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The Regulation of Alternative Splicing by Oncogenic Signaling Pathways.

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THE REGULATION OF ALTERNATIVE SPLICING BY ONCOGENIC SIGNALING PATHWAYS.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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To my husband, Michael,

for your love, friendship, patience, and humor.

And to my wonderful daughter, Valentina,

for letting me experience the kind of love

people freely die for.

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List of Abbreviations

AIG	anchorage-independent growth
Akt	v-akt murine thymoma viral oncogene
Apaf-1	apoptotic protease activating factor 1
ASF/SF2	alternative splicing factor/splicing factor 2 (SRp30a)
Bcl-2	B-cell lymphoma/leukemia-2
BH	Bcl-2 homology
CAMs	cell-to-cell adhesion molecules
CARD	caspase recruitment domain
c-FLIP	c-FLICE inhibitory protein
CRCE 1	ceramide-responsive RNA *cis*-element 1
DAG	diacylglycerol
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERBB	epidermal growth factor receptor family
ERK	extracellular signal-related kinase
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FADD	Fas-associated death domain
FGFR	fibroblast growth factor receptor
FLICE	FADD-like interleukin-1 beta converting enzyme
FOXO	forkhead family of transcription factors
GAPs	GTPase-activating proteins
GEFs	guanine nucleotide exchange factors
HBEC-3KT	human bronchial epithelial cells immortalized with hTERT and cdk4
HGF	hepatocyte growth factor
hnRNP	heterogeneous ribonuclear protein
H-Ras	Harvey Ras
IAP	inhibitors of apoptosis protein
IGF-1	insulin-like growth factor-1
ILK	integrin-linked kinase
IL-3	interleukin 3
IP3	inositol-3-phosphate
ISE	intronic splicing enhancer
ISS	intronic splicing silencer
JNK/SAPK	c-jun N-terminal kinase/stress-activated protein kinase

K-Ras	Kirsten Ras
MAPK	mitogen-activated protein kinase
MDM2	murine double minute 2
MEK	MAP kinase kinase
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor-κB
NHBE	normal human bronchial epithelial cells
NSCLC	non-small cell lung cancer
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent kinase 1
PI3-Kinase	phosphoinositide 3-kinase
PI(4,5)P2	phosphatidylinositol 4,5-bisphosphate
PI(3,4,5)P3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PLCγ	phospholipase C-γ
PP1	protein phosphatase-1
RNA	ribonucleic acid
RNAi	RNA interference
RRM	RNA recognition motif
RTK	receptor tyrosine kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SF1	splicing factor 1
shRNA	short hairpin RNA
siRNA	small interfering RNA
snRNP	small nuclear ribonucleoprotein
snRNA	small nuclear RNA
SR	serine/arginine-rich
TGF-α	transforming growth factor-α
TSGs	tumor suppressor genes
VEGFR	vascular endothelial growth factor receptor
WT	wild type

Abstract

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In the presented study, we demonstrate that the alternative splicing of caspase 9 was dysregulated in a large percentage of NSCLC tumors and cell lines. These findings led to the hypothesis that survival pathways activated by oncogenic mutation regulated this mechanism. Indeed, the oncogenic PI3-Kinase/Akt pathway was demonstrated to regulate the alternative splicing of caspase 9. Further mechanistic studies demonstrate that multiple Akt isoforms can regulate the alternative splicing of caspase 9 in NSCLC. Akt was additionally shown to mediate the exclusion of the exon 3,4,5,6 cassette of caspase 9 via the phospho-state of the RNA *trans*-factor, SRp30a. Mutagenesis studies identified serine ^{199}, serine ^{201}, serine ^{227}, and serine ^{234} as critical residues regulating the alternative splicing of caspase 9, as well as playing a role in the anchorage-independent growth of A549 cells. Since dysregulation of this splicing mechanism correlated with NSCLC tumors/cell lines

and constitutively active Akt, oncogenic factors for NSCLC known to activate the PI3-Kinase/Akt pathway were examined in HBEC-3KT cells. In contrast to k-ras^{V12} expression, the overexpression/mutation of EGFR affected the alternative splicing of caspase 9 in a pro-oncogenic manner, dramatically lowering the caspase 9a/9b mRNA ratio. Stable downregulation of caspase 9b by shRNA blocked the ability of E746-A750 del EGFR expressing HBEC-3KTs to induce anchorage-independent growth, suggesting a role for caspase 9b as a cooperative oncogenic factor. These findings were further corroborated by the ability of caspase 9b expression to completely block the inhibition of clonogenic colony formation by erlotinib. Therefore, this study demonstrates that oncogenic factors activating the PI3-Kinase/Akt pathway regulate the alternative splicing of caspase 9, to produce caspase 9b, via a coordinated mechanism involving the phosphorylation of SRp30a. In additional studies, we demonstrate that the PI3-Kinase/PKCι pathway, a pathway important for cancer cell survival and transformation of lung epithelial cells, regulates the alternative splicing of Bcl-x pre-mRNA via modulation of SAP155 expression to produce an anti-apoptotic phenotype in NSCLC. Therefore, these studies link oncogenic mechanisms in NSCLC to the therapeutically relevant and distal target mechanisms of caspase 9 and Bcl-x pre-mRNA splicing.

CHAPTER 1

INTRODUCTION

1.1. The pathways to cancer

Cancerous cells develop from healthy cells in a complex process called malignant transformation or tumorigenesis. Several lines of evidence indicate that tumorigenesis in humans is a multiple step process characterized by the loss of function of cellular mechanisms that control normal proliferation and differentiation. It is predicted that by the time a cancerous tumor becomes clinically evident, 10 to 20 genetic alterations of tumor suppressor genes and/or proto-oncogenes have occurred, allowing for the transformation of normal human cells into highly malignant derivatives[1]. While there are many distinct types of cancer, six essential alterations to normal cell physiology are believed to be responsible for the progression of most human malignancies: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastases[2]. Five physiologies are described with examples to illustrate the functional importance.

1.1.1. Self sufficiency of growth signals

Mitogenic growth signals are required for a normal cell to enter an active proliferative state. These signals are transmitted into the cell through transmembrane

receptors which are often targets of deregulation during tumorigenesis. While most mitogenic growth factors are produced by one cell in order to simulate a neighboring cell, tumor cells generate many of their own growth signals to which they are responsive, reducing their dependency on stimulation from the normal tissue microenvironment. This creates a positive feedback signaling loop often termed autocrine stimulation.

Mitogenic signaling cascades are commonly upregulated in cancer via the overexpression or mutation of growth factor receptors, often carrying tyrosine kinase activities in their cytoplasmic domains. Both gross over-expression and mutational alteration of receptors can result in ligand-independent signaling leading to uncontrolled cellular growth. The ERBB family is a group of transmembrane receptor tyrosine kinases which, together with their ligands, constitutes a potential growth stimulatory loop, particularly for non-small cell lung cancer (NSCLC). The epidermal growth factor receptor (EGFR) is one member of this family important in lung cancer. EGFR regulates epithelial proliferation and differentiation, with ligands including epidermal growth factor (EGF) and transforming growth factor-α (TGFα).

In addition to growth factors themselves, components downstream of growth factor receptor signaling also play a pivotal role in a cell's ability to sustain growth signals. Point mutations of Ras family proto-oncogenes (usually at *k-Ras* codons 12, 13, or 61) are detected in approximately 30% of lung adenocarcinomas with a *k-Ras* mutation at codon 12 being most frequent[3,4]. Mutated Ras proteins are able to release a flux of mitogenic signals without ongoing stimulation by their upstream regulators[5]. Transgenic mice carrying oncogenic alleles of *k-Ras*, activated only by spontaneous recombination events in

the whole animal, were highly predisposed to a range of tumor types, predominantly early onset lung cancer[6]. Clinically speaking, NSCLC patients with specific *k-Ras* point mutations are associated with a significantly increased risk of tumor recurrence and death, independent of tumor stage and histology[7].

The three major Myc family members (c-Myc, N-Myc, and L-Myc) comprise yet another important positive growth-regulatory system for lung cancer. The most frequently dysregulated Myc family member in NSCLC is c-Myc, as opposed to N-Myc and L-Myc which are generally activated only in SCLC[8,9]. Aberrant activation of c-Myc usually occurs as a result of protein overexpression caused by gene amplification or by transcriptional dysregulation[10] and is found in 8 to 20% of NSCLCs[8,11]. Elevated or deregulated expression of c-Myc has been detected in a wide range of human cancers and is associated with aggressive and poorly differentiated tumors[12-15].

1.1.2. Insensitivity to anti-growth signals

Within normal tissue, multiple anti-proliferation signals exist to maintain cellular quiescence and tissue homeostasis. These growth-inhibitory signals can block proliferation by either forcing cells into the G_0 phase of the cell cycle or inducing terminal differentiation resulting in cellular senescence. Developing cancers cells need to acquire resistance to these anti-proliferative signals for further expansion.

At the molecular level, most anti-proliferative signals are funneled through the protein products of tumor suppressor genes (TSGs). The tumor suppressor p53 has been described as "the guardian of the genome," referring to its role in conserving stability by

preventing genome mutation[16]. In response to DNA damage, p53 can induce growth arrest by holding the cell cycle at the G_1/S regulation checkpoint, allowing DNA repair proteins to act. If DNA damage proves to be irreparable, p53 will initiate apoptosis. Therefore, deregulation of p53 can allow for inappropriate survival of genetically damaged cells.

The *p53* gene is the most frequently mutated TSG in human malignancy, detected in 47% of 4,684 NSCLC tumors[17]. In 1999, Roth JA et al. provided clinical evidence of p53's critical role in tumorigenesis[18]. Direct injection of wild-type p53 gene vectors into human non-small cell lung tumors resulted in tumor regression and tumor growth stabilization in some patients[18]. These observations provided proof of principle that correcting the *p53* defect in the midst of a variety of genetic alterations could negate the malignant phenotype.

1.1.3. Evasion of apoptosis

The expansion of tumor cell populations is not only the result of an increased rate in cell proliferation but also of a decreased rate of cell removal. Apoptosis represents a major source of the elimination of unwanted or unhealthy cells. The evidence is mounting, principally from studies in mouse models and cultured cells, that acquired resistance toward apoptosis is a hallmark of most and perhaps all types of cancer. A cell's fate to proliferate or to engage in apoptosis is influenced by genes that contribute to the development of cancer (oncogenes) and those that act to suppress tumor formation (tumor suppressor genes). Oncogenes are mutated forms of normal cellular genes known as proto-

oncogenes, which tend to operate as positive growth regulators and are involved in promoting the differentiation and proliferation of normal cells. Tumor-suppressor genes tend to have a dampening or repressive effect on the regulation of the cell cycle and/or function to promote apoptosis.

Virtually all cancer cells harbor mutations that provide resistance to apoptosis, which can be acquired through multiple strategies involving interplay between oncogenes and tumor suppressor genes. The p53 tumor suppressor gene is the most commonly mutated gene found in cancer, leaving more than 50% of all cancers with a loss of function of the key pro-apoptotic regulator p53[19,20]. Other oncogenes such as Myc, Ras, and E1A drive cellular proliferation in response to persistent and/or elevated signaling. However, studies of Myc overexpression suggest that these oncogenes may affect apoptosis in different ways, depending on certain circumstances. Myc, under conditions of severe cellular stress or DNA damage, can act as a powerful inducer of apoptosis. However, the pro-apoptotic effects of Myc is eliminated by exogenous survival factors such as IGF-1, by overexpression of anti-apoptotic factors Bcl-2 and Bcl-X_L, as well as by disruption of the FAS death signaling circuit[21]. Collectively, the data suggest that although a cell's apoptotic program can be initiated by an overexpressed oncogene, other compounding factors can attenuate such effects.

Another means by which cancer cells overcome apoptosis is through hyperactivation of cell survival signaling. The anti-apoptotic PI3-Kinase/Akt survival pathway is likely involved in mitigating apoptosis in a substantial fraction of human

cancers. This mitogenic signaling pathway can be stimulated through extracellular factors such as IGF-1/2, EGF, or IL-3, by intracellular signals triggered by oncogene Ras, or by loss of the tumor suppressor gene PTEN, a negative regulator of the PI3-Kinase pathway. Collectively, these observations argue that altering components of the apoptotic machinery can dramatically affect the dynamics of tumor progression, providing a rationale for the inactivation of this machinery during tumor development.

1.1.4. Limitless replicative potential

Research performed over the past 35 years indicate that acquired growth signal autonomy, insensitivity of anti-growth signals, and a resistance to apoptosis is not enough to ensure tumor growth. Many cells carry an intrinsic, cell-autonomous program that limits infinite replication and this program must be disrupted to allow for large tumor masses to develop. Senescence is the term used to describe the halt in proliferation that most cell populations reach once progressed through a certain number of doublings. Normal human cells, when cultured, have the capacity for 60-70 doublings. Mutations in the tumor suppressor genes encoding pRb and p53 proteins are needed in order to circumvent the cellular senescence of cultured human fibroblasts. These mutations enable cultured human fibroblasts to continue multiplying for additional generations until a "crisis" state is entered. The crisis state is characterized by massive cell death, karyotypic disarray associated with end-to end fusion of chromosomes, and the occasional emergence of a variant (1 in 10^7) cell that has acquired the ability to multiply without limit, termed immortalization[22]. Most tumor cells that are cultured *in vitro* appear to be immortalized

and have limitless replicative potential, suggesting that this phenotype was acquired *in vivo* and was necessary to the development of the malignant growth[23].

Telomeres are regions of a short 6 base pair sequence element located at the ends of chromosomes. For each passage through the cell cycle, there is a 50-100 base pair loss of telomeric DNA from the ends of every chromosome, attributing to the inability of DNA polymerases to completely replicate the 3' ends of chromosomal DNA. Telomerase is an enzyme that adds TTAGGG nucleotide repeats onto the ends of telomeric DNA. Normal somatic cells do not posses telomerase activity and senesce when telomeres reach a critical length. However, 85-90% of malignant cells upregulate the expression of the telomerase enzyme, providing a mechanism for infinite replicative potential[24]. Previous studies reveal that ectopically expressing telomerase induces immortalization onto a variety of normal early passage, pre-senescent cell in vitro[25,26]. Additionally, late passage cells about to enter the crisis state continued to proliferate when provided with telomerase enzyme[27-29]. While telomerase activation is not the earliest step in the pathogenesis of cancer, it does occur early enough to potentially serve as a biomarker detectable in pre-neoplastic cells.

1.1.5. Tissue invasion and metastases

It is inevitable, at some point in time a primary tumor mass will acquire the capability to invade surrounding tissues and travel to distant sites where a secondary or metastatic tumor will form. Tumor metastases, the spreading of tumor cells from one tissue to a non-adjacent tissue, are the cause of 90% of human cancer deaths[30]. Like the formation of the primary tumor, metastases rely on the acquisition of all five hallmark

capabilities (four are discussed above). However, what additional changes must occur to induce tissue invasion?

Cancerous cells exhibiting metastatic capabilities have altered cell-to-cell and cell-to-environment interactions. Most notably, the functions of cell-to-cell adhesion molecules (CAMs) and the expression of integrins are modulated in cancer to support tissue invasion. For example, the loss of function of E-cadherin, a cell-to-cell adhesion molecule ubiquitiously expressed in epithelial cells, is commonly seen in cancers of epithelial origin[31]. Downregulation of E-cadherin decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. Furthermore, recent reports have demonstrated that ectopic expression of E-cadherin in cultured cancer cells and in a transgenic mouse model of carcinogenesis impairs invasion and metastatic phenotypes[31].

Invading cancer cells encounter a variety of different tissue microenvironments, which all present different extracellular matrix (ECM) components. Accordingly, successful metastasis is achieved through shifts in the expression of integrins on the migrating cancer cell. Integrins are a family of heterodimeric transmembrane adhesion receptors that mediate cellular attachment to the ECM and to adjacent cells[32]. Research has demonstrated that carcinoma cells switch their integrin profiles from those that favor the ECM present in normal epithelium, to those that preferentially bind the degraded stromal components produced by extracellular proteases[33,34]. Moreover, ectopic expression of specific integrin subunits in cultured cells can inhibit or induce invasive behavior[33]. Overall, it is well established that integrins, due to aberrant adhesive events and cellular

signals that alter gene expression and influence cell survival, contribute to malignant transformation, tumor growth and progression, and invasion and metastasis[35-37]. Thus, the functional loss of E-cadherin and the switching of integrin profiles represent two key steps in the acquisition of the ability of a cancer cell to metastasize.

1.2. Oncogenic pathways dysregulated in NSCLC

According to the World Health Organization, cancer is the leading cause of death worldwide. In 2007 alone, it was estimated 7.9 million people died from cancer, a number that is projected to increase 45% by 2030 (due in part to an increasing and aging population)[38]. Of all cancers diagnosed, lung cancer is by far the most fatal, accounting for 29% of cancer deaths[38].

The overwhelming majority of lung cancers diagnosed are classified as carcinomas – malignancies that arise from epithelial cells. Depending of the size and appearance of the malignant tumors, carcinomas can be characterized as non-small cell or small cell. Non-small cell lung cancers (NSCLC) account for 80% of all lung cancers diagnosed. The most common histologies of NSCLC are adenocarcinomas, squamous cell carcinomas (also called epidermoid carcinoma), and large cell carcinomas. Adenocarcinomas are the most frequent form of lung cancer and originate in the cells that line the alveoli; squamous cell carcinomas originate in squamous cells and are generally found in the center of the lung; and large cell carcinomas may begin in several types of large cells of the lung. Other less common types of non-small cell lung cancer are: pleomorphic, carcinoid tumor, salivary gland carcinoma, and unclassified carcinoma.

Lung cancer is characterized by the accumulation of multiple genetic alterations resulting in the activation of oncogenes or inactivation of tumor suppressor genes[39]. Dysregulation of epidermal growth factor receptor (EGFR) has been observed in multiple cancers including NSCLC[40]. Recently, Hirch et. al. reported that 62% of NSCLCs of adenocarcinoma and squamous cell subtypes exhibited EGFR protein overexpression[41], which is often associated with poor prognosis[42]. In addition to EGFR expression, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), two of its cognate ligands, are also freely expressed in NSCLCs, leading to over-stimulation of downstream mitogenic signaling pathways[43,44]. Three prominent signaling networks linking EGFR activation to cell proliferation and survival are the Ras/Raf/MEK/ERK (Ras/MAPK), the PI3-Kinase/Akt pathway, and the PLCγ/PKC pathway. These pathways, with emphasis on the PI3-Kinase/Akt pathway, and their role in NSCLC will be discussed.

1.2.1. The Ras/Raf/MEK/ERK pathway in NSCLC

MAPK (mitogen-activated protein kinase) pathways play critical roles in regulating cellular proliferation, differentiation, and apoptosis[45]. Three major types of MAP kinase cascades have been reported that respond synergistically to different upstream signals: the classical MAPK (also known as ERK), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 kinase. The extracellular signal-related kinase (ERK) has

been the best characterized MAPK and the Raf-MEK-ERK pathway represents one of the best characterized MAPK signaling pathways.

Activation of MEK or ERK results from stimulation of receptor tyrosine kinases by growth factors such as EGF, HGF, and PGDF. It is also known that oncogenes such as Ras can consititutively activate ERK, leading to malignant transformation *in vitro* and *in vivo*[46]. Ras associates and activates Raf-1, which subsequently phosphorylates MEK, which in turn phosphorylates ERK1/2. Activated ERK1/2 can then translocate to the nucleus to modulate gene expression[47,48]. Ras signaling upon EGF stimulation has been associated with increased cell growth and proliferation.

Constitutive activation of ERK and/or increased levels of ERK have been observed in many tumor cell lines[49] and in epithelial cancer tissues including breast[50,51], kidney[52], colon[53], and head and neck cancers[54]. Additionally, there is some evidence of ERK activation in NSCLC primary cancers as compared to normal lung cells[49,55]. However, the role of ERK activation in patients with NSCLC remains to be defined.

1.2.2. The PI3-Kinase/Akt pathway in NSCLC

In addition to the Ras/MAPK pathway, the PI3-Kinase pathway represents another major signaling network downstream of EGFR activation. PI3-Kinases have been linked to an extraordinarily diverse group of cellular functions, including cell growth, proliferation, differentiation, motility, survival and intracellular trafficking[56-59]. Although

three classes of PI3-Kinases exist (classes I, II, III) and signaling through all classes are connected to key growth-regulatory processes, thus far a central role in cancer has been demonstrated selectively for class IA PI3-Kinases. Therefore, the discussion will focus on signaling through class IA PI3-Kinases.

The PI3-Kinase/Akt pathway is a central regulator of cell proliferation and survival and is dysregulated by oncogenic events in a substantial fraction of malignant tumors. It is targeted by genomic abnormalities including mutation, amplification and rearrangement more frequently than any other pathway in human cancer, with the possible exception of the p53 and retinoblastoma (Rb) pathways. A clear link between the PI3-Kinase pathway and cancer was initially established in the 1980s when two viral oncoproteins, the src protein of Rous sarcoma virus and the middle-T protein of polyma virus, were associated with the pathway's lipid kinase activity[60,61]. Binding of the p85 subunit of PI3-Kinase to phospho-tyrosines on the viral oncoproteins leads to the recruitment of the p110α catalytic subunit, thereby activating the PI3-Kinase signaling pathway. Since then, it has been demonstrated that *PI3KCA* (the gene encoding the p110α subunit of PI3-Kinase) and *PTEN* are two of the most frequently mutated genes in human cancers resulting in deregulated activation of PI3-Kinase signaling. Specifically, *PI3KCA* is frequently amplified or mutated in head and neck, cervical, gastric, ovarian, breast, and lung cancers[62-68].

Recently, it has been demonstrated that Akt, the critical downstream effector of PI3K, is constitutively active in most NSCLC cell lines and in 51% of NSCLC patient samples[69,70]. Similar work has also revealed Akt as a crucial mediator of NSCLC cell

survival and therapeutic resistance[69,71]. Additionally, studies have shown that high levels of phosphorylated Akt in NSCLC are a marker for a poor prognosis[72]. These studies provide evidence for a central role of PI3-Kinase/Akt in tumorigenesis.

1.2.3. Signaling through the PI3-Kinase/Akt pathway

Genetic studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have enlightened our understanding of PI3-Kinase regulation. PI3-Kinase can be activated in response to the binding of growth factors to their respective receptor protein tyrosine kinases (RTKs). RTKs include epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR), as well as many others. In response to growth factors, RTKs interact with the p85 regulatory subunit of PI3-Kinase and localize it to the plasma membrane. Upon activation, PI3-Kinase phosphorylates $PI(4,5)P_2$ to generate the lipid second messenger $PI(3,4,5)P_3$. The lipid phosphatase PTEN antagonizes PI3-Kinase signaling by dephosphorylating $PI(3,4,5)P_3$ into $PI(4,5)P_2$, thus acting as the central negative regulator of the pathway. $PI(3,4,5)P_3$ is now available to recruit and bind a subset of proteins containing pleckstrin-homology (PH) domains. These proteins include Akt (PKB), Tec family tyrosine kinases, guanine nucleotide exchange factors (GEFs) for Rac, and GTPase-activating proteins (GAPs). However, genetic screens in model organisms have identified Akt as the primary downstream mediator of the effects of PI3-Kinase.

Akt, or protein kinase B, is a critical downstream effector of PI3-Kinase and is located at a crucial crossroads of various signaling events. In humans, there are three

isoforms of Akt which are produced from three separate genes: Akt1, Akt2, and Akt3. The N-terminal region of Akt harbors the PH domain, which is recruited to and interacts with PI(3,4,5)P3 at the plasma membrane. Recruitment of Akt to the membrane induces a conformational change, exposing two crucial amino acids that are phosphorylated and necessary for activation. Threonine 308 is located in the kinase domain of Akt and is phosphorylated by active phosphoinositide-dependent kinase 1 (PDK1). This phosphorylation event stabilizes the activation loop whereas phosphorylation of serine 473 in the hydrophobic C-terminal domain by PDK2 is necessary for full Akt activation[73,74]. The identity of PDK2 is still unknown. However, recent reports have proposed several different possibilities, including the mTOR rictor complex (separate from the mTOR raptor complex inhibited by rapamycin), integrin-linked kinase (ILK), PKCβII and even AKT itself, thereby allowing the pathway potential for feedback control[75-77].

