenic networks in cancer

7.2. WCP acts in a non-linear fashion in different cancer models

Historically, most of the studies of the WCP focused on WCP signalling as being functioning in a linear fashion (Klaus and Birchmeier, 2008). However, in my studies I showed that various members of the WCP act in a linear manner as well as acting in a horizontal manner interacting with other proteins not included in the WCP.

In chapter 2 and 3, I tried to identify whether WCP activation was the main contributing event for GSK-3β inactivation *in vivo* in two cancer models MCL and breast cancer. The decision to use pGSK-3β (the inactive form of GSK-3β) as a marker in my studies came for various reasons: First, the antibody used in my immunohistochemical studies was totally novel and was not used in any *in vivo* studies before, I had to do all the optimization for it. Second, until now β-catenin antibodies are the only accepted marker used for *in vivo* studies as a marker for WCP activation in patient samples. Third, although pGSK-3β have been linked to WCP activation, nevertheless it have also been linked to other signalling pathways mainly PI3K/Akt activation (Pap and Cooper, 1998). In both chapter 2 and 3 I found evidence of inactivated GSK-3β (pGSK-3β) in both breast cancer and MCL (47.2% and 67.5% respectively). In chapter 2, I found that in breast cancer, the expression of pGSK-3β *in vivo* correlated with the activation of PI3K/Akt pathway. On the other hand in chapter 3, I found that in MCL, the expression of pGSK-3β *in vivo* correlated with the activation of the WCP. However, in both studies inactivated GSK-3β (pGSK-3β) correlated with a poor outcome in patients, which points towards the importance of pGSK-3β in cancer biology regardless of the event initiating its inactivation. In these studies I presented the non-linear interaction between WCP and PI3K/Akt pathway with pGSK-3β acting as a hub between the two pathways.

In chapter 4, I showed that β-catenin, the main player in the WCP, is transcriptionally regulated by STAT3. I decided to study this interaction in breast cancer model as both STAT3 and WCP was shown to be activated in a subset of breast cancer (Kunigal et al., 2009; Lin et al., 2000). This study clearly shows the non-linear interaction of the WCP member β-catenin acting as hub molecule in both STAT3 and WCP. This finding is also of interest as a recently published study showed that STAT3 can be transcriptionally regulated by β-catenin, which may suggest the presence of a possible feedback loop between the two pathways (Yan et al., 2008).

In chapter 5, the aim at the start of this study was to elucidate the possible cross talk between ADAM10 and β-catenin. The background rationale behind this aim was; first, ADAM10 was shown in colon cancer to be a target gene of β-catenin/TCF signalling as *ADAM10* promoter carried TCF/LEF binding sites (Gavert et al., 2007). Second, β-catenin itself was shown to be regulated by ADAM10 as cleaving of cadherin by ADAM10 led to release of membranous β-catenin and increase in its nuclear translocation (Maretzky et al., 2005; Reiss et al., 2005). At the beginning, I decided to use MCL cancer model for investigating this possible dual interaction between ADAM10 and β-catenin as in a recent study our lab was the first to report the activation of WCP in MCL (Gelebart et al., 2008). Interestingly, the first goal of the study, which is detecting presence and activation of ADAM10, was achieved, as I found ADAM10 to be activated in MCL cell lines and patients' samples examined. The second goal of my study was to elucidate the dual interaction between β-catenin and ADAM10 in MCL. However, using siRNA to inhibit β-catenin level I did not observe any change in ADAM10 protein or mRNA levels, similarly using siRNA to inhibit ADAM10 I did not detect and change in nuclear β-catenin

levels or its transcriptional activity. Accordingly, I could not detect in this study any cross talk between WCP and ADAM10.

In chapter 6, I reported the activation of the Wnt pathway in ALK^{+}ALCL and its cross talk with the oncogenic protein NPM-ALK. The cross talk was found to be at the level of CK2α where downregulation of ALK using siRNA led to downregulation of CK2α protein levels and the downregulation of CK2α led to decrease in serine phosphorylation of NPM-ALK. In this study WCP was shown to interact with NPM-ALK an oncogenic tyrosine kinase known for its multiple effectors and binding partners (Fujimoto et al., 1996). This study shows CK2α as a hub in this interaction.

7.3. Models of cross talk between WCP members and other signalling pathways

As discussed in chapter 1, signalling pathways are said to be cross talking if they are connected in at least one of three ways (Figure 7.2) (Guo and Wang, 2009). Through my studies in this thesis I represented examples of each of these models of cross talk. In chapter 2 and 3 I showed pGSK-3β as a hub where one pathway i.e. PI3K/Akt can modulate a key molecule (pGSK-3β in this case) of the other pathway i.e. WCP. In chapter 4, I showed β-catenin acting as a hub between WCP and STAT3 where the component of one pathway i.e. β-catenin as member in the WCP is the transcriptional target of the other pathway i.e.STAT3. In chapter 6, I showed a third model of cross talk where CK2α a component of the WCP physically interacted with another signalling molecule i.e. NPM-ALK. These different models of cross talk shows the diversity by which WCP members are cross talking in non-linear fashion with other pathways in different cancer models.