The PI3-Kinase/Akt pathway is a key regulator of cell survival through multiple downstream targets[78-80]. It has been clearly demonstrated that Akt can function to enhance cell proliferation and survival through inhibition of the forkhead (FOXO) family of transcription factors, including AFX, FKHR, and FKHRL1. These transcription factors are known to mediate apoptosis by activating transcription of *FasL* and *Bim*, two well documented pro-apoptotic genes[81]. However, Phosphorylation of the FOXO proteins by Akt forces their retention in the cytoplasm through interactions with 14-3-3 proteins, thereby sequestering them from activating transcription of their gene targets. Similarly, Akt can phosphorylate the proapoptotic Bcl-2 family member Bad, preventing its translocation to the mitochondrial membrane through interactions with 14-3-3 proteins[82].

Additionally, reports have demonstrated that, under certain conditions, Akt can act to promote cell survival by phosphorylating I-κB kinase, which activates the pro-survival transcription factor nuclear factor-κB (NF-κB)[83,84].

1.2.4. The PLCγ/PKC pathway in NSCLC

A final key pathway involved in activation of EGFR involves activation of phospholipase C-γ (PLCγ). Four PLC subfamilies have been identified, with PLCγ being the only isoform known to be downstream of receptor tyrosine kinases (RTKs), such as EGFR. Activation of PLCγ results in hydrolyzation of PI(4,5)P2 to generate inositol-3-phosphate (IP3) and diacylglycerol (DAG), allowing for subsequent release of calcium ions from intracellular stores and activation of Protein Kinase C[85].

Protein kinase C (PKC) is a family of ubiquitously expressed, phospholipid-dependent enzymes involved in signal transduction associated with cell proliferation, differentiation, and apoptosis[86]. It is now well established that the PKC is a family of at least 12 serine threonine kinases[87]. Each PKC isozyme differs in structure, biochemical properties, tissue distribution, subcellular localization, and substrate specificity. The mammalian PKCs are classified on the basis of their enzymatic properties as either classical, novel, or atypical. The classical PKCs (α, βI, βII, γ) are Ca^{2+}-dependent and are activated by diaclyglycerol (DAG). The novel PKCs (δ, ε, θ, η) are Ca^{2+}-independent and are activated by DAG. Lastly, the atypical PKCs (ι, ζ) do not require Ca^{2+} for their activation nor are activated by DAG.

PKCs have been demonstrated to act as key cellular components in major signaling pathways involving Ras, Myc, and Fos[88] and altered PKC activity has been linked with various types of malignancies. Higher levels of PKC and differential activation of various PKC isozymes have been reported in breast, renal, pancreatic, and colon cancers[89-91]. Several studies have also presented evidence that overexpression of different PKC isoforms induces cell proliferation and enhanced tumorigenicity. For example, fibroblasts overexpressing PKCβI, PKCε, and PKCγ resulted in an increased proliferation rate, anchorage-independent growth, and enhanced tumorigenicity in nude mice[92-95]. Furthermore, the overexpression of PKCα in MCF-7 breast cancer cells led to a more aggressive neoplastic phenotype[96]. However, there are a number of conflicting reports on whether overexpression of PKC increases or suppresses tumorigenicity in various cells[97,98]. For example, colon carcinoma cells overexpressing PKCβI resulted in tumor growth inhibition[99]. These observations have led researchers to believe that each PKC isozyme plays critical, yet differing roles in tumorigenesis.

The role of PKC isozymes in cancer have been studied for the past 20 years. However, it was only in 2005 that the first PKC isozyme, PKCι, was demonstrated to be a human oncogene[100]. PKCι is at the hub of several major oncogenic signaling pathways and has been directly implicated in Ras-mediated siganaling[101,102]. Overexpression of PKCι is found in NSCLC cell lines and primary tumors, and correlates with poor clinical outcome in NSCLC patients[100]. However, the related atypical PKC, PKCζ, is expressed at low levels in malignant lung tissue indicating PKCι is selectively targeted in

oncogenesis[103]. These data provide a rationale for PKCι-targeted therapy in the treatment of lung cancer.

1.3. RNA Splicing

In cancer, the control of alternative RNA splicing can be dysregulated as a consequence of alterations within signaling cascades, such as the PI3-Kinase/Akt pathway[104]. RNA splicing is the process by which introns, or intervening sequences within protein-coding genes, are removed from unprocessed RNA (pre-mRNA) to produce "mature messenger RNA" (mRNA) prior to translation into protein. A central regulatory mechanism in mammalian cells is the alternative splicing of pre-mRNA in which multiple mRNA transcripts are generated from a single mRNA precursor. Research on the human genome has demonstrated that alternative splicing is an important mechanism responsible for regulating a multitude of biological processes via production of variant protein products, therefore playing a role in a wide range of human diseases. The following sections will discuss the current understanding of pre-mRNA splicing and the mechanisms that regulate mammalian pre-mRNA splicing. It also will discuss the role of alternative splicing in lung cancer.

1.3.1. Pre-mRNA splicing

In the late 1970s, the laboratories of Phillip Sharp and Richard Roberts both independently discovered that genes are not continuous strings but contain non-coding regions of DNA, later termed introns. Furthermore, deletion of these introns through

splicing of pre-mRNA can occur in multiple ways, yielding different protein isoforms from the same DNA sequence[105,106]. Along with 5′ capping and 3′ polyadenylation, RNA splicing is a critical post-transcriptional processing step.

The removal of introns from pre-mRNA, and subsequent joining of exons, is governed by the recognition of sequences at the intron/exon junctions known as splice sites. The exon/intron junction at the 5' end of the intron marks the 5' splice site and includes a GU dinucleotide surrounded by a less conserved consensus sequence. The 3' splice site is located at the opposite end of the same intron and contains three conserved sequence elements: the branch point, followed by a polypyrimidine tract, followed by an intronic terminal AG dinucleotide. A two-step transesterification reaction removes the unnecessary introns. In the first step, the 2' hydroxyl group of the branch site adenosine attacks the phosphate at the 5' splice site of the intron, leading to reaction intermediates: the 5' exon and an intron/3' exon lariat. In the second step, the free 3'-hydroxyl of the 5' exon attacks the phosphate at the 3' end of the intron. This results in release of the inton, still in lariat form, and ligation of the two flanking exons.

The splicing reaction is catalyzed by the spliceosome, a macromolecular complex consisting of five small nuclear ribonucleoprotein particles (U snRNPs) and over 150 non-snRNP splicing factors[107]. Each snRNP is a complex containing a short RNA molecule (U1, U2, U4, U5, and U6 snRNAs) and Sm (or Sm-like) proteins, along with several other specific proteins[108]. Formation of the spliceosome involves an ordered, stepwise assembly, during which complexes E, A, B, and C are formed on the pre-mRNA[109]. Formation of the early (E) complex occurs following U1 snRNP binding to the 5'end splice site, binding of

splicing factor 1 (SF1) to the branch site, and recognition of the 3' splice site by U2 snRNP auxiliary factor (U2AF)[110,111]. This commitment complex is independent of ATP and commits the pre-mRNA to splicing. U2 snRNP interacts with U2AF at the branch site in an ATP-dependent reaction forming the pre-spliceosome complex A. Next, the pre-assembled U4/U5/U6 tri-snRNP is recruited to form Complex B. Following extensive structural and compositional rearrangements, Complex B becomes catalytically activated[112]. The catalytic core of the spliceosome is formed on pre-mRNA by U2, U5, and U6 snRNPs. Both steps of the splicing reaction (described above) are catalyzed in the activated complex B and complex C. Lastly, Spliceosome disassembly is initiated by the release of the newly spliced mRNA and lariat intron.

1.3.2. Mechanisms of Alternative spicing

Alternative splicing occurs as a normal phenomenon and is a major source of protein diversity from the human genome. Through the process of alternative splicing, multiple mRNAs are produced from a single pre-mRNA which encodes structurally and functionally different proteins. Initial estimates suggested that only a small number of genes underwent alternative splicing[113]. However, current research using large-scale oligonucleotide-based microarrays now suggests that at least 70%-80% of human genes have splice variants[114,115].

In the typical pre-mRNA, the splicing pattern can be modified in a variety of ways depending on splice site selection. In addition to the splice signals flanking the exon/intron

junctions (i.e. the 5'splice site, branch site, polypyrimidine tract, and the 3'splice site), other auxiliary signals have recently been identified as playing an important role in repressing or enhancing splice site selection. These elements are short, conserved sequences and are classified according to their location and function. Splicing silencers are regions on the pre-mRNA to which splicing repressor proteins bind, reducing the probability that a nearby site will be used as a splice site. Splicing silencers can be located in an intron (intronic splicing silencer, ISS) or a neighboring exon (exonic splicing silencers, ESS). Splicing enhancers are regions of the pre-mRNA to which splicing activator proteins bind, increasing the probability that a nearby site will be used as a splice site. Like splicing silencers, enhancers may also occur in introns (intronic splicing enhancers, ISE) or exons (exonic splicing enhancers, ESE). In the majority of cases, splicing silencers and enhancers exert their effects through the binding of regulatory proteins, most notably members of the serine-arginine-rich (SR) protein family[116] and members of the heterogenous nuclear ribonucleoproteins (hnRNP) family[117].

1.3.3. SR proteins

Serine/arginine-rich (SR) proteins are a family of highly conserved splicing factors with functions in both constitutive and alternative pre-mRNA splicing[118-123]. SR proteins contain a characteristic RS domain rich in arginine and serine dipeptide repeats, and one or two RNA recognition motifs (RRMs). The RRM provides RNA-binding specificity whereas the C-terminal RS domain promotes protein-protein interactions that facilitate spliceosomal assembly[124,125]. More recently, studies have shown that the RS domain can

also function as a nuclear localization signal (NLS) by mediating the interaction with the SR protein nuclear import receptor, transportin-SR[126-128]. In humans, the 'classical' SR protein family members are defined based on the following criteria: (i) structural similarity, (ii) dual function in constitutive and alternative splicing, (iii) precipitate in the presence of 20mM MgCl2, and (iv) the presence of a conserved phosphoepitope within the RS domain and recognized by mAb104. Additional RS domain-containing proteins exist but lack one or more of the requirements of a 'classical' SR protein.

SR proteins are primarily localized within nuclear speckles. However, studies have demonstrated that their subcellular localization can be modified by the phosphorylation status of the RS domain[129-131]. The serine residues in the RS domain are extensively phosphorylated and, in addition to subcellular localization, the phosphorylation status can also regulate protein-protein interactions[132,133], both of which may regulate the ability of SR proteins to function in splicing. In fact, both hyper- and hypo-phosphorylated SR proteins are unable to support splicing[134-136].

Three protein kinases are widely accepted as directly phosphorylating the RS domains of SR proteins. These include the SR protein kinase family SRPK1[137] and SRPK2[132], the Clk/Sty family[138], and DNA topoisomerase 1[139]. Experiments performed in *D. melanogaster* have provided the most direct evidence for an *in vivo* role of SR protein kinases. DOA is the homolog of Clk/Sty found in *Drosophila*. DOA phosphorylates the Drosophila SR protein RBP1 as well as Tra and Tra2[140]. It was demonstrated that mutations in *Doa* disrupt the splicing of *doublesex* pre-mRNA, but have no effect on the

splicing of *fruitless* pre-mRNA – both regulated by RBP1, Tra, and Tra2[140]. Interestingly, these SR proteins control alternative 3' splice site selection in *doublesex* pre-mRNA and alternative 5' splice site selection in *fruitless* pre-mRNA, suggesting that 5' and 3' splice sites regulated by SR proteins have different requirements for RS domain phoshorylation[140].

Recently the PI3-Kinase/Akt pathway has been suggested to alter the activity of SR proteins. Specifically, Srebrow and colleagues have provided evidence for a direct involvement of Akt in modifying the activity of the SR proteins, SRp30a and 9G8[141]. Independent of this study, Akt was also demonstrated to phosphorylate SRp40 and modify the alternative splicing of PKCβII[142].

Due to their critical roles as essential splicing factors and as splicing regulators, disruption of SR proteins can lead to human disease. It has been reported that 15% of mutations that result in genetic disease affect pre-mRNA splicing[143], disrupting 5' splice sites, 3' splice sites, enhancer sequences, and silencer sequences. Recent analyses demonstrate that more than 50% of single-base substitutions associated with exon-skipping disrupted at least one of the target motifs for the SR proteins SRp30a, SRp40, SRp55 and SC35[144,145]. Recent reports have also established a link between the mis-expression of SR proteins and cancer. Specifically, SRp30a, SC35, and SRp20 were found to be overexpressed in malignant ovarian tissue[146] and SRp30a upregulated in lung, colon, kidney, liver, pancreatic, and breast tumors[147]. These data suggest that SR proteins play a role in cancer development.

1.4. Apoptosis

Apoptosis is a tightly regulated execution of signaling pathways leading to the removal of cells. In 1970, John Foxton Ross Kerr at University of Queensland was able to identify and distinguish apoptosis (Greek: *apo* - from, *ptosis* - falling) from traumatic cell death, known as necrosis. The end result of apoptosis, as opposed to necrosis, is cell death without inflammation of the surrounding tissue. Apoptosis is characterized by a variety of morphological changes, including changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[148-150]. Apoptosis plays a crucial role in developing and maintaining health by eliminating old, unnecessary, or unhealthy cells. In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy.

In mammalian cells, apoptosis occurs via two main signaling pathways. The "extrinsic" or receptor-mediated pathway, is triggered when pro-apoptotic ligands bind to pro-apoptotic receptors on the cell surface. In contrast, the "intrinsic" or mitochondrial pathway is initiated from within the cell as a result of cellular developmental cues or significant cellular stress (e.g., DNA damage), and when stimulated leads to the release of cytochrome c from the mitochondria and activation of the death signal. Both pathways converge to a final common pathway in which the destruction of the cell is ultimately carried out by caspases - intracellular protease enzymes that, upon activation, destroy cellular proteins vital for cell survival and stimulate fragmentation of chromosomal DNA.

1.4.1. Extrinsic Pathway

The extrinsic pathway is triggered in response to external pro-apoptotic ligands, such as endogenous Apo2L/TRAIL and CD95L/FasL. Binding of these ligands to their pro-apoptotic cell surface receptors DR4/DR5 and CD95/Fas, respectively, may lead to apoptosis. Unlike the intrinsic pathway, the extrinsic pathway triggers apoptosis independently of the p53 protein. When a death stimulus triggers the pathway, the membrane-bound FasL interacts with adaptor protein Fas-associated death domain (FADD) and initiator caspases 8 or 10 as procaspases, forming the death-inducing signaling complex (DISC). Formation of DISC facilitates the autocatalytic processing and release of caspase 8 into the cytoplasm where it, in turn, activates caspases 3, 6, and/or 7. Upon DISC activation, the extrinsic pathway converges with the intrinsic pathway and adopts the same effector caspase machinery.

Several pathways and proteins regulate the activation of the extrinsic pathway. c-FLICE inhibitory protein (c-FLIP) acts to inhibit DISC formation by interacting with FADD to block initiator caspase activation. Other inhibitors of the pathway include FAP-1 and the soluble decoy receptors such as DcR3, TRAIL R-3/DcR1, and TRAIL R-4/DcR2 which can act to block FasL binding and Fas receptor stimulation.

1.4.2. Intrinsic pathway

As its name suggests, the intrinsic pathway is initiated from within the cell, usually in response to cellular signals resulting from DNA damage, a defective cell cycle,

detachment from the extracellular matrix, hypoxia, or loss of cell survival factors. When stimulated, the intrinsic pathway, leads to the release of cytochrome c from the mitochondria and activation of caspases. This process ultimately triggers apoptosis[151-154].

One of the most important regulators of the intrinsic pathway is the members of the Bcl-2 superfamily of proteins, which act to regulate the permeability of the mitochondrial membrane[152]. The Bcl-2 family includes pro-apoptotic members such as Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim, and Hrk, and antiapoptotic members such as Bcl-2, Bcl-x(L), Bcl-W, Bfl-1, and Mcl-1. Members of the Bcl-2 family contain signature domains of homology called Bcl-2 homology (BH) domains (termed BH1, BH2, BH3, and BH4)[155]. Pro-apoptotic Bcl-2 members act as promoters of apoptosis by allowing permeabilization of the mitochondrial membrane. In contrast, anti-apoptotic Bcl-2 members function to repress apoptosis by acting to prevent permeabilization of the mitochondrial membrane and blocking cytochrome-c release.

Once the mitochondrial membrane becomes permeable (triggered by apoptotic stimuli), cytochrome-c, second mitochondria-derived activator of caspase (also known as direct IAP-binding protein), and the pro-apoptotic protein SMAC/DIABLO are released into the cytosol. SMAC/DIABLO protein promotes apoptosis by directly interacting with inhibitors of apoptosis proteins (IAPs) and disrupting their ability to inactivate the caspase enzymes. Cytochrome-c is now able to interact with adaptor apoptotic protease activating factor-1 (Apaf-1) and (d)ATP, forming a large multiprotein structure known as the apoptosome. The apoptosome is assembled when seven Apaf-1:cytochrome *c* heterodimers

oligomerize to form a symmetrical "wheel" and procaspase-9 molecules become associated non-covalently to Apaf-1 via caspase-9 CARD/Apaf-1 CARD heterophilic interaction[156,157]. The primary function of the apoptosome is theorized to be multimerization and allosteric regulation of the catalytic activity of caspase 9. Initiator caspase 9 is recruited into the apoptosome and activated, which in turn initiates the caspase cascade involving effector caspases 3, 6, and/or 7[158].

1.4.3. Caspase 9

The caspase cascade plays a vital role in the induction, amplification, and execution of apoptotic signals within the cell. The caspases, or *c*ysteine-*asp*artic acid prote*ases,* are a family of intracellular proteases that — upon activation through the intrinsic and/or extrinsic pathways — destroy essential cellular proteins, leading to apoptosis. Robert Horvitz initially established the importance of caspases in apoptosis through his discovery of the *ced-3* gene and its requirement for cell death in the development of nematode *C. elegans*. Horvitz and his colleague Junying Yuan found in 1993 that the protein encoded by the ced-3 gene was cysteine protease, now known as caspase 1[159]. Since then, eleven caspases have been identified in humans and are classified as either initiator (apical) caspases or effector (executioner) caspases.

Initiator caspases (caspases 2, 8, 9, and 10) are activated through either the intrinsic or extrinsic apoptosis-signaling pathways and are responsible for the activation of the effector caspases (caspases 3, 6, and 7) which, in an expanding cascade, carry out

apoptosis[153,160]. The effector caspases induce cleavage of protein kinases, cytoskeletal proteins, DNA repair proteins, and ultimately destroy "housekeeping" cellular functions, leading to the morphologic expression of apoptosis.

At cytosolic concentration in human cells (<50 nM), procaspase-9 is monomeric and resides as a zymogen[161,162]. In contrast to executioner caspases, caspase 9 does not require proteolysis in the linker region to become active[161,163-166]. Instead, caspase 9 is activated by small-scale rearrangements of its active site[162]. Currently, two opposing theories exist in regards to the mechanism of caspase 9 activation; the allosteric model and the proximity-induced model. In the allosteric model, a single monomer of caspase 9 interacts with the apoptosome; an interaction which induces rearrangements of the active site. In contrast the induced proximity model suggests that as the local concentration of caspase 9 increases, a kinetic barrier is overcome to induce dimerization and monomer/monomer contacts within the catalytic domain of the caspase-9 dimer cause the activating rearrangements.

In 1999, two groups independently identified an alternatively splice variant of caspase 9, termed caspase 9b, which acts as a dominant-negative inhibitor of caspase 9 activation[167,168]. Sequence analysis revealed that the caspase 9b splice variant lacked the exon 3,4,5,6 cassette (residues 140-289) encoding the catalytic domain of caspase 9. Further studies demonstrated caspase 9b antagonized caspase 9a activity by 1) competing with caspase 9a for Apaf-1 binding and/or 2) caspse 9b binds with caspase 9a and blocks its auto-proteolysis[167,168]. These results suggest that caspase-9b functions as an

endogenous apoptosis inhibitory molecule by interfering with the formation of a functional Apaf-1-caspase-9 complex.

Additional findings by Alnemeri and colleagues illustrated that the ratio of caspase 9a to caspase 9b can greatly influence a cell's decision to undergo apoptosis. In these studies, MCF-7 cells transiently transfected with caspase 9b were significantly desensitized to apoptotic stimuli[168]. Consistent with these findings, Weller and colleagues demonstrated transient overexpression of caspase 9b protected LN-229 astrocytoma cells from Fas-mediated apoptosis[169]. The above findings suggest caspase 9b can negatively regulate apoptosis by a dominant-negative mechanism. Therefore, regulation of the inclusion of this four exon cassette is a possible critical determinant to decide whether a cell is susceptible or resistant to apoptosis.

Numerous studies in the literature demonstrate a role for caspase 9 in both the response of cells to undergo chemotherapy-induced cell death and the suppression of oncogenic transformation[167,168,170-178]. As to the latter, Lowe and co-workers demonstrated that p53 null cells as well as caspase 9 -/- cells were resistant to apoptosis induced by c-Myc and oncogenic Ras (h-Ras^{V12}) expression [179]. Furthermore, mouse embryonic fibroblasts cells devoid of either caspase 9 or p53 demonstrated dramatic enhancement of cellular transformation by c-Myc and oncogenic Ras as assayed by colony formation in soft agar and tumorigenesis in nude mice [179]. Studies to orient the pathway by the same laboratory group also demonstrated that caspase 9 was downstream of p53 in this pathway [179]. Thus, caspase 9 acts as a tumor suppressor of c-Myc and H-Ras^{V12} transformation by playing a major role in the p53-dependent pathway of apoptosis/senescence [179].

1.4.4. Bcl-x

In 1993 Boise, L.H. et al. indentified and characterized a Bcl-2 related gene, Bcl-x, which functions independently of Bcl-2 in regulating apoptosis. This report indentified two distinct isoforms of the gene, termed Bcl-x(L) and Bcl-x(s). Bcl-x(L), the larger of the two isoforms, was demonstrated to serve as an inhibitor of apoptosis whereas Bcl-x(s) possesses an antagonistic function, to promote apoptosis. Specifically Bcl-x(s) was demonstrated to inhibit the ability of Bcl-2 to promote apoptosis[180]. Additional studies revealed that Bcl-x(L) and Bcl-x(s) are both generated from the *Bcl-x* gene via alternative splicing of two distinct 5' splice sites located within exon 2 of the Bcl-x pre-mRNA transcript[180]. Processing at the upstream (intron distal) 5' splice site results in processing of the smaller (170 amino acids) Bcl-x(s) isoform, while processing at the downstream (intron proximal) 5' splice site results in processing of the larger (233 amino acids) Bcl-x(L). Bcl-x(L) contains an additional 63 amino acids, encoding for two Bcl-2 homology domains, BH1 and BH2 that are lacking in Bcl-x(s)[181]. The presence and absence of the BH1 and BH2 domains is responsible for the antagonizing functions of Bcl-x(L) and Bcl-x(s)[181]. Research has demonstrated that mutations in either BH1 or BH2 domain of Bcl-x(L) compromise its ability to interact with other Bcl-2 family members, such as Bad and Bax, and prevent it from carrying out its anti-apoptotic functions[182,183].

The antagonistic behaviors of Bcl-x(L) and Bcl-x(s) have been demonstrated to influence the regulation of apoptotic signaling. Numerous studies have shown that overexpression of Bcl-x(L) in cells confers resistance to many apoptotic stimuli, and cooperates with oncogenic factors (e.g. c-Myc) in tumorigenesis[180,181,184-191]. Furthermore,

many cell types spontaneously resistant to chemotherapeutic agents also demonstrate increased levels of Bcl-x(L)[192-197]. The regulation of Bcl-x(L) expression is a complex mechanism consisting of both transcriptional and post-transcriptional processes. Several studies have demonstrated that the Bcl-x splice variant, Bcl-x(s), in contrast to Bcl-x(L), promotes apoptosis[181,198-201], and the mechanism of alternative 5' splice site selection of Bcl-x pre-mRNA has emerged as a potential target for anti-cancer therapeutics in non-small cell lung cancer (NSCLC).

CHAPTER 2

THE PI3-KINASE/AKT PATHWAY REGULATES THE ALTERNATIVE SPLICING OF CASPASE 9 IN A PRO-ONCOGENIC MANNER.

2.1. Introduction

Regulation of pre-mRNA splicing plays an important role in human disease[202]. Although a major source of cancer-causing errors in gene expression arise from mutations in enhancer or silencer elements located in pre-mRNA, research over the past two decades have reported cancer-specific alternative splicing in the absence of genomic mutations. For example, the alternatively spliced variant of MDM2 oncoprotein, MDM2b, is unable to bind p53 due to the exclusion of the p53-binding domain. MDM2b PCR products were exclusively detected in osteosarcomas and not normal bone and their levels overexpressed in astrocytomas, ovarian cancers, and bladder cancers[203-205].

In order to influence the development and progression of cancer, the alternatively spliced variant must likely be expressed in significant amounts in relation to its predominantly spliced variant. Unpublished reports from our laboratory demonstrate that a 46% reduction in the caspase 9a/9b mRNA ratio corresponds to a 62% reduction in caspase 9 activity. Conversely, a two-fold increase in the caspase 9a/9b mRNA ratio corresponds to a 2.7-fold increase in the caspase 9 activity. Thus, modulation of the

alternative splicing of caspase 9 could greatly influence the cell's decision to undergo apoptosis.

Caspase 9 is a critical regulator in the apoptotic pathway and its activation is required for cell death induced by various chemotherapies, stress agents, and radiation[167,168,170-176]. Research published by Soengas et al. in 1999 revealed that caspase 9 (caspase 9a) plays a distinct role in oncogenesis, acting as a tumor suppressor[179]. Specifically, caspase 9 was shown to act downstream of p53 and have a profound effect on suppressing the tumorigenicity of MEFs expressing oncogenic ras (h-Ras^{V12}) and c-Myc[179]. The data presented demonstrates that knockout of caspase 9a can facilitate oncogenic transformation and tumor development.

In 1999, a novel isoform of caspase 9 was cloned from human liver cDNA, termed caspase-9S[167]. In the same year, an independent study by Srinivasula et al. also identified the transcriptional variant of caspase 9, designating it caspase 9b[168]. Both reports demonstrated that caspase 9b functions as an endogenous dominant-negative isoform of the full-length caspase 9, acting to inhibit recruitment and activation of procaspase 9[167,168]. In addition, Seol and Billiar were the first to demonstrate that the alternative spliced caspase 9 isoform, caspase 9b, is expressed in significant levels in various cancer cell lines[167]. In this study, we tested the hypothesis that the alternative splicing of caspase 9 was dysregulated in a pro-survival manner (lower caspase 9a/9b mRNA ratio) in NSCLC patient tumor samples consisting of adenocarcinoma, squamous cell carcinoma, and large cell carcinoma histologies. Additionally, we hypothesize that one of the major mitogenic

signaling pathways regulates the alternative splicing of caspase 9 to favor the production of caspase 9b, thereby decreasing the caspase 9a/9b mRNA ratio.

2.2. Materials and Methods

2.2.1. Cell Culture

A549, H2347, H358, H226, H2170, H596, H1792, H1299, H520, H1703 and H292 cells were grown in 50% RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) and 50% Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with L-glutamine, 10% (v/v) fetal bovine serum (Sigma), 100 units/ml penicillin G sodium (Invitrogen Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Carlsbad, CA). H2030 and H838 cells were grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum (Sigma), 100 units/ml penicillin G sodium (Invitrogen Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Carlsbad, CA). All adenocarcinoma and squamous carcinoma cell lines were purchased from ATCC (Rockville, MD, USA). HBEC-3KT cells, a generous gift from J.D. Minna, were cultured with keratinocyte serum-free medium containing 50μg/ml bovine pituitary extract and 5ng/ml recombinant epidermal growth factor (Life Technologies, Gaithersburg, MD). NHBE cells, purchased from Clonetics (Cambrex Bio Science, Lonza Group Ltd, Switzerland) were maintained in basal bronchial epithelial growth media (BEGM) supplemented with SingleQuot Kits-growth factors, cytokines and supplements (Cambrex, Lonza, Lonza Group Ltd, Switzerland). All cells were maintained at less than 80% confluency under standard

incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37 °C). For all comparison studies between cell lines, 1.2 x 10^5 cells were plated in tissue culture plates (6 well dish). The following day, media was removed, cells were washed with 1 x PBS and plated in keratinocyte serum-free medium without supplements overnight. Total RNA and/or protein was then isolated for analysis.

2.2.2. Quantitative/Competitive RT-PCR

Total RNA from cell lines were isolated using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen™, Carlsbad, CA) and oligo (dT) as the priming agent. After 50 min of incubation at 42°C, the reactions were terminated by heating at 70°C for 15 min. Template RNA was then removed using RNase H (Invitrogen, Carlsbad, CA). To quantitatively evaluate the expression of endogenous caspase 9 splice variants, an upstream 5' primer to caspase-9 (5'-GCT CTT CCT TTG TTC ATC TCC-3') and a 3' primer (5'-CAT CTG GCT CGG GGT TAC TGC-3') (Integrated DNA Technologies, Inc., Coralville, IA) were used. Using these primers, 20% of the reverse transcriptase reaction was amplified for 25 cycles (94°C, 30 s melt; 57°C, 30 s anneal; 72°C, 1 min extension) using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). Gene products produced from endogenous caspase 9 PCR resulted in a 1248 base pair caspase 9a splice variant and 798 base pair caspase 9b splice variant. The primers used to evaluate caspase 8 splice variants are sense (5'-GCA TTA GGG ACA GGA ATG GA-3') and

antisense (5'-GAA GGG GAC TTC AAA CCA GTG-3'). The PCR conditions for caspase 8 were 95ºC for 5 mins followed by 25 cycles of 94ºC for 30s, 60ºC for 30s and 72ºC for 1min. The primers used to evaluate caspase 2 splice variants are sense (5'-ACT TGC TGC CTA AGA GGG GTC- 3') and antisense (5'-CTT GGG CAG TTG GCG TTG TC -3'). The PCR conditions for caspase 2 were 95ºC for 5 mins followed by 25 cycles of 94ºC for 30s, 64ºC for 30s and 72ºC for 1min. The primers used to evaluate Bcl-x splice variant were (5'-GAG GCA GGC GAC GAG TTT GAA-3') and (5'-TGG GAG GGT AGA GTG GAT GGT-3'). The PCR conditions for analyzing the expression of Bcl-x splice variants were 95ºC for 5 mins followed by 25 cycles of 94ºC for 30s, 58ºC for 30s and 72ºC for 1min. The final PCR products were resolved on a 5% TBE acrylamide gel electrophoresis, stained with SYBR® Gold (Invitrogen™, Carlsbad, CA) and visualized using a Molecular Imager® FX (Bio-Rad) with a 488 nm EX (530 nm BYPASS) laser.

2.2.3. Western Immunoblotting

Cells were lysed using CelLytic™ lysis Buffer (Sigma-Aldrich) supplemented with protease Inhibitor cocktail (Sigma-Aldrich). Protein samples (5 μg) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PDVF) (Bio-Rad) and blocked in 5% milk/1 X PBS – 0.1% Tween (M-PBS-T) for 2 h. Primary antibodies were anti-caspase 9 (1:1,000, Assay Designs), anti-Akt1 (1:1,000, Santa Cruz), anti-Akt2 (1:1,000, Santa Cruz), anti-PDK-1 (1:1,000, Santa Cruz), anti-T7 tag (1:10,000, Novagen), and anti-β-actin (1:5,000, Sigma-Aldrich). Secondary antibodies were HRP-conjugated

goat anti-mouse or anti-rabbit (1:5,000, Sigma-Aldrich). Immunoblots were developed using Pierce enhanced chemiluminescence (ECL) reagents and Bio-Max film.

2.2.4. Inhibitor studies and adenoviral infection

For Inhibitor studies, 1.2 x 10^5 cells were plated into 6-well tissue culture plates. The following day media was removed and replaced with the appropriate complete growth media. Cells were subsequently treated with sham control (1:1000) or the appropriate concentration of active inhibitor (1:1000) (Calbiochem). Twenty-four hours post-treatment, total RNA and/or protein was isolated. For adenoviral infection, 1.2 x 10^5 cells were plated into 6-well tissue culture plates. Two hours later, cells were infected with either constitutively active AKT2 adenovirus, PTEN adenovirus, or Null adenovirus (50MOI) (Vector Biolabs). Forty-eight hours later, total RNA and/or protein was isolated.

2.2.5. Silence interfering RNA transfection

For inhibition of Akt1, Akt2, and PDK-1 expression, cell lines were transfected with either Akt1 SMARTpool multiplex, Akt2 SMARTpool multiplex, PDK-1 SMARTpool multiplex or scrambled control siRNA (Dharmacon; Lafayette, CO) using Dharmafect 1 transfection reagent (Dharmacon; Lafayette, CO) following the manufacturer's protocol. Briefly, cell lines were plated in six-well tissue culture dishes and allowed to rest overnight. At 50% confluence, cells were transfected with siRNA [100nM] using Dharmafect 1 in Opti-Mem I reduced-serum medium. Forty-eight hours post-transfection, RNA and/or protein were isolated.

2.3. Results

2.3.1. The alternative splicing of caspase 9 is dysregulated in a variety of NSCLC tumors and transformed lung cancer cells.

In this study, we examined the hypothesis that the alternative splicing of caspase 9 was dysregulated (in a pro-survival manner (e.g. lower C9a/C9b mRNA ratio)) in NSCLC tumors, including adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. Utilizing total RNA from pathologist-verified human NSCLC tumor samples, quantitative/competitive RT-PCR analysis was performed to determine the degree of dysregulation in the caspase 9a/9b mRNA ratio as compared to matched, normal lung tissue controls (Table 2-1). Tumor samples were categorized into three groups respectively: normal, a caspase 9a/9b mRNA ratio of >3.3; moderately dysregulated, a caspase 9a/9b mRNA ratio of 2.3-3.3; and highly dysregulated, a caspase 9a/9b mRNA ratio <2.3 (Figure 2-1a,b). A caspase 9a/9b mRNA ratio of ≥4.0 is observed in non-tansformed cells. Of the NSCLC samples examined, 36% demonstrated a moderately dysregulated caspase 9a/9b mRNA ratio (*N*=149). Even more dramatic, 42% of tumors demonstrated a > 50% decrease in the caspase 9a/9b mRNA ratio. Thus, the ratio of caspase 9a/9b mRNA is significantly lower in a high percentage of NSCLC tumors correlating with loss of activity of the tumor suppressor, caspase 9a.

Table 2-1. Pathology-verified patient normal tissue and tumor tissue samples analyzed for their caspase 9a/9b mRNA ratio. Each sample is detailed with diagnosis, group, and ratio of caspase 9a/9b mRNA.

Pathology-verified patient tumor samples.

Sample	Diagnosis	Group	Caspase 9a/9b ratio
1-I	Normal	Normal	4.0
2-I	Normal	Normal	4.0
3-I	Normal	Normal	3.5
5-I	Normal	Normal	4.0
6-I	Normal	Normal	3.9
7-I	Normal	Normal	4.0
9-I	Squamous cell carcinoma	High	2.0
11-I	Squamous cell carcinoma	Moderate	3.0
13-I	Squamous cell carcinoma	High	2.0
14-I	Large cell carcinoma	Moderate	2.3
17-I	Non-small cell carcinoma	High	2.0
19-I	Large cell carcinoma	Moderate	2.5
20-I	Squamous cell carcinoma	High	1.0
21-I	Squamous cell carcinoma	Normal	4.0
24-I	Squamous cell carcinoma	Normal	3.7
26-I	Squamous cell carcinoma	Moderate	3.2
28-I	Large cell carcinoma	Normal	3.4
30-I	Squamous cell carcinoma	High	1.8
31-I	Non-small cell carcinoma	Moderate	3.0
32-I	Squamous cell carcinoma	Moderate	3.0
38-I	Squamous cell carcinoma	High	1.0
40-I	Squamous cell carcinoma	Normal	4.0
41-I	Large cell carcinoma	Normal	3.5
43-I	Squamous cell carcinoma	Moderate	2.3
44-I	Squamous cell carcinoma	Moderate	2.6
46-I	Adenocarcinoma	High	1.2
47-I	Squamous cell carcinoma	Moderate	2.8
48-I	Non-small cell carcinoma	Moderate	2.3
2-II	Large cell carcinoma	High	1.5
6-II	Squamous cell carcinoma	High	2.0
10-II	Large cell carcinoma	Moderate	2.6
11-II	Squamous cell carcinoma	Moderate	3.0
14-II	Squamous cell carcinoma	Moderate	2.7
17-II	Squamous cell carcinoma	High	2.0
18-II	Squamous cell carcinoma	High	1.1
21-II	Squamous cell carcinoma	Moderate	2.3
23-II	Non-small cell carcinoma	Moderate	2.3
24-II	Squamous cell carcinoma	Moderate	2.4
27-II	Squamous cell carcinoma	High	2.0
31-II	Squamous cell carcinoma	Normal	4.5
32-II	Squamous cell carcinoma	Normal	3.6
35-II	Squamous cell carcinoma	High	1.0
36-II	Squamous cell carcinoma	High	1.1
37-II	Squamous cell carcinoma	High	1.3
38-II	Large cell carcinoma	High	1.7
3-III	Normal	Normal	3.9
7-III	Normal	Normal	**3.8**
9-III	Squamous cell carcinoma	Normal	3.6
10-III	Large cell carcinoma	High	2.0
12-III	Bronchioloalveolar carcinoma	Normal	3.7
15-III	Squamous cell carcinoma	Normal	4.5
16-III	Squamous cell carcinoma	Moderate	2.3
19-III	Large cell carcinoma	High	1.0
20-III	Non-small cell carcinoma	High	2.0
23-III	Squamous cell carcinoma	Moderate	3.2
25-III	Squamous cell carcinoma	Moderate	2.7
27-III	Squamous cell carcinoma	High	1.8
30-III	Squamous cell carcinoma	Normal	4.0
31-III	Squamous cell carcinoma	High	1.1
32-III	Squamous cell carcinoma	Normal	3.5
33-III	Adenosquamous carcinoma	High	1.7
34-III	Large cell carcinoma	High	0.9
39-III	Adenosquamous carcinoma	Normal	3.7
43-III	Non-small cell carcinoma	Normal	3.9
44-III	Non-small cell carcinoma	High	1.6
45-III	Squamous cell carcinoma	High	1.0

Matched-pair Analysis (Normal tissue vs. Tumor tissue)

1-IV	Normal 1	Normal	4.0
2-IV	Squamous cell carcinoma 1	High	0.9
5-IV	Normal 2	Moderate	2.8
6-IV	Large cell carcinoma 2	High	1.8
9-IV	Normal 3	Normal	4.7
10-IV	Large cell carcinoma 3	High	1.7
11-IV	Normal 4	Normal	3.7
12-IV	Large cell carcinoma 4	Normal	3.7
17-IV	Normal 5	Normal	4.5
18-IV	Squamous cell carcinoma 5	High	1.0
21-IV	Normal 6	Normal	4.0
22-IV	Non-small cell carcinoma 6	High	2.1
23-IV	Normal 7	Normal	3.8
24-IV	Squamous cell carcinoma 7	Normal	3.5
25-IV	Normal 8	Normal	3.5
26-IV	Squamous cell carcinoma 8	Moderate	2.9
31-IV	Normal 9	Normal	3.6
32-IV	Squamous cell carcinoma 9	Moderate	2.4

Table 2-1 (cont.) Pathologist-verified patient normal tissue and tumor tissue samples analyzed for their caspase 9a/9b mRNA ratio. Each sample is detailed with diagnosis, group, and ratio of caspase 9a/9b mRNA.

Pathology-verified patient tumor samples.

Sample	Diagnosis	Group	Caspase 9a/9b ratio
1-I	Normal	Normal	4.0
7-I	Normal	Normal	4.0
10-I	Not reported	High	1.5
12-I	Not reported	High	1.5
15-I	Adenocarcinoma of lung	High	1.5
16-I	Adenocarcinoma of lung	High	1.5
25-I	Adenocarcinoma of lung	Moderate	3.1
27-I	Adenocarcinoma of lung	Moderate	2.8
33-I	Adenocarcinoma of lung	High	2.0
34-I	Adenocarcinoma of lung	High	1.8
36-I	Adenocarcinoma of lung	High	1.0
37-I	Adenocarcinoma of lung	High	2.1
39-I	Adenocarcinoma of lung	High	1.8
42-I	Adenocarcinoma of lung	High	1.6
45-I	Adenocarcinoma of lung	High	1.7
4-II	Adenocarcinoma of lung	Moderate	2.3
5-II	Adenocarcinoma of lung	High	1.2
7-II	Adenocarcinoma of lung	Normal	4.0
8-II	Adenocarcinoma of lung	Moderate	3.1
9-II	Adenocarcinoma of lung	High	1.8
12-II	Adenocarcinoma of lung	Moderate	2.3
13-II	Adenocarcinoma of lung	High	1.7
15-II	Adenocarcinoma of lung	High	1.1
16-II	Adenocarcinoma of lung	High	1.0
19-II	Adenocarcinoma of lung	High	2.1
22-II	Adenocarcinoma of lung	High	2.1
25-II	Adenocarcinoma of lung	Moderate	2.7
26-II	Adenocarcinoma of lung	Moderate	3.3
28-II	Adenocarcinoma of lung	Normal	5.1
29-II	Adenocarcinoma of lung	Moderate	2.8
30-II	Adenocarcinoma of lung	Normal	4.0
33-II	Adenocarcinoma of lung	Moderate	2.4
34-II	Adenocarcinoma of lung	High	1.1
40-II	Adenocarcinoma of lung	Moderate	2.3
41-II	Adenocarcinoma of lung	Normal	3.5
43-II	Adenocarcinoma of lung	High	1.7
44-II	Adenocarcinoma of lung	Moderate	3.1
45-II	Adenocarcinoma of lung	Moderate	2.3
46-II	Adenocarcinoma of lung	High	1.1
47-II	Adenocarcinoma of lung	High	1.7
48-II	Adenocarcinoma of lung	High	2.2
K-1	Adenocarcinoma of lung	Moderate	2.9
K-3	Adenocarcinoma of lung	Moderate	3.2
K-4	Adenocarcinoma of lung	Moderate	3.4
K-5	Adenocarcinoma of lung	Moderate	3.1
K-6	Adenocarcinoma of lung	Moderate	3.3
K-7	Adenocarcinoma of lung	Normal	4.4
K-8	Adenocarcinoma of lung	Moderate	2.8
K-9	Adenocarcinoma of lung	Moderate	2.4
K-10	Adenocarcinoma of lung	Normal	4.0
K-11	Adenocarcinoma of lung	Moderate	3.1
K-19	Adenocarcinoma of lung	Normal	4.5
K-20	Adenocarcinoma of lung	Moderate	2.7
K-21	Adenocarcinoma of lung	Moderate	3.1
K-24	Adenocarcinoma of lung	High	2.2
K-26	Adenocarcinoma of lung	High	0.9
4-III	Normal	Normal	4.0
5-III	Normal	Normal	3.6
11-III	Adenocarcinoma of lung	High	2.2
13-III	Adenocarcinoma of lung	Normal	3.7
14-III	Adenocarcinoma of lung	High	2.1
18-III	Adenocarcinoma of lung	Normal	3.9
21-III	Adenocarcinoma of lung	Normal	4.1
24-III	Adenocarcinoma of lung	High	1.6
26-III	Adenocarcinoma of lung	Moderate	3.0
28-III	Adenocarcinoma of lung	High	1.3
29-III	Adenocarcinoma of lung	Moderate	2.4
36-III	Adenocarcinoma of lung	Normal	4.3
38-III	Adenocarcinoma of lung	Moderate	3.1
40-III	Adenocarcinoma of lung	Normal	3.9
42-III	Adenocarcinoma of lung	Normal	3.9
46-III	Adenocarcinoma of lung	High	1.5
47-III	Adenocarcinoma of lung	Moderate	2.3
48-III	Adenocarcinoma of lung	Moderate	2.4

Matched-pair Analysis (Normal tissue vs. Tumor tissue)

3-IV	Normal 1	Normal	4.0
4-IV	Adenocarcinoma of lung 1	High	0.9
7-IV	Normal 2	Normal	3.6
8-IV	Adenocarcinoma of lung 2	High	1.1
13-IV	Normal 3	Normal	3.9
14-IV	Adenocarcinoma of lung 3	High	1.5
15-IV	Normal 4	Normal	3.7
16-IV	Adenocarcinoma of lung 4	Moderate	2.6
27-IV	Normal 5	Normal	3.5
28-IV	Adenocarcinoma of lung 5	High	1.0
33-IV	Normal 6	Normal	4.0
34-IV	Adenocarcinoma of lung 6	Normal	4.0
37-IV	Normal 7	Normal	4.3
38-IV	Adenocarcinoma of lung 7	High	2.2
39-IV	Normal 8	Normal	4.0
40-IV	Adenocarcinoma of lung 8	Moderate	2.3
41-IV	Normal 9	Normal	3.8
42-IV	Adenocarcinoma of lung 9	High	2.0
45-I	Normal 10	Normal	3.7
46-IV	Adenocarcinoma of lung 10	Normal	3.9

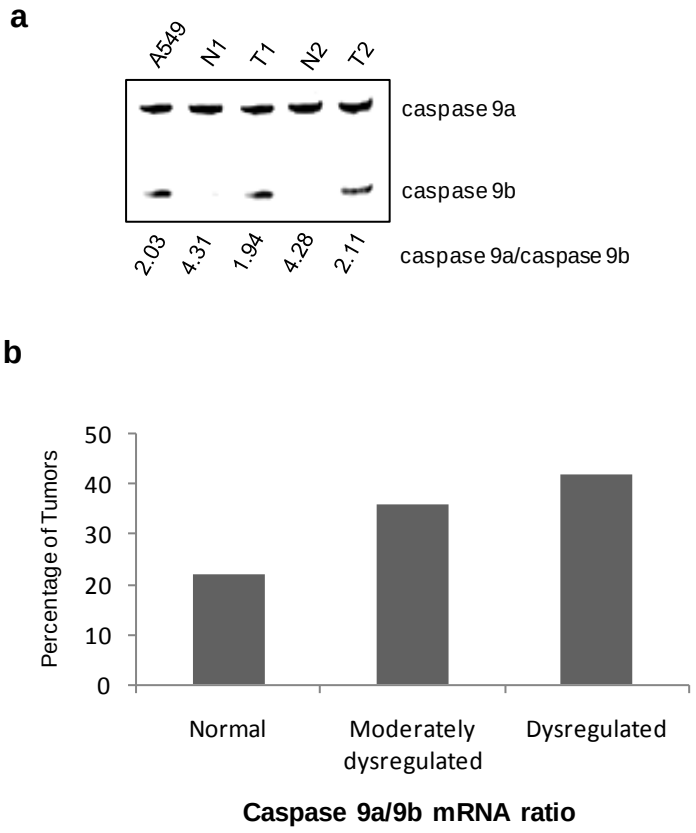

Figure 2-1. The caspase 9a/9b mRNA ratio is dysregulated in NSCLC tumors. A population of cDNAs from pathologist-verified lung adenocarcinomas, squamous cell carcinomas, and large cell carcinomas (Origene; Rockville, MD) underwent quantitative/competitive PCR for expression of caspase 9 pre-mRNA splice variants. **a)** Representation of the matched pair analysis used to determine the degree of caspase 9a/9b dysregulation in NSCLC tumor samples where N is normal tissue and T is tumor tissue. A549 samples were analyzed in parallel to provide a caspase 9a/9b mRNA ratio control. Each cDNA is detailed with diagnosis, tumor grade, caspase 9a/9b ratio group, and caspase 9a/9b mRNA ratio in Table 2-1. **b)** Quantitative/competitive PCR analysis of caspase 9 splice variants demonstrate that 36% of NSCLC tumors present a moderately dysregulated caspase 9a/9b mRNA ratio (C9a/9b ratio of 2.3-3.3) and 42% of NSCLC tumors present a highly dysregulated caspase 9a/9b mRNA ratio (C9a/9b ratio <2.3) (N=147), as determined by densitometric analysis of PCR products.