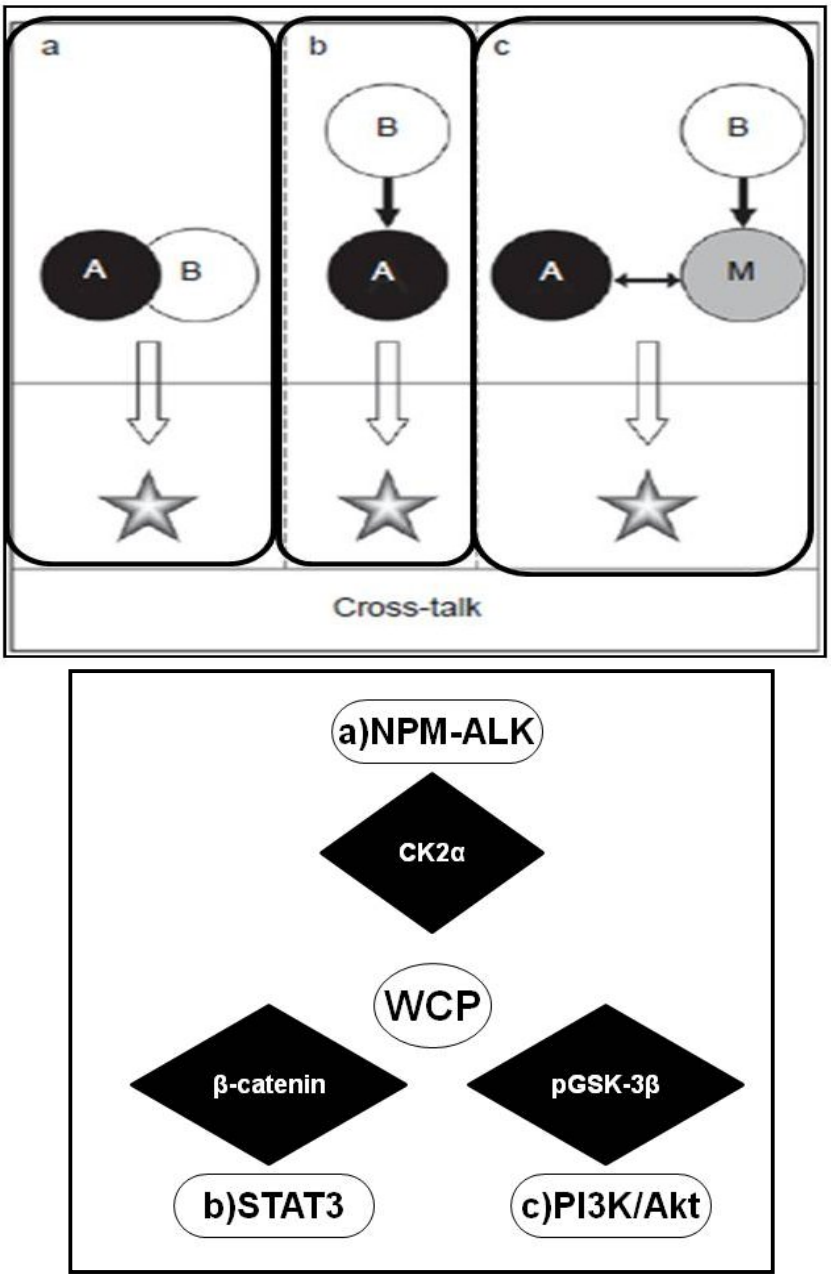

Figure 7.2. Summary of different models of cross talk studied in the current thesis. A) WCP member CK2α physically interacts with NPM-ALK. B) WCP member β-catenin is the transcriptional target for STAT3. C) WCP member pGSK-3β is modulated by the PI3K/Akt pathway.

7.4. Biologic importance of WCP members involved in the cross talk

In all my studies I have demonstrated biologic importance of different WCP members involved in the cross talk. In chapter 2 and 3, inactivated GSK-3β (pGSK-3β) correlated with a poor outcome in patients both in breast cancer and MCL which points towards the possibility of using it as a prognostic marker. In chapter 4, I demonstrated the biologic importance of β-catenin in breast cancer as inhibition of β-catenin using siRNA led to decrease in cell growth. In chapter 6, I showed the importance of CK2α in the biology of ALK^{+}ALCL as its inhibition caused decrease in cell growth and cell cycle arrest. Given the biologic importance shown of all the WCP members acting as a hub for interaction with other pathways, this further emphasises the importance of identifying these hubs as therapeutic targets.