Since solid tumors and normal tissue controls are in many cases a combination of cell types, we then examined a pure population of non-transformed lung epithelial cells, specifically normal human bronchial epithelial cells (NHBE) and immortalized HBEC-3 cells, for the ratio of caspase 9a/9b mRNA in comparison to the transformed lung epithelial cell lines, A549, H838, H2347, H358, H2030, H226, H2170, H596, H1792, H1299, H520, H1703, H292 cells (Table 2-2). HBEC-3 cells present with a normal caspase 9a/9b mRNA ratio of 4.02±0.15 as do NHBE cells which present with a caspase 9a/9b mRNA ratio of 4.15±0.23 (Figure 2-2a). In contrast, 9 of 11 transformed lung epithelial cells grown under the exact same culture conditions demonstrated a significant decrease in the caspase 9a/9b mRNA ratio. Importantly, the disproportionate ratio of caspase 9a/9b mRNA observed in the transformed lung epithelial cell lines translated to a disproportionate ratio of caspase 9a/9b protein expression (Figure 2-2b). These data indicate that a significant portion of lung adenocarcinoma tumors and transformed lung epithelial cells demonstrate severe dysregulation of the alternative splicing of caspase 9 to favor a pro-survival/pro-oncogenic phenotype.

Table 2-2. Characterization of NSCLC cell lines

Cell line	NSCLC Histology	Mutational Status	Capase 9a/9b ratio	Degree of Dysregulation
A549	AD	WT p53, k-Ras ^{V12} mutation	1.92 ± 0.16	D
H520	SQ	p53 mutation, WT Ras	2.25 ± 0.15	D
H1703	ADSQ	p53 mutation, WT Ras	3.32 ± 0.08	N
H292	SQ	WT p53, WT Ras	1.72 ± 0.20	D
H2030	AD	WT p53, k-Ras^{V12} mutation	2.06 ± 0.11	D
H838	AD	p53 mutation, WT Ras	1.42 ± 0.18	D
H596	ADSQ	p53 mutation, WT Ras	2.73 ± 0.09	M
H2170	SQ	p53 mutation, WT Ras	3.65 ± 0.09	N
H2347	AD	WT p53, N-Ras mutation	3.68 ± 0.10	N
H226	SQ	WT p53, WT Ras	2.15 ± 0.12	D
H358	BA	WT p53, k-Ras^{V12} mutation	3.02 ± 0.16	M

AD, adenocarcinoma; SQ, squamous cell carcinoma; ADSQ, adenosquamous cell carcinoma; BA, Bronchioalveolar carcinoma; D, dysregulated; M, moderately dysregulated; N, normal.

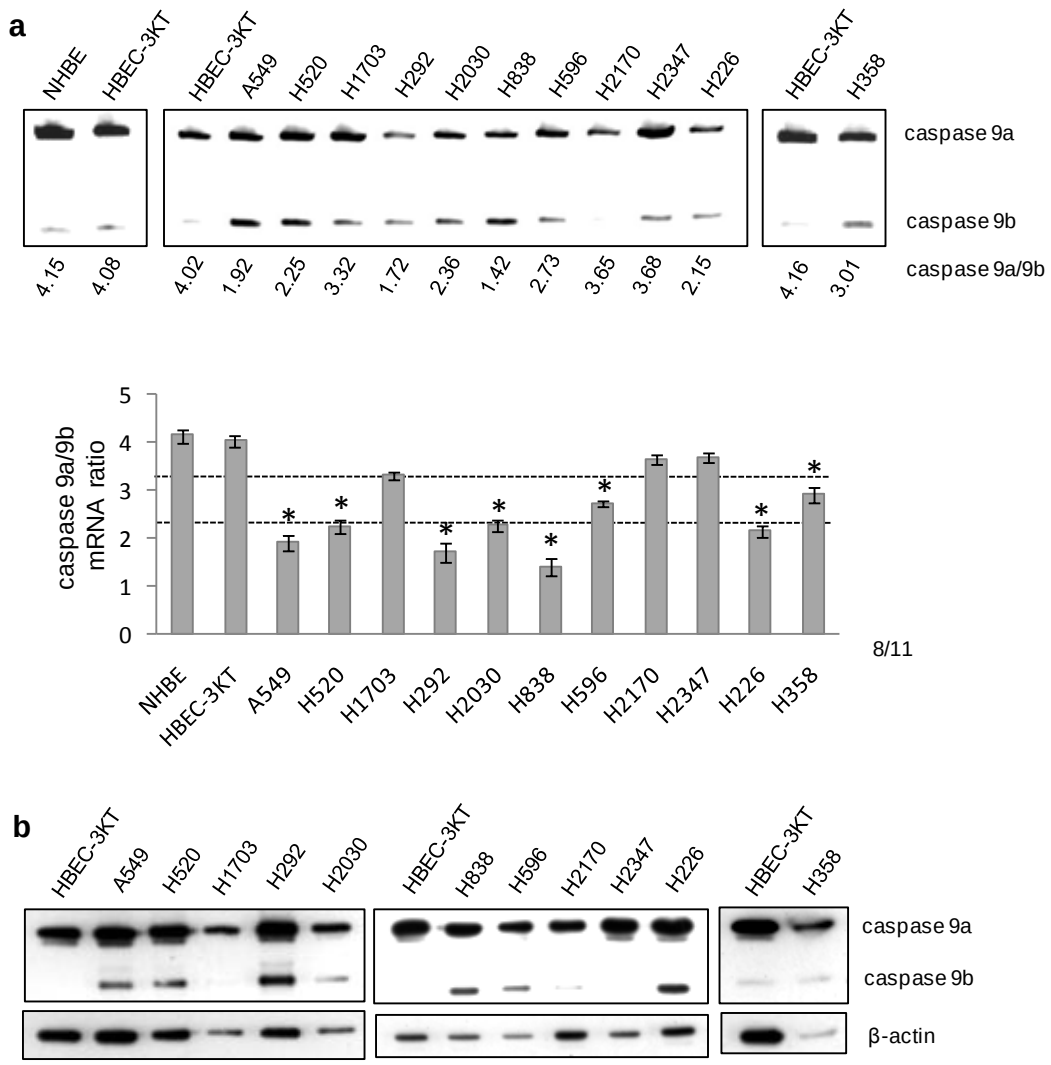

Figure 2-2. The caspase 9a/9b mRNA ratio is dysregulated in NSCLC cell lines. Lung adenocarcinoma and squamous cell carcinoma cell lines were analyzed for the expression of caspase 9 splice variants. **a)** Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratio of total RNA isolated from a variety of NSCLC cell lines and non-transformed HBEC-3KT cells. Below each cell line and their corresponding caspase 9a/9b mRNA ratio is depicted graphically. Eight of eleven NSCLC cell lines present with a dysregulated caspase 9a/9b mRNA ratio. **b)** In parallel, total protein was isolated and analyzed by western blotting with anti-caspase 9 antibody to compare the expression of caspase 9a and caspase 9b splice variants.

2.3.2. The PI3-Kinase pathway regulates the alternative splicing of caspase 9.

Since the ratio of caspase 9a/9b mRNA is highly dysregulated in NSCLC, we hypothesized that one of the major mitogenic signaling pathways activated by oncogenic mutation regulates the alternative splicing of caspase 9 pre-mRNA to favor the production of anti-apoptotic caspase 9b. To investigate this hypothesis, these pathways were examined for effects on the ratio of caspase 9a/9b mRNA utilizing small-molecule inhibitors at doses and times well characterized in the scientific literature and previously utilized in studies on A549 cells[206-212]. Treatment of A549 cells with the following inhibitors, the MAP Kinase inhibitor PD98059 [50μM] and SB202190 [10μM], MEK 1/2 inhibitor U0126 [10μM], Rho-kinase inhibitor Y-27632 [10μM], Casein Kinase II inhibitor DMAT [10μM], Src kinase inhibitor I [25μM], and JNK Inhibitor II, SP600125, [10μM] had no effect on the alternative splicing of caspase 9 (Table 2-3). In contrast, treatment of A549 lung adenocarcinoma cells with the PI3-K inhibitor, LY294002 [50μM], resulted in a significant increase in the ratio of caspase 9a/9b splice variants compared to the inactive, structurally-related compound, LY303511 (Figure 2-3a,b). Specifically, the caspase 9a/9b mRNA ratio increased from 2.10 ± 0.18 for control samples to 3.91 ± 0.08 for inhibitor treated samples ($p < 0.0008$ (n=6), corresponding to approximately a 2-fold increase in the caspase 9a/9b ratio. Since PI3-Kinase inhibition increases caspase 9a with a parallel decrease in caspase 9b, these data demonstrate that PI3-Kinase regulates the alternative splicing of caspase 9 in an anti-apoptotic/pro-survival manner. Furthermore, the inhibition of PI3Kinase effectively returned the caspase 9a/9b mRNA ratio to the ratio observed in non-transformed lung

epithelial cells. Importantly, this effect on the caspase 9a/9b ratio translated to the protein level (Figure 2-3b).

Table 2-3. Mitogen signaling pathways demonstrating no involvement in the alternative splicing of caspase 9 as analyzed by small molecule inhibitors.

Mitogenic Pathways Investigated	Inhibitor	Concentration
Mitogen Activated Protein Kinase (MAPK)	U0126	10μM
	PD98059	50μM
	SB202190	10μM
Rho Kinase (ROCK)	Y-27632	10μM
Casein Kinase (CK)	DMAT	10μM
Src Family Tyrosine Kinases	Src Kinase Inhibitor I	25μM
Protein Kinase C	Go6983	10μM
	Go6976	10μM
mTOR	Rapamycin	10μM
Glycogen Synthase Kinase (GSK)	SB216763	10μM
c-Jun-N-Terminal Kinase (JNK/SAP Kinase)	SP600125	10μM

To determine the translatability of the mechanism, H838, H2030, H358, H292, and H520 cells (all presenting a dysregulated caspase 9a/9b mRNA ratio) were also treated with LY294002 (Figure 2-3c). As with the A549 cells, the caspase 9a/9b mRNA ratio was dramatically increased (H838 cells from 1.24 ± 0.05 to 3.29 ± 0.19; H2030 cells from 2.30 ± 0.08 to 3.74 ± 0.08; H358 cells from 3.11 ± 0.09 to 4.13 ± 0.12; H292 cells from 2.01 ± 0.09 to 4.11 ± 0.11; and H520 cells from 2.35 ± 0.08 to 3.91 ± 0.13). Therefore, the ability of the PI3-Kinase pathway to regulate the alternative splicing of caspase 9 translates to multiple NSCLC cell lines.

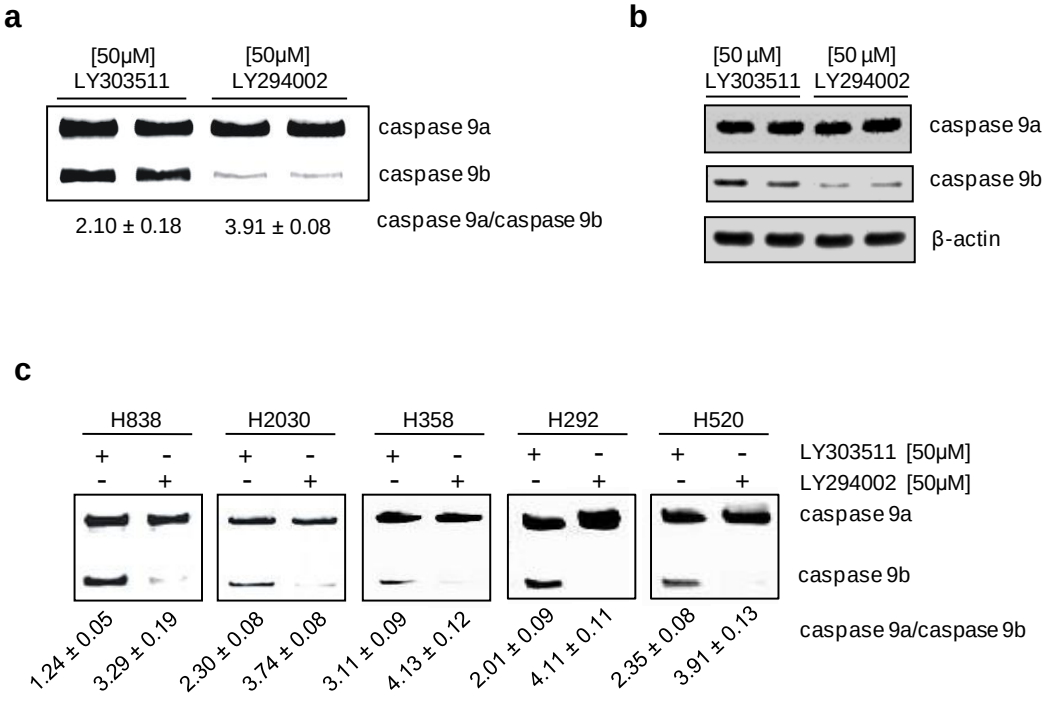

Figure 2-3. Effect of PI3-Kinase inhibition on the alternative splicing of caspase 9 pre-mRNA. a) Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A549s treated with either LY294002 [50uM] or structurally inactive control LY303511 [50uM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densotimetric analysis of RT-PCR fragments. **b)** Twenty four hours post-treatment, total protein was isolated and analyzed by western blotting with anti-caspase 9 antibody to compare the expression of caspase 9a and caspase 9b splice variants. **c)** To determine the translatability of the mechanism, H838s, H2030s, H358s, H292s, and H520s were treated with either LY294002 [50uM] or structurally inactive control LY303511 [50uM]. Twenty four hours post-treatment, total RNA was isolated and quantitative/competitive RT-PCR performed. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control, p values < 0.01 were considered significant. Data are representative of three separate determinations on two separate occasions.

2.3.3 Akt, downstream of PI3-Kinase, regulates the alternative splicing of caspase 9.

Akt/PKB, SGK, PKCξ, and PKCδ are downstream of PI3-Kinase, all of which have been well described in the literature[213]. To investigate the downstream effector of PI3-Kinase responsible for regulating the alternative splicing of caspase 9, well-characterized small-molecule inhibitors in conjunction with RNAi technology was employed. Treatment of A549 cells with the Protein Kinase C inhibitors GÖ6976 [10μM] and GÖ6983 [10μM] (Table 2-3; Figure 2-4a,b), resulted in no significant change in the caspase 9a/b mRNA ratio in A549 cells. Conversely, treatment of A549 cells with the Akt inhibitor, AKT VIII [25μM] and the phosphatidylinositol analog, SH-5 [10μM], which inhibits the activation of Akt, resulted in an increased caspase 9a/9b mRNA ratio to the same extent as PI3-Kinase inhibition (from 2.10 ± 0.11 for DMSO control samples to 4.03 ± 0.13 for Akt inhibitor VIII-treated samples (n=6) $p<0.01$; and from 2.10 ± 0.08 for DMSO control samples to 3.73 ± 0.13 for SH-5-treated samples (n=4) $p<0.01$) (Figure 2-5a,b). In addition, treatment of A549 cells with the PDK1 inhibitor OSU03012 [10μM] or downregulation of PDK1 using siRNA (Figure 2-5c,d) resulted in a significantly increased caspase 9a/9b mRNA ratio (from 2.25 ± 0.13 for control samples to 4.05 ± 0.22 for inhibitor treated samples (n=4), $p<0.01$; and from 2.13 ± 0.09 for siRNA control samples to 3.78 ± 0.21 for siPDK1 treated samples (n=4) $p<0.01$).

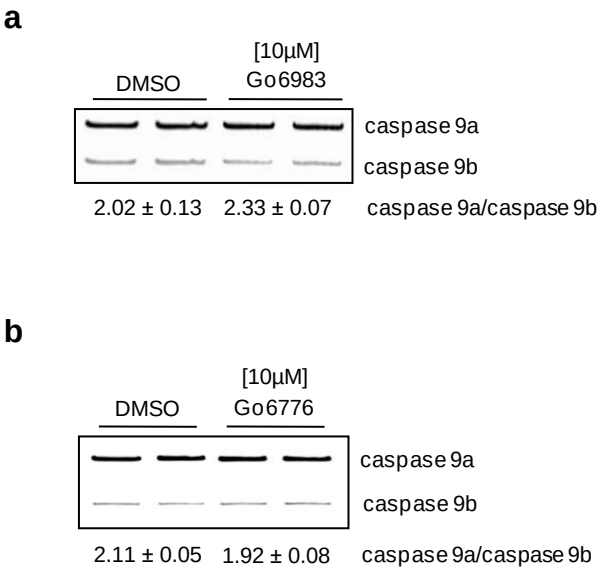

Figure 2-4. Inhibition of Protein Kinase C (PKC) does not affect the alternative splicing of caspase 9 pre-mRNA. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A549s treated with either DMSO control (0.1%), **a)** Gö6983 [10μM] or **b)** Gö6776 [10μM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as mewereans ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 considered significant. Data are representative of three separate determinations on two separate occasions.

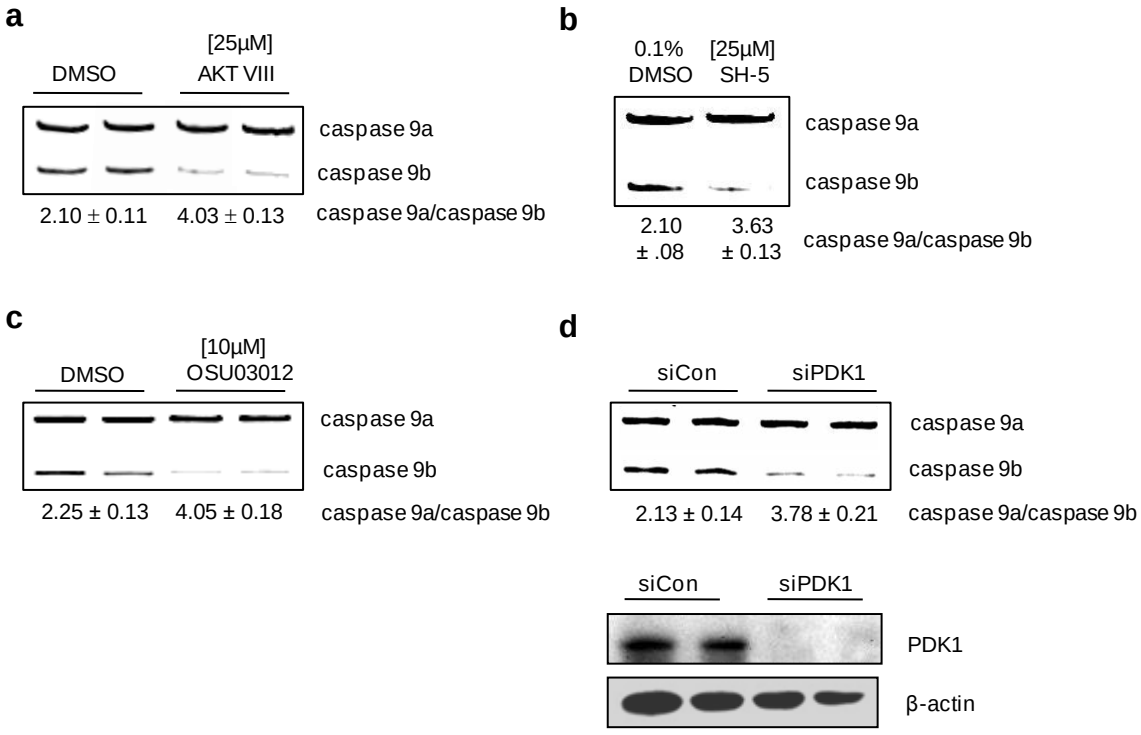

Figure 2-5. Inhibition of Akt activation regulates the alternative splicing of caspase 9 pre-mRNA. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A459 cells treated DMSO control or **a)** Akt inhibitor VIII [25μM], **b)** SH-5 [25μM], or **c)** OSU03012 [10μM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. **d)** Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A459**s** transfected with scrambled siRNA [100nM] or PDK1 siRNA [100nM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. To confirm protein knockdown, total protein lysate was subjected to western immunoblotting against anti-PDK1 antibody. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant. Data are representative of three separate determinations on two separate occasions.

Since Akt inhibition increases caspase 9a with a parallel decrease in caspase 9b, these data demonstrate that an Akt isoform regulates the alternative splicing of caspase 9 in an anti-apoptotic/pro-survival manner. This effect was specific for the alternative splicing of caspase 9 as treatment of A549 cells with AKT VIII inhibitor demonstrated no effect on the alternative splicing of caspase 8, caspase 2, and Bcl-x pre-mRNA (Figure 2-6). Furthermore, co-treatment of A549 cells with both AKTVIII and LY294002 could not further increase the caspase 9a/9b mRNA ratio demonstrating a linear pathway and Akt as the downstream effector for PI_3 kinase (Figure 2-7).

To examine the Akt isoform responsible for regulating the alternative splicing in A549 cells, small interference RNA (siRNA) technology was again utilized. Western immunoblotting demonstrated that A549 cells were essentially devoid of Akt3, but expressed both Akt1 and Akt2, thus siRNA was utilized for only Akt1 and Akt2. Downregulation of Akt1 using multiplex siRNA resulted in no change in the caspase 9a/9b mRNA ratio with respect to control siRNA treated cells ($p>0.34$) (Figure 2-8a). In contrast, downregulation of Akt2 using multiplex siRNA resulted in a dramatic increase in the caspase 9a/b ratio from 2.10 ± 0.05 to 4.09 ± 0.22, $p<0.01$ ($n=5$) (Figure 2-8b). As with the previous studies on PI3-Kinase, this effect on the caspase 9a/9b mRNA ratio translated to the protein level (Figure 2-8c).

To further validate a role for Akt in regulating the alternative splicing of caspase 9, ectopic expression of a constitutively-active Akt2 (always membrane bound via myristoylation) induced a significant decrease in the caspase 9a/9b mRNA ratio (Figure 2-9a). Furthermore, ectopic expression of PTEN, a negative regulator of the Akt signaling

pathway, induced the contrasting effect (Figure 2-9b). Therefore, Akt2 regulates the alternative splicing of caspase 9 via PIP_3 membrane interactions in A549 cells, and also requires PDK-1 phosphorylation.

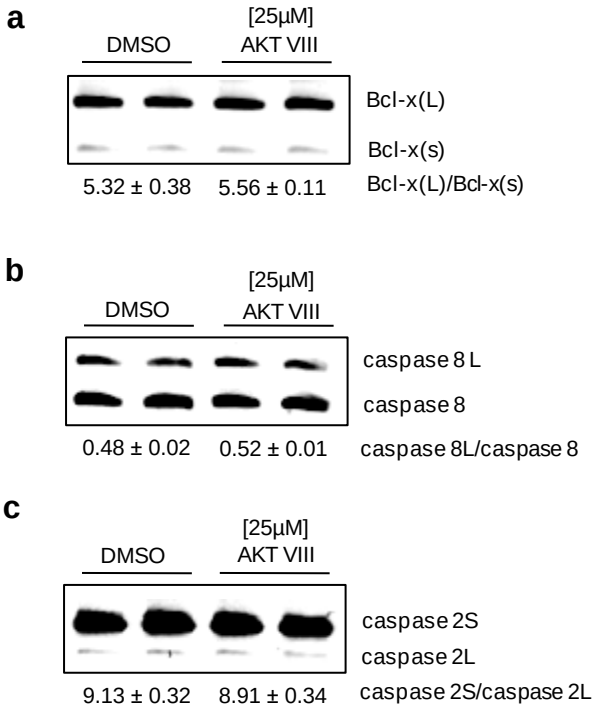

Figure 2-6. Inhibiton of Akt does not affect the alternative splicing of Bcl-x, capase 8, or caspase 2. A459 cells were treated with Akt inhibitor VIII [25µM]. Twenty-four hours post-treatment, RNA was isolated and quantitative/competitive RT-PCR performed for **a)** Bcl-x splice variants, **b)** caspase 8 splice variants, and **c)** caspase 2 splice variants. The ratio of PCR products was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d.

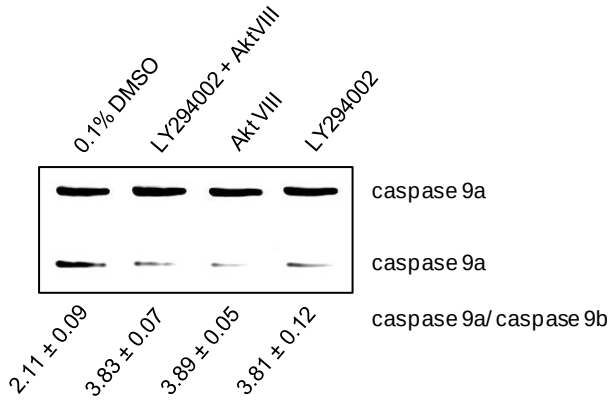

Figure 2-7. The alternative splicing of caspase 9 is regulated by PI3-Kinase/Akt in a linear manner. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A459s treated with DMSO control (0.1%), LY294002 [50μM], Akt VIII [25μM], or both LY294002 [50μM] and Akt VIII [25μM] in combination. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant. Data are representative of three separate determinations on two separate occasions.

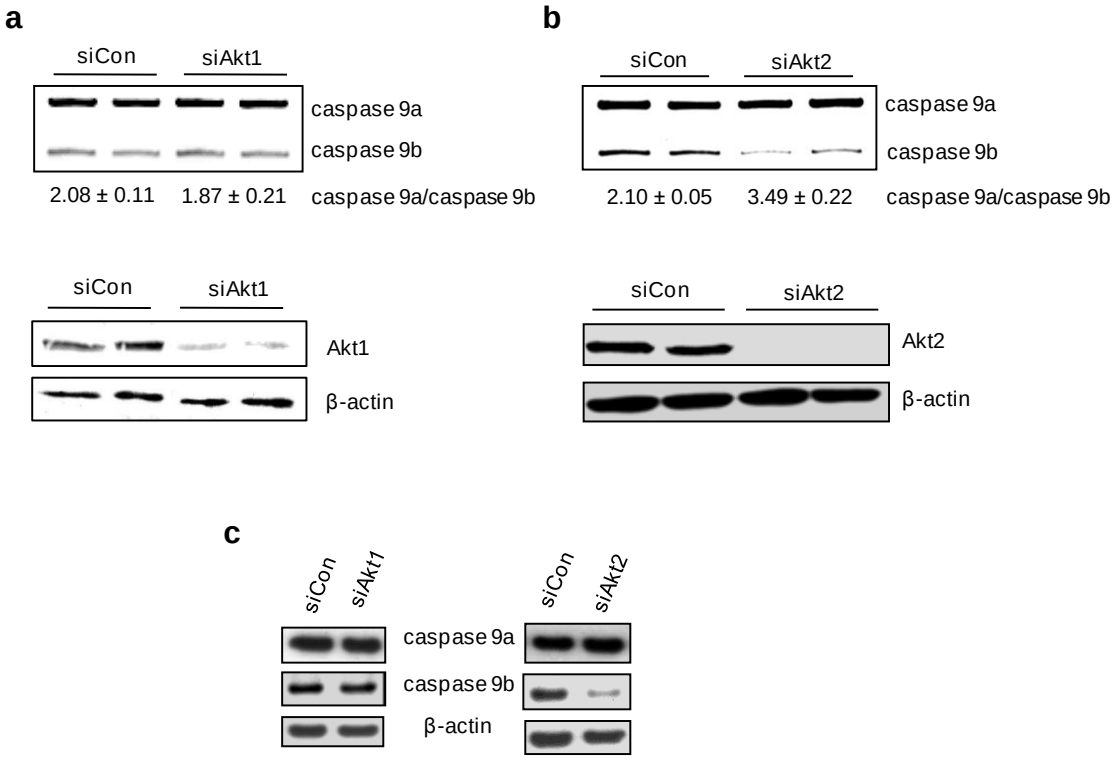

Figure 2-8. Downregulation of Akt2 increases the caspase 9a/9b mRNA ratio in A549 cells. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from A549s transfected with control siRNA, **a)** AKT1 siRNA, or **b**) AKT2 siRNA. Knockdown of AKT1 and AKT2 were confirmed by western blot. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant. **c**) Total proteins from A549s transfected with the indicated siRNAs were also subjected to western blot analysis to demonstrate the change in the ratio of caspase 9a to caspase 9b protein expression.

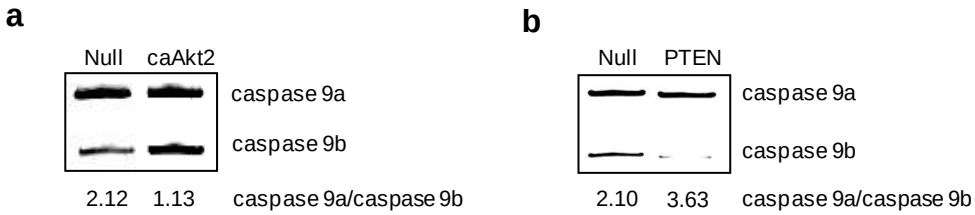

Figure 2-9. Akt2 regulates the alternative splicing of caspase 9 via PIP_3 membrane interactions in A549 cells. A459 cells were infected with either a) caAkt2 adenovirus (25MOI) or b) PTEN adenovirus (25MOI). Fourty-eight hours post-infection, RNA was isolated and quantitative/competitive RT-PCR performed. The ratio of caspase 9a to caspase 9b PCR products was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

To determine the translatability of this mechanism to other NSCLC cell lines, H2030, H292, H358, and H520 cells were also treated with the AKT VIII inhibitor [50μM]. As with the A549s, all four cell lines also demonstrated a dramatic increase in the caspase 9a/9b mRNA ratio in the presence of this inhibitor (Figure 2-10). Additionally, Akt1 and Akt2 was downregulated via siRNA in H358 cells to determine the Akt isoform responsible for regulating the alternative splicing of caspase 9. In contrast to A549s, downregulation of Akt1 but not Akt2 induced a significant increase in the caspase 9a/9b mRNA ratio (Figure 2-11 and data not shown). Therefore, multiple Akt isoforms have the potential to regulate the alternative splicing of caspase 9 in NSCLC cells.

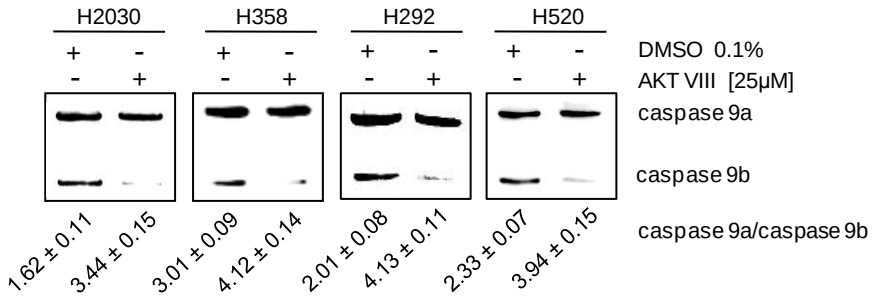

Figure 2-10. Inhibition of Akt regulates the alternative splicing of caspase 9 in multiple NSCLC cell lines. Quantitative/competitive RT-PCR of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios of H2030s, H358s, H292s, and H520s treated with Akt inhibitor VIII [25μM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

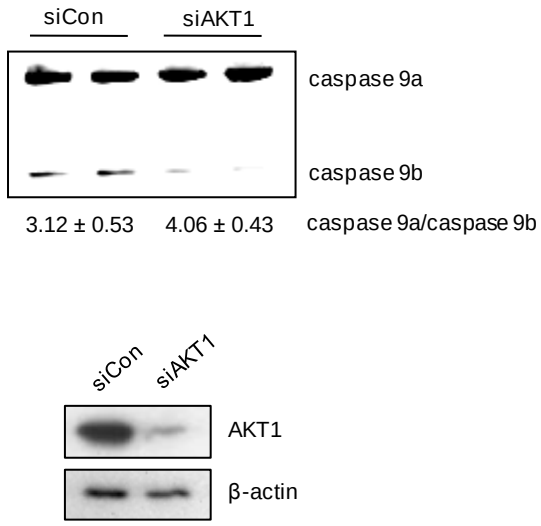

Figure 2-11. Knockdown of Akt1 regulates the alternative splicing of caspase 9 in H358 cells. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from H358s transfected with control siRNA and AKT1 siRNA. Knockdown of AKT1 and AKT2 were confirmed by western blot analysis. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

2.4. Discussion

Lung cancer is the leading cause of cancer mortality in the Western world and the 5-year survival rate for all stages is only ~15% [214,215]. From a histological point of view, lung cancer can be classified as non-small cell (NSCLC) or small cell lung cancer (SCLC); 80% of lung cancers diagnosed are NSCLCs, and 20% are SCLCs[216]. NSCLCs can be further subdivided to include adenocarcinomas of the lung, squamous cell carcinomas of the lung, and large cell carcinomas of the lung.

The data presented in this chapter demonstrates that the alternative splicing of caspase 9 is dysregulated in a pro-survival manner (lower caspase 9a/9b mRNA ratio) in a large population of pathologist-verified human NSCLC tumor samples and transformed lung cancer cells. Specifically, 79% of tumors samples examined presented with a moderate to severely dysregulated caspase 9a/9b mRNA ratio. Similarly, 8 of 11 transform lung cancer cell lines presented with a moderate to severely dysregulated caspase 9a/9b mRNA ratio. These findings prompted us to investigate the signaling pathways responsible for regulating the alternative splicing of caspase 9 in this pro-survival fashion. Utilizing small-molecule inhibitors and siRNA technologies, the PI3-Kinase/Akt pathway was identified as the major mitogenic signaling pathway responsible for regulating the inclusion/exclusion of the exon 3,4,5,6 cassette of caspase 9. This finding translated to multiple NSCLC cell lines, including adenocarcinomas and squamous cell carcinomas.

CHAPTER 3

THE ROLE OF EGFR IN THE ALTERNATIVE SPLICING OF CASPASE 9

3.1. Introduction

Lung cancer is characterized by the accumulation of multiple genetic alterations resulting in the activation of oncogenes or inactivation of tumor suppressor genes[39]. In essentially all epithelial cancers, including NSCLC, one or more members of the family of epidermal growth factor receptor (EGFR) genes are either overexpressed or dysregulated[40]. Aberrant signaling of EGFR family members can occur by the following three fundamental mechanisms: activating gene mutations, increased gene copy number (via amplification or polysomy), and altered ligand expression (potentially leading to autocrine stimulatory loops)[217]. Mutations in exons encoding the tyrosine kinase domains of EGFR are found in 10% of lung adenocarcinomas[218]. Of those EGFR mutations, nearly 90% are either short deletions in exon 19 (i.e. ΔE746-A750 del) or point mutations that result in substitution of arginine for leucine at amino acid 858 (L858R)[218]. Recently, Hirch et. al. reported that 62% of NSCLCs of adenocarcinoma and squamous cell subtypes exhibited EGFR protein overexpression[41], which is often associated with poor prognosis[42]. In addition to EGFR expression, epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), two of its cognate ligands, are also freely expressed in NSCLCs, leading to over-stimulation of downstream mitogenic signaling pathways[43,44].

The PI3-Kinase/Akt pathway is one of the major signaling networks downstream of EGFR signaling. Since the PI3-Kinase/Akt pathway was demonstrated to regulate the alternative splicing of caspase 9 pre-mRNA in a pro-survival manner, we investigated the role of EGFR signaling on the alternative splicing of caspase 9 pre-mRNA. Aberrant EGFR signaling in cancer cells can accelerate tumor progression and much effort has focused on the development of drugs that specifically inhibit EGFR tyrosine kinase activity[217,219,220]. Erlotinib (Tarceva, OSI-774) is one of the most extensively evaluated tyrosine kinase inhibitors of the EGFR currently in clinical trials[221]. This year, Jackman, D.M. et al. reported on the impact of EGFR mutations on the clinical outcomes in previously untreated NSCLC patients[222]. Interestingly, patients harboring an exon 19 deletion in EGFR had a longer median prolonged time to progression (14.6 versus 9.7 months) and longer overall survival (30.8 versus 14.8 months) compared to patients harboring the 858R mutation[222]. Therefore, we investigated the effect of modulating the caspase 9a/9b mRNA ratio on the sensitivity of A549 cells to erlotinib, and on the ability of the E746-A750 del EGFR-expressing HBEC3-KT cells to confer anchorage-independent growth.

3.2. Materials and Methods

3.2.1. Cell Culture

A549 cells were grown in 50% RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) and 50% Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with L-glutamine, 10% (v/v) fetal bovine

serum (Sigma), 100 units/ml penicillin G sodium (Invitrogen Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Carlsbad, CA). A549s were purchased from ATCC (Rockville, MD, USA). HBEC-3KT cell lines were a provided by John D. Minna and previously described[223]. Briefly, the HBEC-3 (HBEC3-KT) immortalized normal cell line was established by introducing mouse Cdk4 and hTERT into normal HBECs obtained from a 65 year old woman without cancer[223]. KRASV12, wild-type EGFR and mutant EGFRs were introduced into the HBEC3-KT cell line as previously described[223]. HBEC-3KT cell lines were cultured with keratinocyte serum-free medium (K-SFM) (Invitrogen Life Technologies, Carlsbad, CA) containing 50μg/ml bovine pituitary extract with or without 5ng/ml recombinant epidermal growth factor (Invitrogen Life Technologies, Carlsbad, CA). All cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37 °C).

3.2.2. Quantitative/Competitive RT-PCR

Total RNA from cell lines were isolated using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen™, Carlsbad, CA) and oligo (dT) as the priming agent. After 50 min of incubation at 42°C, the reactions were terminated by heating at 70°C for 15 min. Template RNA was then removed using RNase H (Invitrogen, Carlsbad, CA). To quantitatively evaluate the expression of endogenous caspase 9 splice variants, an

upstream 5’ primer to caspase-9 (5’-GCT CTT CCT TTG TTC ATC TCC-3’) and a 3’ primer (5’-CAT CTG GCT CGG GGT TAC TGC-3’) (Integrated DNA Technologies, Inc., Coralville, IA) were used. Using these primers, 20% of the reverse transcriptase reaction was amplified for 25 cycles (94°C, 30 s melt; 57°C, 30 s anneal; 72°C, 1 min extension) using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). Gene products produced from endogenous caspase 9 PCR resulted in a 1248 base pair caspase 9a splice variant and 798 base pair caspase 9b splice variant. The final PCR products were resolved on a 5% TBE acrylamide gel electrophoresis, stained with SYBR® Gold (Invitrogen™, Carlsbad, CA) and visualized using a Molecular Imager® FX (Bio-Rad) with a 488 nm EX (530 nm BYPASS) laser.

3.2.3. Western Immunoblotting

Cells were lysed using CelLytic™ lysis Buffer (Sigma-Aldrich) supplemented with protease Inhibitor cocktail (Sigma-Aldrich). Protein samples (5 μg) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PDVF) (Bio-Rad) and blocked in 5% milk/1 X PBS – 0.1% Tween (M-PBS-T) for 2 h. Primary antibodies were anti-caspase 9 (1:1,000, Assay Designs), anti-Akt1 (1:1,000, Santa Cruz), anti-Akt2 (1:1,000, Santa Cruz), and anti-β-actin (1:5,000, Sigma-Aldrich). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit (1:5,000, Sigma-Aldrich). Immunoblots were developed using Pierce enhanced chemiluminescence (ECL) reagents and Bio-Max film.

3.2.4. Small interfering RNA transfection

For inhibition of Akt1 and Akt2 expression, cell lines were transfected with either Akt1 SMARTpool multiplex, Akt2 SMARTpool multiplex, or scrambled control siRNA (Dharmacon; Lafayette, CO) using Dharmafect 1 transfection reagent (Dharmacon; Lafayette, CO) following the manufacturer's protocol. Briefly, cell lines were plated in six-well tissue culture dishes and allowed to rest overnight. At 50% confluence, cells were transfected with siRNA [100nM] using Dharmafect 1 in Opti-Mem I reduced-serum medium. Forty-eight hours post-transfection, RNA and/or protein were isolated.

3.2.5. Inhibitor studies

For Inhibitor studies, 1.2 x 10^5 cells were plated into 6-well tissue culture plates. The following day media was removed and replaced with the appropriate complete growth media. Cells were subsequently treated with sham control (1:1000) or the appropriate concentration of active inhibitor (1:1000) (Calbiochem). Twenty-four hours post-treatment, total RNA and/or protein was isolated.

3.2.6. Clonogenic and soft agar assays

For clonogenic assays, 150 viable cells were seeded into 6-well tissue culture dishes with complete growth media. Two hours post-plating, cells were treated with the appropriate concentration of erlotinib (1:1,000 dilution). The following day, cells were rinsed with 1xPBS and allowed to grow in complete growth media for 12 days. Colonies were visualized and counted following fixation with methanol and staining with 0.1%

crystal violet. For soft agar assays using HBEC-3KT cell lines, 1,000 viable cells were suspended and plated in 0.37% agar (Fisher Scientific) in K-SFM medium supplemented with 20% of fetal bovine serum and 50 µg/mL bovine pituitary extract into 6-well tissue culture plates, and were layered over a 0.6% agar base in the same medium used to suspend the cells. The number of microscopically visible colonies (>40 cells) were counted 4 weeks later. For soft agar assays using NSCLC cell lines following cell sorting (described below), 5,000 viable cells were suspended and plated in 0.3% agar (Fisher Scientific) in their appropriate growth media with all supplements (described above) into 6-well tissue culture plates, and were layered over a 0,6% agar base in the same media used to suspend cells. The number of microscopically visible colonies (>40 cells) were counted 2 weeks later.

3.2.7. Stable transfections

To generate a HBEC3-KT, E746-A750 del EGFR clonal cell line stably expressing shRNA knockdown of caspase 9b, 1.5 x 10^4 cells were plated and infected with either shRNA caspase 9b lentiviral particles (20MOI) or control shRNA lentiviral particles (20MOI) (Lentigen). The following day, lentivirus medium was removed and replaced with complete KSFM medium (described above). Forty-eight hours after infection, drug selection was started with 20ug/mL hygromycin (Sigma-Aldrich) and continued for 11 days. To generate a "batch culture" A549 cell line stably expressing shRNA knockdown of caspase 9b, 1.5 x 10^4 cells were plated and infected with either shRNA caspase 9b lentiviral particles (20MOI) or control shRNA lentiviral particles (20MOI) (Lentigen).

The following day, lentivirus medium was removed and replaced with complete A549 medium (described above). Forty-eight hours after infection, drug selection was started with 50ug/mL hygromycin (Sigma-Aldrich) and continued for 11 days. To generate a "batch culture" A549 cell line stably expressing ectopic caspase 9b cDNA, 1.5 x 10^4 cells were plated in 35mm tissue culture dishes. The following day, 0.5ug of pcDNA3.1 (-) zeocin containing caspase 9b was transfected into the cells using Effectene (Qiagen Inc., Valencia, CA) following the manufacture's protocol. Twenty-four hours after transfection, cells were rinsed with 1xPBS and selection started with 50µg/mL zeocin (Invitrogen Life Technologies, Carlsbad, CA) and continued for 11 days.

3.3. Results

3.3.1. EGFR overexpression or mutation affects the alternative splicing of caspase 9 via activation of the PI3-Kinase/Akt pathway.

The PI3-Kinase pathway is constitutively activated by a number of oncogenic mutations including those common in NSCLC, such as k-ras (e.g. k-Ras^{V12}) and EGFR overexpression/mutation. Therefore, we examined whether these two common oncogenes in NSCLC affected the alternative splicing of caspase 9 in non-transformed human bronchial epithelial cells (HBEC-3KT cells). Whereas low expression of k-Ras^{V12} in HBEC-3KT cells[223] had no discernable effect on the ratio of caspase 9a/9b mRNA, the overexpression of wild-type EGFR, the expression of L858R mutation in EGFR, and the expression of the del E746-A750 EGFR mutation induced a significant reduction in the caspase 9a/9b mRNA ratio (Figure 3-1). Importantly, the del E746-A750 mutation of

EGFR induced the most significant decrease in the caspase 9a/9b mRNA ratio correlating with the ability of this mutant to induce anchorage independent growth[223]. As with the lung adenocarcinoma and squamous cell lines, the ability of EGFR mutation to induce a lowering of the caspase 9a/9b ratio was dependent on the PI3-Kinase/Akt pathway. Treatment of E746-A750 del expressing HBEC-3KT cells with LY294002 and Akt inhibitor VIII also increased the ratio of caspase 9a/9b mRNA to levels comparable with normal HBEC-3KT cells (Figure 3-2a,b). These data suggest that EGFR overexpression/mutation correlates with Akt activation affecting the alternative splicing of the tumor suppressor, caspase 9.

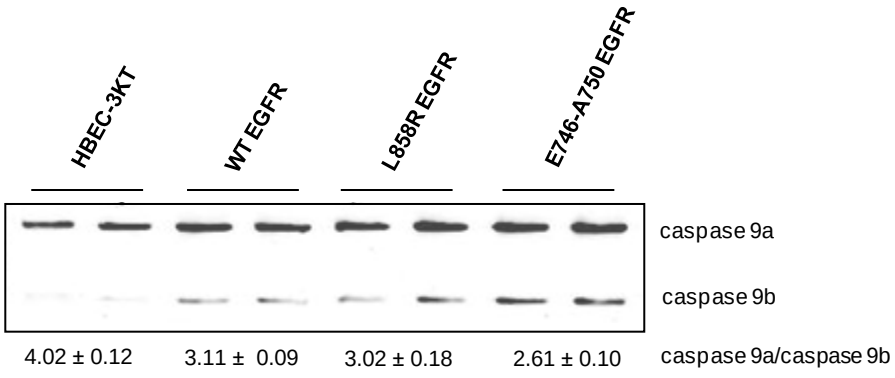

Figure 3-1. EGFR overexpression or mutation affects the alternative splicing of caspase 9 pre-mRNA. Total RNA was isolated from vector control-, wild-type EGFR-, L858R EGFR-, and E746-A750 del EGFR-expressing HBEC-KT cells. Quantitative/competitive RT-PCR analysis was performed of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios depicted. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

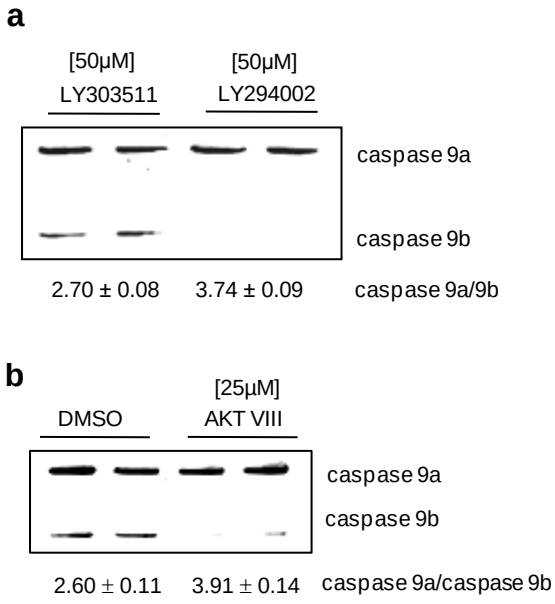

Figure 3-2. The PI3-Kinase/Akt pathway regulates the alternative splicing of caspase 9 in E746-A750 del EGFR-expressing HBEC-3KT cells. Quantitative competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from E746-A750 del EGFR-expressing HBEC-3KT cells treated with **a)** LY303511 control compound [50μM] or LY294002 [50μM], and **b)** DMSO control (0.1%) or Akt inhibitor VIII [25μM]. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

We extended these results by examining the AKT isoform responsible for regulating the alternative splicing of caspase 9 pre-mRNA in HBEC-3KT cells harboring the del E746-A750 mutation in EGFR. Interestingly, siRNA against both AKT1 and AKT2 (Figure 3-3), but not AKT3 (data not shown), resulted in a significantly increase caspase 9a/caspase 9b mRNA ratio as compared to control siRNA (from 2.81 ± 0.05 for

siRNA control samples to 4.01 ± 0.13 for siAKT1 samples (n=3), p<0.01; and to 3.72 ± 0.11 for siAKT2 treated samples (n=3) p<0.01). Therefore, multiple AKT isoforms are responsible for regulating the alternative splicing of caspase 9 in NSCLC.

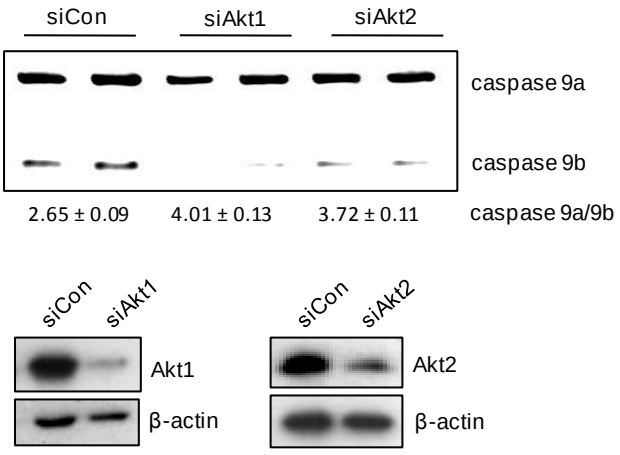

Figure 3-3. Knockdown of Akt1 or Akt2 in E746-A750 del EGFR-expressing HBEC-3KT cells results in an increased caspase 9a/9b mRNA ratio. Quantitative/competitive RT-PCR analysis of caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios from E746-A750 del EGFR-expressing HBEC-KT cells treated with either scrambled control siRNA, Akt1 siRNA, or Akt2 siRNA. Knockdown of Akt1 and Akt2 were confirmed by western blot analysis. The ratio of caspase 9a to caspase 9b mRNA was determined by densitometric analysis of RT-PCR fragments. Data are expressed as means ± s.d. *P*-values in pairwise comparisons to the control; p values < 0.01 were considered significant.