7.5. Limitations and future studies

The different studies in this thesis each have its own limitations and possible future directions. In chapter 2, the limited number of patients' sample i.e. 72 patients of breast cancer, hindered the statistical analysis of certain categories. Specifically, in this cohort the number of ER negative patients was 22 cases and although they had a huge impact in the survival data I could not perform correlational statistical analysis of immunohistochemical markers in this group due to small sample size. Accordingly, future studies can be done using larger cohort of breast cancer patient samples, especially the ones representing various molecular subtypes of breast cancer. Also, it will be of interest to plan further studies to identify the implication of GSK-3β inactivation in patient treatment. For instance, patients could be stratified into two groups according to their pGSK-3β status. Given that the positive group will have possible activation of pAkt pathway, small molecule inhibitors targeting

pAkt can be used in positive versus negative groups in combination with conventional chemotherapy. According to this hypothesis the ones with positive pGSK-3β will possibly benefit from treatment with pAkt inhibitors in contrast to the negative ones.

In chapter 2 and 3 in this thesis I identified that inactivated GSK-3β (pGSK-3β) correlated with a poor outcome in patients. This finding should warrant caution in using new small molecule inhibitors developed to inhibit GSK-3β as they could be a double edged sword. While GSK-3β inhibitors may have beneficial effect in treating diseases as Alzheimer disease and diabetes, however they may carry a potential hazard for cancer development (Phukan et al., 2010). Further studies on the effect of these newly developed small molecule inhibitors of GSK-3β in cancer are warranted.

In chapter 4, I demonstrated a novel mechanism of regulation of β-catenin by STAT3 in breast cancer. As β-catenin is the main player in the WCP, so regulation of its transcriptional and protein levels would consequently have an effect on the WCP signalling. In this study I identified a subset of breast cancer which has activation of both STAT3 and β-catenin. This subset of patients would greatly benefit from the combination therapy between STAT3 inhibitors and small molecule inhibitors of β-catenin. Of note, this study was conducted in the context of breast cancer; it would be tempting to see whether this interaction is occurring in normal mammary cells. Furthermore, the investigation of the cross talk between the WCP and STAT3 carries potential interest especially in field of ES cell research as both pathways are implicated in maintaining self renewal in murine stem cells (Okita and Yamanaka, 2006).

In chapter 5, the aim at the start of this study was to elucidate the possible cross talk between ADAM10 and β-catenin. However, although I did not detect any cross talk between ADAM10 and β-catenin in this cancer model i.e. MCL; this does not totally exclude the existence of this cross talk in other cancer models. In fact, the discovery of ADAM10 regulation by β-catenin was made in CRC model which represent the paradigm of WCP activation. Moreover, activation of different target genes of the Wnt pathways have been suggested to be cell type specific, as not all genes will be activated in all cell types, rather than specific genes are activated in specific cell types (Logan and Nusse, 2004). Importantly, a recently published study showed that SFRPs, which are known as Wnt inhibitors, can bind to and inactivate ADAM10 (Esteve et al., 2011). Accordingly, the next stage of this research will be to further evaluate the relationship between ADAM10 and other components of the WCP in different cancer models. Also, it would be of interest to study the possible cross talk between WCP and TNFα/NFκB pathway with ADAM10 acting as a hub between the two pathways, especially that both pathways (i.e. TNFα/NFκB and WCP) and ADAM10 are implicated in cancer pathogenesis.

In chapter 6, I reported the activation of the Wnt pathway in ALK^+ALCL and its cross talk with the oncogenic protein NPM-ALK. The Wnt oligonucleotide used in this study, showed expression of other WCP members besides CK2α, among them were Dvl2, Dvl3 and Wnt3a (unpublished data). These various Wnt members could be studied each separately to find their biologic significance in ALK^+ALCL. It would also be of interest to study the cross talk of WCP with other oncogenic tyrosine kinases implicated in cancer.

7.6. Closing remarks

Overall, this thesis provided insight into the novel non-linear interactions of the WCP in cancer. To this point, I have identified novel important cross talk between WCP and oncogenic networks in different cancer models i.e. solid and hematopoietic tumors. In the first and second *in vivo* studies I conducted I showed that GSK-3β inactivation is likely attributed to WCP in MCL, however it is likely contributed to PI3K/Akt in breast cancer. Furthermore, I identified the clinical importance of this event in both cancer models. In the third study I conducted I showed crosstalk between WCP and STAT3 at the level of β-catenin, being a transcriptional target for both pathways. In the fourth study I did not find any cross talk between the WCP and ADAM10 in MCL. In the fifth study I identified the cross talk between WCP and NPM-ALK at the level of CK2α.

I believe that the work done in this thesis furthers our knowledge of the implication and importance of WCP in cancer pathogenesis. This in-depth understanding of the dynamics of signalling pathway interactions allows us to identify therapeutic targets. It gives us rationale for the combination of different pathway inhibitors as an approach in cancer therapeutics, recognizing the complexity of cross talk between signalling pathways in cancer. The work in this thesis provides a framework for further studies identifying critical targets in WCP, encouraging more efforts towards identifying specific molecular hubs and ultimately developing novel strategies to target inherent abnormalities of cancer cells.

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