3.3.2. Modulation of the alternative splicing of caspase 9 regulates the ability of erlotinib to inhibit colony formation of A549s.

Previous results presented demonstrate the alternative splicing of caspase 9 splicing is regulated via the EGFR/PI3-Kinase/Akt pathway. This prompted us to investigate the effect of erlotinib, a tyrosine kinase inhibitor of the human EGFR, on the caspase 9a/9b mRNA ratio. Treatment of A549s with erlotinib led to a dose-dependent increase in the caspase 9a/9b mRNA ratio (Figure 3-4). Next, the effect of lowering the caspase 9a/9b mRNA ratio on the sensitivity of A549 cells to erlotinib was examined in attempt to validate our hypothesis that a reduced ratio of caspase 9a/9b mRNA observed in non-small cell lung carcinomas was relevant in a clinical setting. Ectopic expression of caspase 9b cDNA completely inhibited the ability of erlotinib to suppress cell survival, as determined by clonogenic survival assays (Figure 3-5a). In contrast, downregulation of caspase 9b sensitized A549 cells to erlotinib, dramatically reducing the IC50 from 16.5μM for control shRNA cells to 4.5μM for C9bshRNA cells (Figure 3-5b).

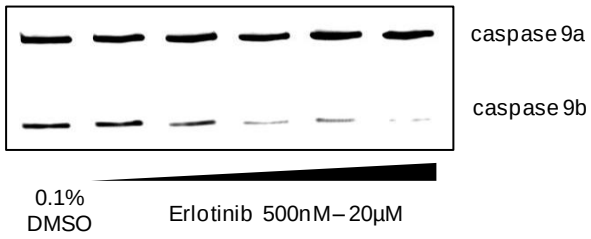

Figure 3-4. Treatment of A549s with erlotinib results in a dose-dependent increase in the caspase 9a/9b mRNA ratio. Quantitative competitive RT-PCR analysis of caspase 9 splice variants from E746-A750 del EGFR-expressing HBEC-3KT cells treated with increasing doses of erlotinib (dose-dependent response).

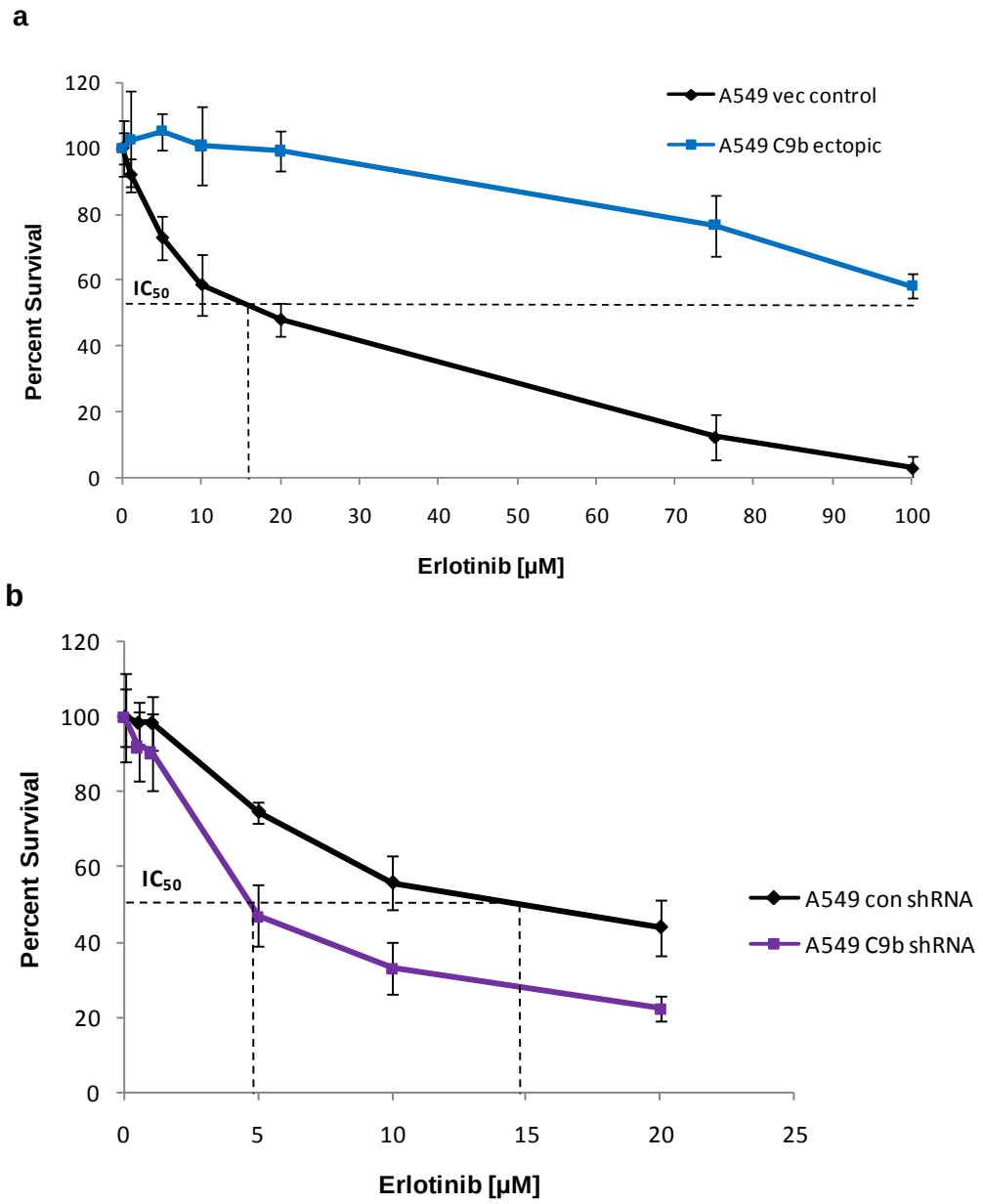

Figure 3-5. Modulation of the alternative splicing of caspase 9 regulates the ability of erlotinib to inhibit liquid colony formation of A549 cells. A549 cells expressing **a)** ectopic caspase 9b cDNA or vector control, and **b)** caspase 9b shRNA or control shRNA, were plated in triplicate 35mm plates in the presence of varying concentrations of erlotinib. The following day, erlotinib-containing medium was removed and replaced with new medium. Cells were cultured for 12 days following staining with crystal violet and colony counting.

3.3.3. The alternative splicing of caspase 9 plays a major role in maintaining the anchorage-independent growth of A549 cells.

Due to the fact that a large majority of NSCLC tumors and cell lines present with a dysregulated caspase 9a/9b ratio, and the knowledge that caspase 9a is a tumor suppressor, we hypothesized that the alternative splicing of caspase 9 is involved in maintaining the transformed phenotype of NSCLC. To determine the biological role of the alternative splicing of caspase 9, A549 "batch culture" cell lines stably expressing either caspase 9b cDNA or caspase 9b shRNA were produced and assayed for their ability to grow in soft agar. Ectopic expression of caspase 9b (a two-fold increase in caspase 9b expression and 50% reduction in the ratio of caspase 9a/9b mRNA) significantly increased the ability of A549 cells to grow in soft agar (Figure 3-6a). In contrast, stable expression of caspase 9b shRNA significantly reduced the ability of A549 cells to grow in soft agar (Figure 3-6b). Thus, these data suggest that the alternative splicing of caspase 9 plays a role in regulating the anchorage-independent growth of A549 cells.

3.3.4. EGFR (E746-A750 del) mutation requires the lowering of the caspase 9a/9b mRNA ratio to confer anchorage-independent growth to HBEC-3KT cells.

The E746-A750 del EGFR mutation in HBEC-3KT cells conferred anchorage-independent growth[223] and demonstrated the greatest effect on lowering the caspase 9a/9b mRNA ratio. In addition, results presented above have provided biological evidence for the role of caspase 9b in regulating the anchorage-independent growth of A549s.Therefore, we hypothesized that increased expression of caspase 9b was a major mechanism in

conferring anchorage-independent growth of HBEC-3KT cells. To test this hypothesis, an E746-A750 del EGFR clonal cell line (HBEC-3KT Δ746/C9b-dr) stably expressing caspase 9b shRNA was produced. The expression of caspase 9b in the HBEC-3KT Δ746/C9b-dr clonal cell line was reduced to normal immunoreactive levels and this cell line presented with a caspase 9a/caspase 9b mRNA ratio comparable to the ratio observed in HBEC-3KT cells (Figure 3-7a,b). Validating our hypothesis, the HBEC-3KT Δ746/C9b-dr cells demonstrated a complete loss of anchorage-independent growth compared to E746-A750 del EGFR cells stably expressing control shRNA (Figure 3-7c). These effects did not require stable expression and were not due to integration artifacts as short term/transient downregulation of caspase 9b using shRNA lentiviral particles also inhibited the anchorage-independent growth of E746-A750 del EGFR cells (Figure 3-8a). Downregulation of caspase 9b was again confirmed in these cells via RT-PCR of caspase 9 splice variants and western blot analysis (Figure 3-8b,c). Thus, the distal mechanism of caspase 9 splicing plays a major role in the ability of EGFR signaling to confer anchorage-independent growth.

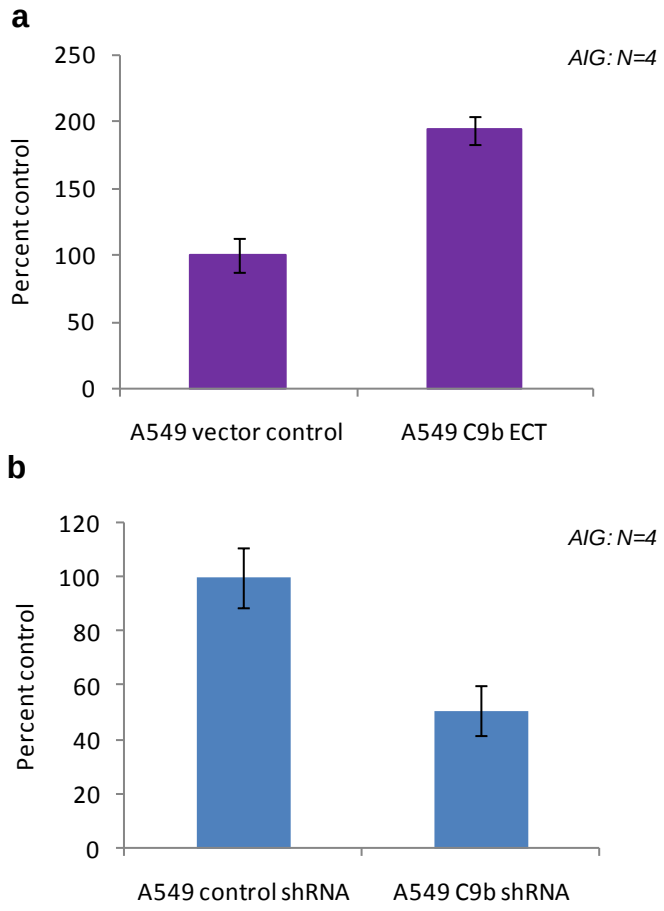

Figure 3-6. The alternative splicing of caspase 9 regulates the anchorage-independent growth of A549 cells. A549 cells stably expressing **a)** ectopic caspase 9b cDNA or vector control, and **b)** caspase 9b shRNA or control shRNA, were assayed for their ability to grow in soft agar. A total of 1,000 cells were plated per dish and cultured for 2 weeks before counting. Depicted is the quantification of the number of colonies counted, represented as percent control.

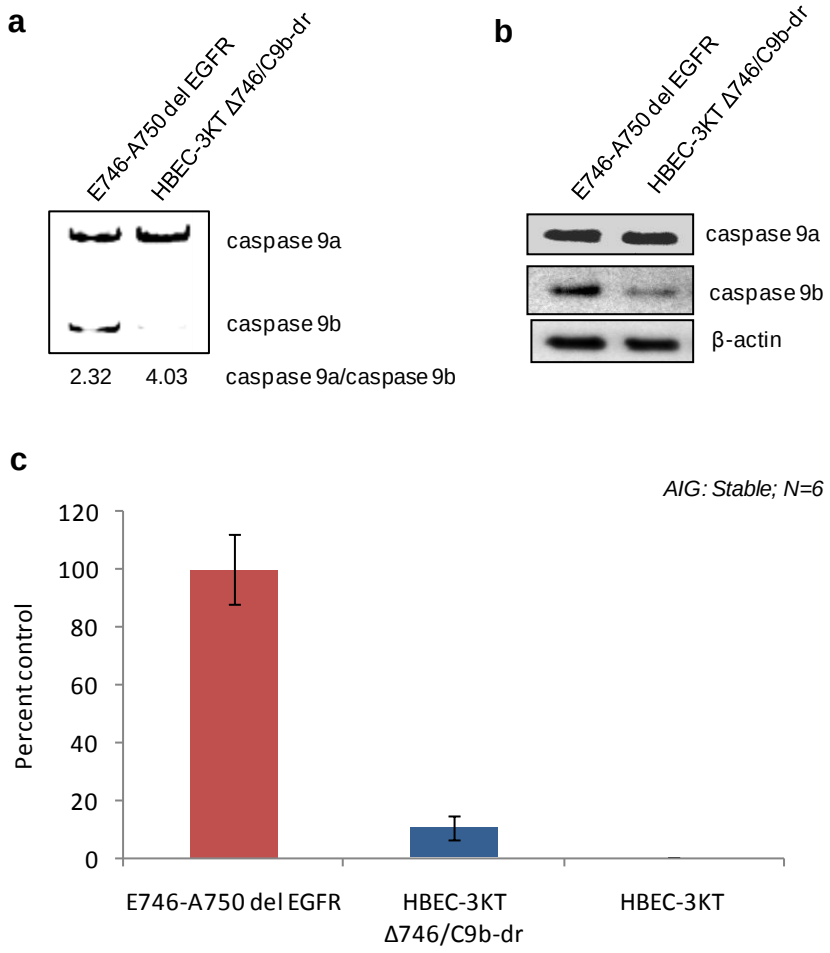

Figure 3-7. E746-A750 del EGFR-expressing HBEC-3KTs require a lowering of the caspase 9a/9b mRNA ratio to confer anchorage-independent growth (stable expression). Characterization of HBEC-3KT Δ746 cells stably expressing caspase 9b shRNA or control shRNA by **a)** quantitative/competitive RT-PCR analysis of caspase 9 splice variants and **b)** western blot analysis for protein expression of caspase 9 splice variants. **c)** Colony formation assay in soft agar of HBEC-3KT cells stably expressing caspase 9b shRNA or control shRNA. Depicted is the quantification of the number of colonies counted, represented as percent control.

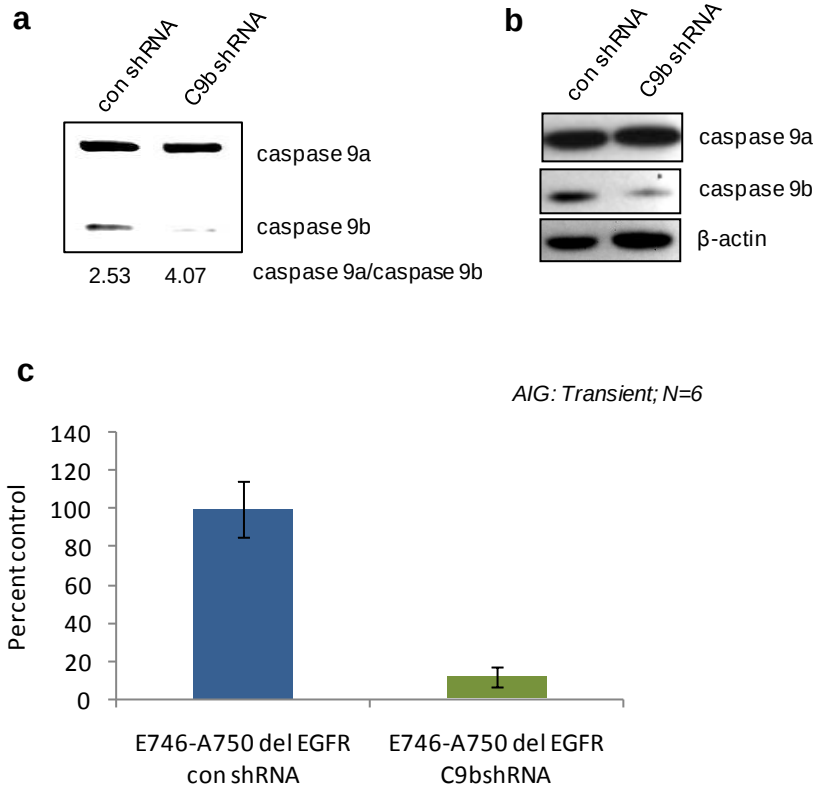

Figure 3-8. E746-A750 del EGFR-expressing HBEC-3KTs require a lowering of the caspase 9a/9b mRNA ratio to confer anchorage-independent growth (transient expression). Characterization of HBEC-3KT Δ746 cells transiently expressing caspase 9b shRNA or control shRNA by **a)** quantitative/competitive RT-PCR analysis of caspase 9 splice variants and **b)** western blot analysis for protein expression of caspase 9 splice variants. **c)** Colony formation assay in soft agar of HBEC-3KT cells transiently expressing caspase 9b shRNA or control shRNA. Depicted is the quantification of the number of colonies counted, represented as percent control.

3.4. Discussion

The PI3-Kinase/Akt pathway is one of the major signaling networks downstream of EGFR signaling. The presented data has demonstrated that the PI3-Kinase/Akt pathway regulates the alternative splicing of caspase 9 pre-mRNA in a pro-survival manner, and that either mutation or overexpression of EGFR in non-transformed human bronchial epithelial cells (HBEC-3KT) also represses the inclusion of the exon 3,4,5,6 cassette of caspase 9. Interestingly, the E746-A750 del EGFR-expressing HBEC3-KT cells, which confer anchorage-independent growth[223], demonstrated the greatest effect on lowering the caspase 9a/9b ratio. This prompted us to investigate the effect of erlotinib, a tyrosine kinase inhibitor of the human EGFR, on the caspase 9a/9b mRNA ratio. As anticipated, treatment of A549 cells with erlotinib led to a dose-dependent increase in the caspase 9a/9b mRNA ratio. We extended these results to show that modulations of the caspase 9a/9b mRNA ratio regulated the ability of erlotinib to inhibit liquid colony formation of A549 cells.

Based on these findings, and the published evidence that caspase 9a is a tumor suppressor[179], we investigated the effect of modulating the caspase 9a/9b mRNA ratio on the anchorage-independent growth of the E746-A750 del EGFR-expressing HBEC3-KT cells. In parallel, the effect of modulating the caspase 9a/9b mRNA ratio on the anchorage-independent growth of A549 cells was also examined. The data presented clearly demonstrates that the alternative splicing of caspase 9 plays a major role in conferring anchorage-independent growth and in regulating the sensitivity of NSCLC cells to erlotinib.

CHAPTER 4

THE PI3-KINASE/AKT PATHWAY REGULATES THE ALTERNATIVE SPLICING OF CASPASE 9 IN A PHOSPHO-SRP30A-DEPENDENT MANNER.

4.1. Introduction

SR proteins are required for constitutive and alternative pre-mRNA processing and are specific substrates for protein phosphatase-1 (PP1), a ceramide-activated protein phosphatase. Previous studies from our laboratory demonstrate that endogenous ceramide modulated the phosphorylation status of SR proteins in a PP1-dependent manner[224]. Additionally, our laboratory has recently described a pathway linking the generation of *de novo* ceramide and the activation of PP1 to the regulation of the alternative splicing of caspase-9 pre-mRNA[225]. Specifically, treatment of A549 lung adenocarcinoma cells with D-e-C_6 ceramide down-regulated the levels of caspase 9b mRNA and immunoreactive protein coupled with an increase in caspase 9a mRNA and immunoreactive protein levels, in a dose- and time-dependent manner[225]. This effect required the generation of endogenous ceramide through the *de novo* pathway and, more importantly, inhibitors of PP1 abolished the ability of ceramide to affect the alternative splicing of caspase 9[225]. Additional studies from our laboratory identified the SR protein, SRp30a, as a critical splicing factor responsible for regulating the alternative splicing of caspase 9 pre-mRNA[226]. Furthermore, SRp30a was shown to be a required for ceramide to affect the

alternative splicing of caspase-9 pre-mRNA, thereby linking *de novo* ceramide generation, SRp30a, and the regulation of caspase-9 alternative splicing[226].

These previous studies from our laboratory prompted us to investigate whether the phosphorylation status of the RS domain of SRp30a modulated the alternative splicing of caspase 9 pre-mRNA. Furthermore, we sought to identify the precise serine residues in the RS domain of SRp30a which regulated the inclusion/exclusion of the exon 3,4,5,6 cassette of caspase 9 pre-mRNA. Lastly, we examined the role of the phospho-state of SRp30a on the anchorage-independent growth of A549 lung adenocarcinoma cells.

4.2. Materials and Methods

4.2.1. Cell Culture

A549 cells were grown in 50% RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) and 50% Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with L-glutamine, 10% (v/v) fetal bovine serum (Sigma), 100 units/ml penicillin G sodium (Invitrogen Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Carlsbad, CA). A549s were purchased from ATCC (Rockville, MD, USA). All cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37 °C).

4.2.2. Quantitative/Competitive RT-PCR Quantitative/competitive RT-PCR

Total RNA from cell lines were isolated using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (SuperScript™ First-Strand Synthesis System for RT-PCR, Invitrogen™, Carlsbad, CA) and oligo (dT) as the priming agent. After 50 min of incubation at 42°C, the reactions were terminated by heating at 70°C for 15 min. Template RNA was then removed using RNase H (Invitrogen, Carlsbad, CA). To quantitatively evaluate the expression of endogenous caspase 9 splice variants, an upstream 5' primer to caspase-9 (5'-GCT CTT CCT TTG TTC ATC TCC-3') and a 3' primer (5'-CAT CTG GCT CGG GGT TAC TGC-3') (Integrated DNA Technologies, Inc., Coralville, IA) were used. Using these primers, 20% of the reverse transcriptase reaction was amplified for 25 cycles (94°C, 30 s melt; 57°C, 30 s anneal; 72°C, 1 min extension) using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). Gene products produced from endogenous caspase 9 PCR resulted in a 1248 base pair caspase 9a splice variant and 798 base pair caspase 9b splice variant. To evaluate the expression of the splice variant products of the caspase 9 minigene, a 5' primer to caspase 9 minigene (5'-CAT GCT GGC TTC GTT TCT G-3') and a 3' primer (5'-AGG GGC AAA CAA CAG ATG G-3') were used. Gene products produced from caspase 9 minigene PCR resulted in an 889 bp caspase 9a splice variant and a 443bp caspase 9b splice variant. Using these primers, 20% of the reverse transcriptase reaction was amplified for 25 cycles (94°C, 30 s denaturation; 60°C, 30 s anneal; 72°C, 1 min extension) using Platinum Taq DNA polymerase (Invitrogen™, Carlsbad, CA). The final PCR products were resolved on a 5%

TBE acrylamide gel electrophoresis, stained with SYBR® Gold (Invitrogen™, Carlsbad, CA) and visualized using a Molecular Imager® FX (Bio-Rad) with a 488 nm EX (530 nm BYPASS) laser.

4.2.3. Western Immunoblotting

Cells were lysed using CelLytic™ lysis Buffer (Sigma-Aldrich) supplemented with protease Inhibitor cocktail (Sigma-Aldrich). Protein samples (5 μg) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PDVF) (Bio-Rad) and blocked in 5% milk/1 X PBS – 0.1% Tween (M-PBS-T) for 2 h. Primary antibodies were anti-αTubulin (1:1000, Santa Cruz), anti-laminA/C (1:1000, Cell Signaling), and anti-T7 tag (1:10,000, Novagen). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit (1:5,000, Sigma-Aldrich). Immunoblots were developed using Pierce enhanced chemiluminescence (ECL) reagents and Bio-Max film.

4.2.4. Expression plasmids and transient transfections

The mammalian expression vectors pCGT7-SRp30a-WT and the SRp30a RS domain mutant, SRp30a-RD, were a gift from J.F. Caceres and previously described[227]. To generate SRp30a phospho-mutants, site-directed mutagenesis was performed using QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacture's protocol. To analyzed caspase 9a and caspase 9b mRNA expression, 1.5 x 10^4 cells were plated into 6-well tissue culture dishes. The following day cells were transfected with 0.5ug of pCGT7-SRp30a plasmids with or without 0.2ug of caspase 9

minigene plasmid DNA using Effectene (Qiagen Inc., Valencia, CA) following the manufacture's protocol. Twenty-four hours post-transfection, total RNA was harvested for RT-PCR analysis. To analyze the anchorage-independent growth of A549 cells expressing SRp30a phospho-mutants, 7.5 x 10^5 cells were plated into 100mm tissue culture dishes. The following day, cells were co-transfected with 0.5 ug of pEGFP-Endo and 2 ug of pCGT7-SRp30a constructs using Effectene (Qiagen Inc., Valencia, CA) following the manufacture's protocol. Twenty-four hours post-plating, cells were sorted using a Beckman CoulterEPICS Elite ESP multilaser flow cytometer. GFP positive cells were collected and plated into soft agar (described below) to demonstrate effects on anchorage-independent growth.

4.2.5. Soft agar assays

For soft agar assays, 5,000 GFP positive A549 cells (described above) were suspended and plated in 0.3% agar (Fisher Scientific) in their appropriate growth media with all supplements (described above) into 6-well tissue culture plates, and were layered over a 0.6% agar base in the same media used to suspend cells. The number of microscopically visible colonies (>40 cells) were counted 2 weeks later.

4.2.6. In vitro phosphorylation assays

A549 cells (10^6) were transfected with 2μg of SRp30a-WT, SRp30a-QD, and SRp30a-QA constructs using Effectene (Qiagen Inc., Valencia, CA) following the

manufacture's protocol. Twenty-four hours after transfection, cells were scraped into 150 uL of NP-40 lysis buffer (50mM Tris-HCL [pH 8.0], 150mM NaCl, 1% NP-40,100ug of phenylmethyl-sulfonyl fluoride [PMSF]/mL, and protease inhibitor cocktail. The lysates were incubated with 500 U of alkaline phosphatase (New England Biolabs, Beverly, Mass.)/mL for 1 hr. at 37ºC. Wild-type and mutant SRp30a proteins were detected by Western blot using anti-T7 tag antibody (1:10,000).

4.2.7. Subcellular fractionation

A549s cells (10^6) were transfected with 2μg of pEGFP-Endo, SRp30a-WT, SRp30a-QD, SRp30a-QA, and SRp30a-RD constructs using Effectene (Qiagen Inc., Valencia, CA) following the manufacture's protocol. Twenty-four hours after transfection, cells were washed with PBS and scraped into lysis buffer (20mM HEPES, 1mM EDTA, 2mM $MgCl_2$, 10mM KCl, and 0.25M sucrose) containing protease inhibitor cocktail. Cells were homogenized with 10 strokes in a douncer and centrifuged for 10 minutes at 1000 x g. Supernatant was collected as cytosolic fraction and cell pellet was resuspended in lysis buffer as nuclear fraction.

4.3. Results

4.3.1. The phosphorylation status of SRp30a regulates the alternative splicing of caspase 9 pre-mRNA.

SR proteins are heavily phosphorylated in their RS domains, and their phosphorylation status can influence 5' splice site selection and exon inclusion (i.e.

splicing activity) for many pre-mRNAs[118,119,134,228,229]. Previously, our laboratory demonstrated that SRp30a was a required enhancer factor for the inclusion of the exon 3,4,5,6 cassette of caspase 9[226]. SRp30a was also shown to be required for ceramide to affect the alternative splicing of caspase 9 pre-mRNA and ceramide treatment led to the dephosphorylation of SR proteins[226]. Additionally, SRp30a has been demonstrated to be a specific target of Akt[141,142]. Therefore, we hypothesize that the phospho-status of SRp30a regulates the inclusion of the exon 3,4,5,6 cassette of caspase 9, downstream of Akt activation. To investigate this hypothesis, we employed an established phospho-mimic of SRp30a, SRp30a-RD, in which the majority of serine residues in the RS domain were mutated into aspartic acid[227]. Co-expression of SRp30a-RD with a functional caspase 9 minigene induced a significant decrease in the caspase 9a/9b ratio compared to wild-type SRp30a and empty vector controls (Figure 4-1a). Importantly, expression of SRp30a-RD also induced a significant decrease in the endogenous caspase 9a/9b mRNA ratio as compared to wild-type SRp30a and empty vector controls (Figure 4-1b).

To determine the serine residue/residues of SRp30a required for regulating the alternative splicing of caspase 9, site-directed replacement mutagenesis was utilized. Multiple serine residues in the RS domain of SRp30a contain recognized motifs for Akt phosphorylation[141]. These serine residues were individually mutated into aspartic acid to produce phospho-mimics (see Table 4-1). Co-expression of only the SRp30a-S199D, SRp30a-S201D, SRp30a-S227D, and SRp30a-S234D mutants with a functional caspase 9 minigene resulted in an decreased caspase 9a/9b ratio compared to wild type SRp30a

control (Figure 4-1c). Mutation of other serine residues in the RS-domain had no effect on the ratio of caspase 9a/9b mRNA (see Table 4-1).

We extended these results to produce a SRp30a double phospho-mutant (SRp30a-S199,201D), a SRp30a triple phospho-mutant (SRp30a-S199,201,234D), and a SRp30a quadruple (SRp30a-QD) mutant harboring serine to aspartic acid mutations at residues 199, 201, 227 and 234. Expression of SRp30a double and triple phospho-mutants with the caspase 9 minigene further decreased the caspase 9a/9b mRNA ratio as compared to the SRp30a single mutants and SRp30a wild type controls (data not shown). Importantly, ectopic expression of SRp30a-QD induced a reduction in the caspase 9a/9b ratio comparable to the SRp30a-RD mutant (Figure 4-1d). Conversely, a quadruple de-phospho-mimic of SRp30a (SRp30a-QA) induced the opposite effect in A549 cells (Figure 4-1d). These effects could not be accounted for by localization issues as both SRp30a quadruple mutants are predominately localized in the nucleus and are expressed in equivalent amounts, as demonstrated via western blot analysis (Figure 4-1e).

Table 4-1. Potential phosphorylation sites in SRp30a involved in regulating the alternative splicing of caspase 9. Listed are the serine residues examined for their role in regulating the alternative splicing of caspase 9 pre-mRNA. The phosphorylation status of the corresponding residues were determined by the indicated method and how they affected the caspase 9a/9b mRNA ratio is depicted.

Possible phosphosite	Determination	ESI-LC-MS/MS confirmed	Affect on C9a/C9b ratio
S199	Mass Spectrometry	Yes	Yes
S201	Mass Spectrometry	Yes	Yes
S205	Mass Spectrometry	Yes	No
S221	Kinase site prediction	No	No
S225	Mass Spectrometry	Yes	No
S227	Mass Spectrometry	Yes	Yes
S231	Mass Spectrometry	Yes	No
S234	Mass Spectrometry	Yes	Yes
S238	Mass Spectrometry	Yes	No
S242	Mass Spectrometry	Yes	No
S244	Mass Spectrometry	Yes	No
S246	Kinase site prediction	No	No
T248	Kinase site prediction	No	No

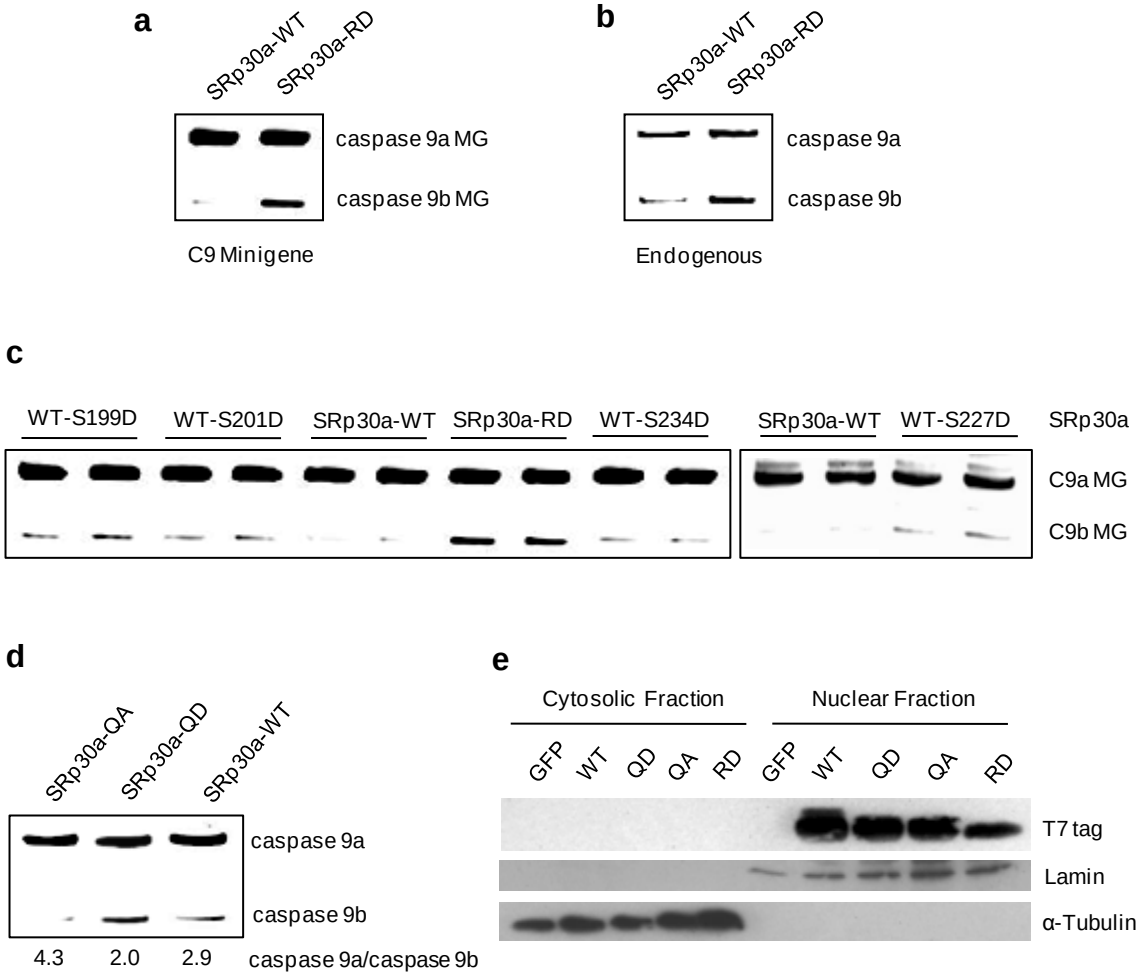

Figure 4-1. The phosphorylation status of SRp30a regulates the alternative splicing of caspase 9 pre-mRNA. a) Quantitative/competitive RT-PCR analysis of capspase 9 minigene-derived transcripts from A549s co-transfected with the caspase 9 minigene and SRp30a-WT or SRp30a-RD. **b)** Quantitative/competitive RT-PCR analysis of endogenous caspase 9 splice variants from A549 cells transfected with SRp30a-WT or SRp30a-RD. **c)** Quantitative/competitive RT-PCR analysis of caspase 9 minigene-derived transcripts from A549s co-transfected with the caspase 9 minigene and the indicated SRp30a phospho-mutants. **d)** Quantitative/competitive RT-PCR of endogenous caspase 9 splice variants and the corresponding caspase 9a/9b mRNA ratios of A549s transfected with SRp30a-QA, SRp30a-QD, or SRp30a-WT. Ratios of caspase 9a to caspase 9b mRNA were determined by densotimetric analysis of RT-PCR fragments. **e)** Western blot analysis of cytosolic and nuclear protien fractions from A549s transfected with GFP, SRp30a-WT, SRp30a-QD, SRp30a-QA, or SRp30a-RD. Fractions were analyzed for expression of the T7 tag, lamin, or α-tubulin.

To demonstrate that these phosphorylation sites are hyperphosphorylated in NSCLC cells, the phoshorylation status of the transiently expressed epitope-tagged SRp30a were analyzed by comparing the electrophoretic migration profiles of the proteins after incubation in the presence of either alkaline phosphatase or denatured alkaline phosphatase. Increased migration of SRp30a-WT was observed after treatment with alkaline phosphatase to levels corresponding to those of SRp30a-QD and SRp30a-QA after treatment with denatured alkaline phosphatase, indicating that serine 199, 201, 227, and 234 exist in a phosphorylated state in A549 cells (Figure 4-2).

4.3.2. The phosphorylation status of SRp30a modulates the effect of Akt signaling on alternative splicing of caspase 9.

Blaustein and co-workers recently provided evidence for a direct involvement of Akt in modifying SRp30a activity[141]. To determine whether the PI3-Kinase/Akt pathway regulates the alternative splicing of caspase 9 in a phospho-SRp30a-dependent manner, SRp30a-QD was expressed in the presence or absence of the Akt inhibitor, AKT VIII. In the presence of SRp30a-QD, AKT VIII inhibitor was unable to return/increase the ratio of the caspase 9a/9b mRNA to the same extent as compared to wild-type SRp30a. This effect was specific for the SRp30a-QD mutant as co-expression of either the empty vector or SRp30a wild-type vector had no effect on the ability of this inhibitor to increase the caspase 9a/9b mRNA ratio (Figure 4-3). Therefore, the Akt pathway regulates the alternative splicing of caspase 9 at least partially via the phospho-state of SRp30a.

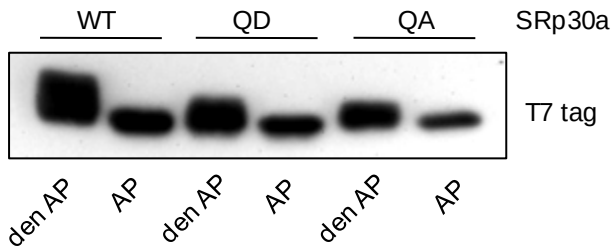

Figure 4-2. Serine residues 199, 201, 227, and 234 of SRp30a exist in a hyper-phosphorylated state in A549 cells. Immunoblot analysis of the phosphorylation state of SRp30a-WT and the SRp30a phospho-mutants. A549s were transfected with the indicated T7-tagged SRp30a constructs, total cell lysates were incubated in the presence of either active alkaline phosphatase (AP) or denatured alkaline phosphatase (den AP), and tagged SRp30a was detected by Western blotting.

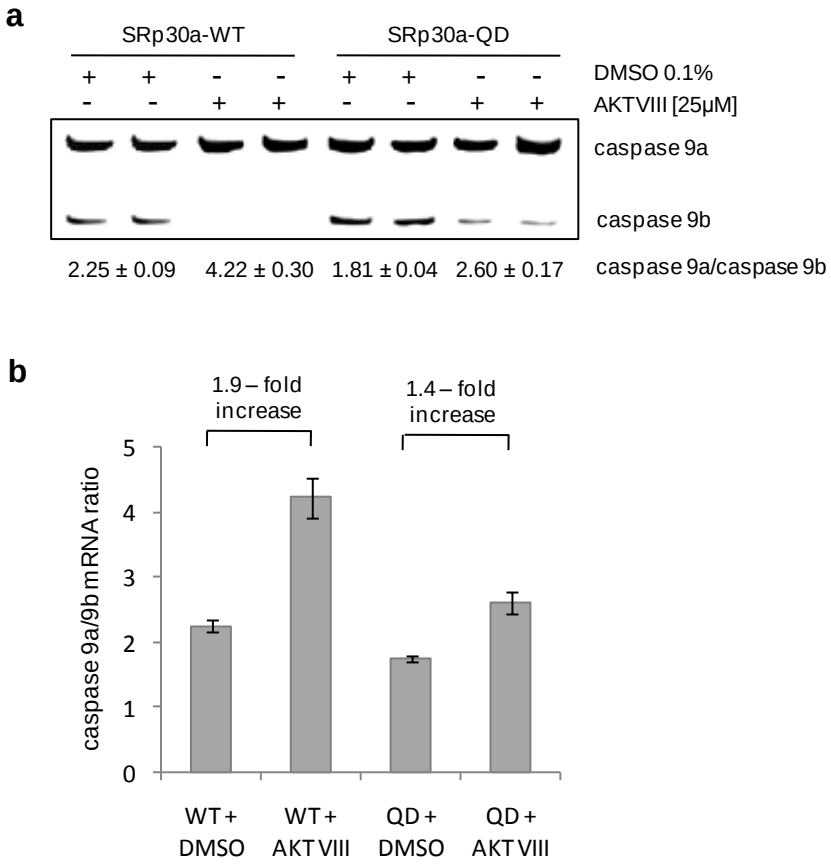

Figure 4-3. The phosphorylation status of SRp30a modulates the effect of Akt signaling on the alternative splicing of caspase 9. a) Quantitative/competitive RT-PCR analysis of endogenous caspase 9 splice variants and their corresponding caspase 9a/9b mRNA ratios from A549s transfected with SRp30a-WT or SRp30a-QD in the presence or absence of AKT inhibitor VIII [25μM]. Ratios of caspase 9a to caspase 9b mRNA were determined by densotimetric analysis of RT-PCR fragments. b) Graphical depiction of the caspase 9a/9b mRNA ratios.

4.3.3. The phosphorylation status of SRp30a regulates the anchorage-independent growth of A549 cells.

Our above findings indentify SRp30a as a critical RNA *trans*-factor regulating the alternative splicing of caspase 9 in a phosphorylation-dependent manner. Specifically, the phosphorylation status of serine 199, 201, 227, and 234 of SRp30a was demonstrated to modulate the caspase 9a/9b mRNA ratio. Importantly, these serine residues were predicted to be EGF stimulated as reported by PHOSIDA, a phosphorylation site database, and Human Protein Reference Database (HPRD). Based on this knowledge, and our findings that the E746-A750 del EGFR-expressing HBEC-3KT cells require a lowering of the caspase 9a/9b mRNA to confer anchorage-independent growth (discussed in chapter 3), we hypothesized that the phospho-status of SRp30a at serine 199, 201, 227, and 234 regulates the anchorage-independent growth of A549 cells. To test this hypothesis, A549s were transiently transfected with GFP-vector control, SRp30a-QD, and SRp30a-QA, and analyzed for their ability to grow in soft agar. Indeed, expression of SRp30a-QD resulted in significantly more colonies in soft agar as compared to A549s expressing SRp30a-QA or GFP-vector control (Figure 4-4). Conversely, expression of SRp30a-QA resulted in significantly fewer colonies in soft agar as compared to A549s expressing SRp30a-QD or GFR-vector control (Figure 4-4). Thus, the phosphorylation status of serine residues 199, 201, 227, and 234 of SRp30a plays an important role in the anchorage-independent growth of A549 cells.

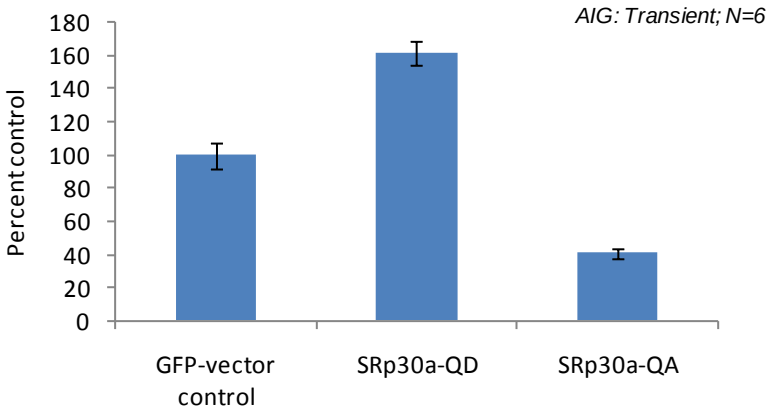

Figure 4-4. The phosphorylation status of SRp30a regulates the anchorage-independent growth of A549 cells. A549 cells transiently expressing GFP-vector control, SRp30a-QD, or SRp30a-QA were assayed for their ability to grow in soft agar. Cells were allowed to grow for two weeks before colonies were counted. Depicted is the quantification (mean ± s.d.) of the number of colonies counted, represented as percent control.

4.4. Discussion

The functional role of the RS domain of SR proteins in constitutive and alternative splicing has been extensively studied. Published reports have demonstrated that RS domains do not contribute to the protein's affinity for pre-mRNAs[230-232] but instead directs protein-protein interactions, functioning as activators of RNA splicing[124,233].

Initial studies presented in this chapter demonstrate that expression of an established phospho-mimic of SRp30a, SRp30a-RD, repressed the inclusion of the exon 3,4,5,6 exon cassette of caspase 9 pre-mRNA. The majority of the serine residues in the

RS domain of SRp30a-RD were previously mutated into aspartic acid[227]. Published reports with SRp30a-RD demonstrated *in vitro* that the RS domain of this mutant could mimic the structural and functional properties of a naturally phosphorylated RS domain that are required for RNA splicing[227].

These initial results were extended to identify the specific serine residues in the RS domain of SRp30a that regulated the inclusion/exclusion of the exon 3,4,5,6 cassette of caspase 9 pre-mRNA. Specifically, the phospho-status of serine residues 199, 201, 227, and 234 of SRp30a were demonstrated to modulate the effect of Akt signaling on the alternative splicing of caspase 9. These data are consistent with published findings by Blaustein and colleagues who have recently provided evidence for Akt directly phosphorylating SRp30a[141]. Similarly, Cooper and colleagues have presented strong data demonstrating translocation of Akt2 to the nucleus in response to insulin or IGF-1 and subsequent phosphorylation of the SR protein, SRp40, which closely resembles SRp30a[142]. Thus, we propose the predominantly expressed Akt isoform translocates to the nucleus and phosphorylates SRp30a at specific regulatory residues leading to the exclusion of the exon 3,4,5,6 cassette from caspase 9 mRNA. Lastly, studies presented in this chapter demonstrate that the phosphorylation status of serine residues 199, 201, 227, and 234 of SRp30a were also important in modulating the anchorage-independent growth of A549s.

CHAPTER 5

REGULATION OF THE ALTERNATIVE SPLICING OF BCL-X IN A549 LUNG ADENOCARCINOMA CELLS.

5.1. Introduction

Numerous studies have shown that overexpression of Bcl-x(L) in cells confers resistance to many apoptotic stimuli, and cooperates with oncogenic factors (e.g. c-Myc) in tumorigenesis[180,181,184-191]. Furthermore, many cell types spontaneously resistant to chemotherapeutic agents also demonstrate increased levels of Bcl-x(L)[192-197]. The regulation of Bcl-x(L) expression is a complex mechanism consisting of both transcriptional and post-transcriptional processes. In regards to post-transcriptional processing, the *BCL-x* gene, via alternative 5' splice site selection within exon 2, produces either the pro-apoptotic Bcl-x(s) (upstream 5'SS selection) or the anti-apoptotic Bcl-x(L) (downstream 5'SS selection). Several studies have demonstrated that the Bcl-x splice variant, Bcl-x(s), in contrast to Bcl-x(L), promotes apoptosis[181,198-201], and the mechanism of alternative 5' splice site selection of Bcl-x pre-mRNA has emerged as a potential target for anti-cancer therapeutics in non-small cell lung cancer (NSCLC). Specifically, Taylor and co-workers showed that Bcl-x alternative splicing was specifically modulated using an antisense oligonucleotide specific for Bcl-x pre-mRNA at a site surrounding the Bcl-x(L) 5' splice site[234]. Hybridization of this oligonucleotide to Bcl-x pre-mRNA induced an

increase in the expression of Bcl-x(s) and a decrease in the expression of Bcl-x(L) resulting in sensitization of the NSCLC cells to chemotherapeutic agents and eventually inducing apoptosis after long term exposure (> 48 hr)[234]. These findings were also demonstrated by Kole and co-workers who also translated these findings to several different cancer types[235]. Thus, regulation of the 5'SS selection within the Bcl-x exon 2 is a critical factor in determining whether a NSCLC cell is susceptible or resistant to apoptosis in response to chemotherapy[234-238].

To this end, previous studies by our laboratory defined the generation of *de novo* ceramide and the activation of protein phosphatase-1 (PP1) as major components of an apoptotic signaling pathway regulating the 5' splice site selection of Bcl-x exon 2 in response to chemotherapeutic agents[224,225]. A recent study by Zhou and co-workers verified these early findings and extended the list of chemotherapeutic agents to emetine, a potent protein synthesis inhibitor[239]. Mechanistic studies from our laboratory identified the ceramide-responsive RNA *cis*-element 1 (CRCE 1) located in exon 2 of Bcl-x pre-mRNA 277-295 bp upstream from intron 2[240]. In further mechanistic studies, the involvement of the splicing factor, SAP155, as an RNA *trans*-factor that interacted with CRCE 1 and regulated the 5'SS selection of Bcl-x pre-mRNA was elucidated[241]. Importantly, this RNA *trans*-factor was required for the effect of ceramide on the alternative 5'SS selection of Bcl-x, and downregulation of SAP155 by siRNA technology was as effective as ceramide in reducing the viability of NSCLC cells[241].

In this regard, we demonstrate that the ratio of Bcl-x(L)/(s) mRNA is increased in a large number of NSCLC tumors. Based in these findings, the goal of these studies was to

examine survival/mitogenic pathways responsible for regulating the alternative 5'SS selection of Bcl-x pre-mRNA in malignant NSCLC cells. Specifically, we identify the PI_3 kinase pathway as a key survival pathway regulating the alternative 5'SS selection of Bcl-x pre-mRNA to produce the anti-apoptotic Bcl-x(L) isoform. Furthermore, we show that PKCι is downstream of PI_3 kinase and regulates this alternative splicing mechanism via the expression of SAP155. Overall, this report defines the survival/oncogenic pathway regulating the alternative splicing of Bcl-x pre-mRNA in A549 lung adenocarcinoma cells.

5.2. Materials and Methods

5.2.1. Cell Culture

A549 cells were grown in 50% RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) and 50% Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with L-glutamine, 10% (v/v) fetal bovine serum (Sigma), 100 units/ml penicillin G sodium (Invitrogen Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin sulfate (Invitrogen Life Technologies, Carlsbad, CA). A549s were purchased from ATCC (Rockville, MD, USA). Cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO2, 37 °C).

5.2.2. Quantitative/Competitive RT-PCR

Total RNA from cell lines were isolated using the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using Superscript III reverse transcriptase (SuperScript™ First-Strand

Synthesis System for RT-PCR, Invitrogen™, Carlsbad, CA) and oligo (dT) as the priming agent. After 50 min of incubation at 42°C, the reactions were terminated by heating at 70°C for 15 min. Template RNA was then removed using RNase H (Invitrogen, Carlsbad, CA). The primers used to evaluate Bcl-x splice variants were (5'-GAG GCA GGC GAC GAG TTT GAA-3') and (5'-TGG GAG GGT AGA GTG GAT GGT-3') (Integrated DNA Technologies, Inc., Coralville, IA). The PCR conditions for analyzing the expression of Bcl-x splice variants were 95ºC for 5 mins followed by 25 cyles of 94ºC for 30s, 58ºC for 30s and 72ºC for 1min using Platinum *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA). The final PCR products were resolved on a 5% TBE acrylamide gel electrophoresis, stained with SYBR® Gold (Invitrogen™, Carlsbad, CA) and visualized using a Molecular Imager® FX (Bio-Rad) with a 488 nm EX (530 nm BYPASS) laser.

5.2.3. Western Immunoblotting

Cells were lysed using CelLytic™ lysis Buffer (Sigma-Aldrich) supplemented with protease Inhibitor cocktail (Sigma-Aldrich). Protein samples (5 µg) were subjected to 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (PDVF) (Bio-Rad) and blocked in 5% milk/1 X PBS – 0.1% Tween (M-PBS-T) for 2 h. Primary antibodies were anti-PKCι (1:1,000, Santa Cruz), anti-PKCζ (1:1,000, Santa Cruz), anti-Sap155 (1:1,000), and anti-β-actin (1:5,000, Sigma-Aldrich). Secondary antibodies were HRP-conjugated goat anti-mouse or anti-rabbit (1:5,000, Sigma-Aldrich). Immunoblots were developed using Pierce enhanced chemiluminescence (ECL) reagents and Bio-Max film.

5.2.4. Silence interfering RNA transfection

For inhibition of PKCι and PKCζ expression, cell lines were transfected with either PKCι SMARTpool multiplex, PKCζ SMARTpool multiplex, or scrambled control siRNA (Dharmacon; Lafayette, CO) using Dharmafect 1 transfection reagent (Dharmacon; Lafayette, CO) following the manufacturer's protocol. Briefly, cell lines were plated in six-well tissue culture dishes and allowed to rest overnight. At 50% confluence, cells were transfected with siRNA [100nM] using Dharmafect 1 in Opti-Mem I reduced-serum medium. Forty-eight hours post-transfection, RNA and/or protein were isolated.

5.2.5. Inhibitor studies

For Inhibitor studies, 1.2 x 10^5 A549 cells were plated into 6-well tissue culture plates. The following day media was removed and replaced with the appropriate complete growth media. Cells were subsequently treated with sham control (1:1000) or the appropriate concentration of active inhibitor (1:1000) (Calbiochem). Twenty-four hours post-treatment, total RNA and/or protein was isolated.

5.3. Results

5.3.1. The alternative splicing of Bcl-x is dysregulated in a variety of NSCLC tumors.

Previously, our laboratory had demonstrated that the alternative splicing of Bcl-x pre-mRNA was regulated by apoptotic stimuli [242,243]. In this study, we expanded our investigations to test the hypothesis that the alternative splicing of Bcl-x was dysregulated

(in a pro-survival manner or increased Bcl-x(L)/(s) mRNA ratio) in cancer phenotypes, specifically NSCLC tumors. Utilizing total RNA samples from pathologist-verified human NSCLC tumor samples, quantitative/competitive RT-PCR analysis was performed to determine the degree of dysregulation in the Bcl-x(L)/(s) ratio as compared to normal lung tissue controls. Tumor samples were categorized into three groups respectively: normal, a Bcl-x(L)/(s) mRNA ratio < 5.8; moderately dysregulated, a Bcl-x(L)/(s) mRNA ratio of 5.8-7.3; and highly dysregulated, a Bcl-x(L)/(s) mRNA ratio > 7.3 (Figure 5-1). These classifications regarding the degree of dysregulation were due to the observation that the normal lung tissue samples had a Bcl-x(L)/(s) ratio of 4.75 ± 0.15 (N=4). Of the NSCLC tumors examined, 70% (N=47) presented with a > 25% increase in the Bcl-x(L)/(s) mRNA ratio as compared to normal lung tissue controls. Even more dramatic, 27% of NSCLC tumors examined presented with a > 50% increase in the Bcl-x(L)/(s) mRNA ratio (Figure 5-1). Thus, the ratio of Bcl-x(L)/(s) mRNA is dramatically increased in a high percentage of NSCLC tumors correlating with an increase in the oncogenic cooperating factor, Bcl-x(L). Therefore, these data indicate that a significant portion of lung adenocarcinoma tumors demonstrate dysregulated alternative splicing of Bcl-x favoring a pro-survival/pro-oncogenic phenotype.

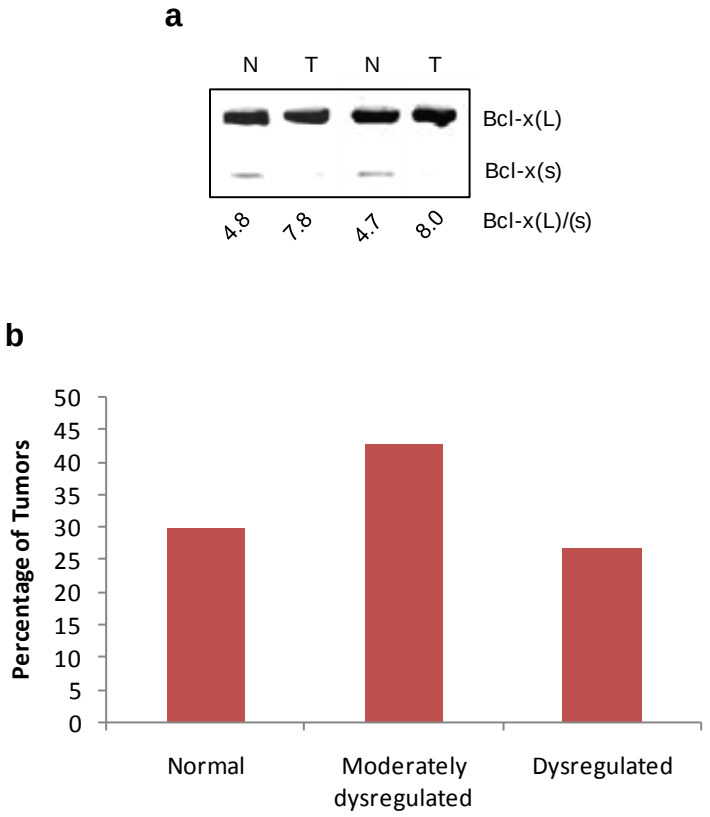

Figure 5-1. The Bcl-x(L)/(s) mRNA ratio is dysregulated in NSCLC tumors. A population of cDNAs from pathologist-verified lung adenocarcinomas, squamous cell carcinomas, and large cell carcinomas (Origene; Rockville, MD) underwent quantitative/competitive PCR for expression of Bcl-x splice variants. **a)** Representation of the RT-PCR analysis used to determine the degree of Bcl-x(L)/(s) dysregulation in NSCLC tumor samples where N is normal lung tissue and T is tumor tissue. **b)** Quantitative/competitive PCR analysis of Bcl-x splice variants demonstrate that 43% of NSCLC tumors present a moderately dysregulated Bcl-x(L)/(s) mRNA ratio (Bcl-x(L)/(s) ratio of 5.9-7.3) and 27% of NSCLC tumors present a highly dysregulated Bcl-x(L)/(s) mRNA ratio (Bcl-x(L)/(s) ratio >7.3) (N=47), as determined by densitometric analysis of PCR products.

5.3.2. The PI3-Kinase pathway regulates the 5' splice site selection of Bcl-x pre-mRNA.

Previously, our laboratory has demonstrated that increases in endogenous ceramide levels via the *de novo* sphingolipid pathway regulates the alternative splicing of Bcl-x pre-mRNA resulting in a decrease in the Bcl-x(L)/(s) mRNA ratio[225]. In this study, we hypothesized that one of the major survival/mitogenic pathways regulates the alternative processing of Bcl-x pre-mRNA to favor the production of anti-apoptotic Bcl-x(L), thereby increasing the Bcl-x(L)/(s) mRNA ratio. To investigate this hypothesis, these pathways were examined for regulation of the alternative splicing of Bcl-x pre-mRNA utilizing small-molecule inhibitors well-characterized in the scientific literature. Treatment of A549 cells with the following inhibitors, the MAP Kinase inhibitor PD 98059 [10μM], MEK 1/2 inhibitor U0126 [10μM], Rho-kinase inhibitor Y-27632 [10μM], Casein Kinase II inhibitor DMAT [10μM], and Src kinase inhibitor [25μM] had no effect on the alternative splicing of Bcl-x pre-mRNA. In contrast, treatment of A549 lung adenocarcinoma cells with the PI3-Kinase inhibitor LY294002 [50 μM] resulted in a significant reduction in the ratio of Bcl-x(L)/(s) splice variants compared to the inactive, structurally related compound, LY303511 [50μM] (Figure 5-2). Specifically, the Bcl-x(L)/(s) mRNA ratio decreased from 6.00 ± 0.16 for LY303511 control-treated samples to 3.40 ± 0.19 for LY294002-treated samples ($p < 0.01$; n=6), corresponding to approximately a ~45% decrease in the Bcl-x(L)/(s) mRNA ratio. Therefore, these data demonstrate that PI3-Kinase regulates the alternative splicing of Bcl-x pre-mRNA in an anti-apoptotic/pro-survival manner.

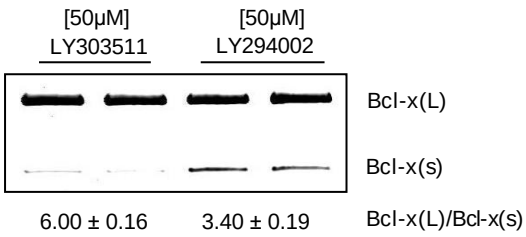

Figure 5-2. Effect of PI3-Kinase inhibiton on the alternative splicing of Bcl-x pre-mRNA. Quantitative/competitive RT-PCR analysis of Bcl-x splice variants and the corresponding Bcl-x(L)/(s) mRNA ratios from A549s treated with either structurally inactive LY303511 [50μM] or LY294002 [50μM]. The ratio of Bcl-x(L) to Bcl-x(s) mRNA was determined by densitometric analysis of RT-PCR fragments ($p<0.01$, N=6). Data are expressed as means ± s.d. Data are representative of three separate determinations on two separate occasions.

5.3.3. PKCι regulates the activation of the Bcl-x(s) 5' splice site in A549 cells.

Akt/PKB, aPKCs (ξ andι), mTOR/S6 kinase, and PKCδ are downstream of PI3Kinase, all of which have been well-described in the literature. To investigate the downstream effector of PI3-Kinase responsible for regulating the alternative splicing of Bcl-x pre-mRNA, well-characterized small-molecule inhibitors and RNAi technology were employed. Treatment of A549 cells with either the small-molecule Akt1/2 inhibitor, AKT VIII [25μM], the classical PKC (cPKC) inhibitor, GF109203X [10μM], the cPKC/novel PKC (nPKC) inhibitor, Gö6976 [10μM], or the mTOR/S6 kinase inhibitor, rapamycin [10μM], had no effect on the Bcl-x(L)/(s) mRNA ratio. On the other hand, treatment of A549 cells with the pan-Protein Kinase C (cPKCs, nPKCs, and atypical PKCs) inhibitor Gö6983 [10 μM] (Figure 5-3), significantly decreased the Bcl-x(L)/(s) mRNA ratio from 6.04 ± 0.18 for control samples to 3.16 ± 0.29 ($p<0.05$; n=6). The effects were specific to

the 5'SS selection of Bcl-x pre-mRNA as the alternative splicing of caspase 9, another *de novo* ceramide target, was unaffected by Gö6983. Therefore, these data demonstrate that the PI3-Kinase survival pathway regulates the alternative splicing of Bcl-x via an atypical PKC.

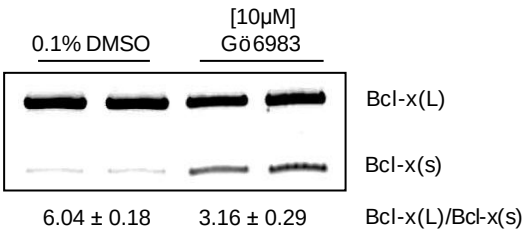

Figure 5-3. Inhibition of PKCs decreases the Bcl-x(L)/(s) mRNA ratio in A549 cells. Quantitative/competitive RT-PCR analysis of Bcl-x splice variants and the corresponding Bcl-x(L)/(s) mRNA ratios from A549s treated with either 0.1% DMSO or Gö6983 [10μM]. The ratio of Bcl-x(L) to Bcl-x(s) mRNA was determined by densitometric analysis of RT-PCR fragments ($p<0.01$, $N=6$). Data are expressed as means ± s.d. Data are representative of three separate determinations on two separate occasions.

The results obtained with the small molecule inhibitors demonstrated that an aPKC was involved in regulating the alternative splicing of Bcl-x pre-mRNA. Specifically, the PKC inhibitors, Gö6976 & GF109203X, are effective toward cPKCs, nPKCs, and PKD/PKCμ, but had no effect on the alternative splicing of Bcl-x pre-mRNA. In contrast, the pan-PKC inhibitor Gö6983 was very effective in lowering the Bcl-x(L)/(s) mRNA ratio. In contrast to both Gö6976 & GF109203X, Gö6983 also inhibits atypical PKCs.

Therefore, process of elimination suggested that an aPKC regulated the 5'SS selection of Bcl-x pre-mRNA. To confirm a role for an aPKC in this mechanism, siRNA to both human aPKC isoforms, PKCι and PKCζ, was employed. In contrast to PKCζ, Downregulation of PKCι, a known downstream target of the PI3-Kinase, induced the activation of the Bcl-x(s) 5'SS, decreasing the Bcl-x(L)/(s) ratio from 6.12 ± 0.12 for DMSO-treated cells to 4.01 ± 0.11 for siPKCι-treated cells (Figure 5-4a). Thus, the atypical PKC, PKCι, regulates the alternative splicing of Bcl-x pre-mRNA in a pro-survival fashion.

Our laboratory has previously reported that the RNA *trans*-factor, SAP155, regulates the 5' splice site selection of Bcl-x pre-mRNA. To determine whether the expression of SAP155 was a distal mechanism regulated by PKCι, siRNA to PKCι was again employed to examine the expression of SAP155. Knockdown of PKCι by siRNA induced a significant decrease in the levels of SAP155 (Figure 5-4b). These data provide preliminary evidence for PKCι regulation of the 5'SS selection of Bcl-x pre-mRNA via upregulation of SAP155.

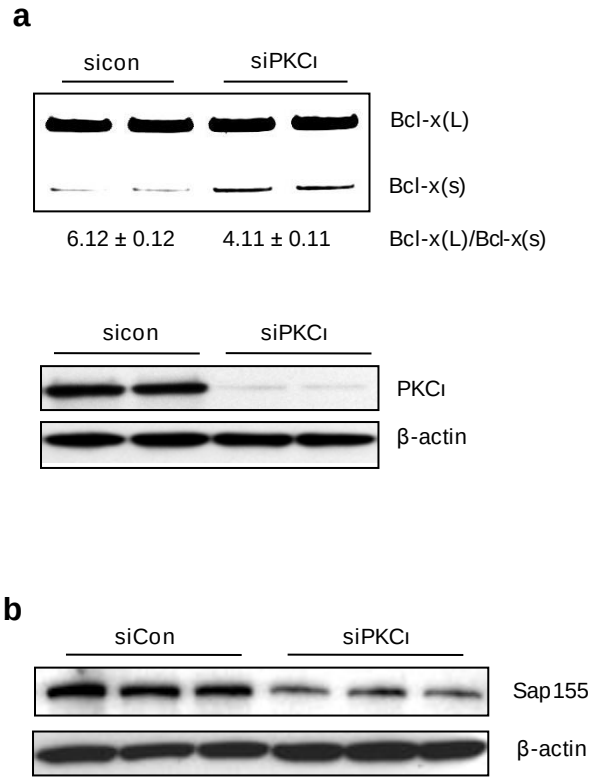

Figure 5-4. Downregulation of PKCι decreases the Bcl-x(L)/(s) mRNA ratio and correlates with a downregulation of Sap155 protein expression in A549 cells. Quantitative/competitive RT-PCR analysis of Bcl-x splice variants and the corresponding Bcl-x(L)/(s) mRNA ratios from A549s transfected with control siRNA or PKCι siRNA. Knockdown of PKCι was confirmed by western blot. The ratio of Bcl-x(L) to Bcl-x(s) mRNA was determined by densitometric analysis of RT-PCR fragments ($p<0.01$, N=6). Data are expressed as means ± s.d. **b**) Total proteins from A549s transfected with the indicated siRNAs were also subjected to western blot analysis to determine expression of SAP155.

5.4. Discussion

The presented study began from our first observation that the alternative splicing of Bcl-x pre-mRNA was dysregulated in a large percentage of transformed lung epithelial cells. This led to the hypothesis that a survival pathway of signal transduction regulated the alternative splicing of Bcl-x pre-mRNA. Indeed, we have previously shown that a contrasting pathway in apoptotic signaling existed for the activation of the Bcl-x(s) 5' splice site. Specifically, our laboratory showed that the production of Bcl-x(s) via alternative splicing was dependent on the generation of *de novo* ceramide and the activation of PP1 [242,244]. Therefore, a survival pathway regulating this key distal mechanism and balancing the cell between apoptosis and survival was likely to exist as well. The data presented demonstrates that the atypical PKC, PKCι, is a major regulator of the alternative splicing of Bcl-x pre-mRNA in A549 cells, acting downstream of the major survival/oncogenic pathway, PI3-Kinase. Therefore, in contrast to published reports demonstrating a classical PKC responsible for regulating the 5' SS selection of Bcl-x pre-mRNA in non-malignant cells[245], it is an oncogenic atypical PKC that regulates the 5'SS selection in a pro-survival fashion in transformed phenotypes.

Once the upstream components in the survival signaling pathway were determined, our laboratory focused on linking these upstream signals to downstream RNA *trans*-factors known to modulate the 5' splice site selection of Bcl-x pre-mRNA. For this purpose, we turned to the literature as well as our own work for these RNA *trans*-factors. In a collaboratory study with Sette and co-workers, SAM68, a well established RNA *trans*-factor with roles in cell signaling and transformation, was shown to regulate the 5' splice

site selection in HEK293 cells. However, this finding did not translate to non-small cell lung cancer cells, as siRNA directed against SAM68 only slightly inhibited the use of the Bcl-x(s) 5' splice site[246].

From the negative results obtained on SAM68, we then examined the expression of the RNA *trans*-factor, SAP155. Our laboratory has previously reported this RNA *trans*-factor functioning as a repressor of the the Bcl-x(s) 5' splice site[241]. The data presented in this chapter provides a preliminary link between the expression of SAP155 to the activation/expression of the NSCLC proto-oncogene, PKCι. As to how PKCι regulates the expression of SAP155 is currently unknown.

As stated in the introduction of this chapter, the physiological significance of the Bcl-x(L)/Bcl-x(s) mRNA ratio has been documented by many reports in the literature demonstrating that the fate of the cell can be determined by the proportion of anti-apoptotic Bcl-x(L) to pro-apoptotic Bcl-x(s) [181,247,248]. Furthermore, the induction of pro-apoptotic Bcl-x(s) has also been shown to sensitize cells to apoptosis and loss of viability to chemotherapeutic agents [247,248]. Published findings from our laboratory corroborate these findings demonstrating that treatment of A549 cells with concentrations of ceramide known to activate the Bcl-x(s) 5' splice site also lowered the IC_{50} of the chemotherapeutic agent, daunorubicin [242]. Taken together, these data suggest a link between signal transduction pathways mediating the 5' splice site selection of Bcl-x pre-mRNA and the sensitivity of cells to apoptosis in response to chemotherapeutics. Specifically, SAP155 expression may be a key link, as we have previously shown that ceramide could not induce apoptosis or sensitize cells to daunorubicin in A549 cells above the extent of SAP155

downregulation by siRNA. Unfortunately, little is known about the PKCι signaling pathway to form a strong hypothesis as to how ceramide may be blocking signals mediating SAP155 expression. The activation of the Bcl-x(s) 5'SS is regulated by a serine/threonine protein phosphatase. Therefore, it is conceivable that a ceramide-activated protein phosphatase such as PP1 may dephosphorylate PKCι effectively "shutting-down" the pro-survival pathway and thus, SAP155 expression.

Outside of the realm of chemotherapy sensitive, the alternative splicing of Bcl-x pre-mRNA may also have roles in oncogenesis. Recently, Evan and co-workers demonstrated that Bcl-x(L) cooperated with c-Myc in oncogenic transformation *in vivo*[188]. These findings correlate well to the observation that a large number of NSCLC tumors demonstrated dysregulation of the alternative 5'SS selection of Bcl-x exon 2 to favor Bcl-x(L) expression. Therefore, PKCι may act as an oncogene via simple removal of Bcl-x(s) with concomitant increase in Bcl-x(L), which promotes oncogenesis.

In conclusion, this study demonstrates several novel findings. First, this study demonstrates that the PI3-Kinase/PKCι pathway regulates the alternative 5'SS selection of Bcl-x exon 2. Second, this study provides preliminary data that the proto-oncogene PKCι regulates this distal mechanism via upregulation of SAP155. Therefore, this study provides a key distal signaling mechanism regulated by a proto-oncogene for NSCLC and provides a plethora of new targets for the development of therapeutics to combat NSCLC.

CHAPTER 6

GENERAL DISCUSSION

RNA splicing is an essential, precisely regulated process and, its regulation has been demonstrated to play a critical role in human disease[202]. Research has clearly demonstrated that dysregulation of cell signaling pathways has a significant impact on alternative RNA splicing in human pathologies, including cancer[249-251]. In this study, the oncogenic signaling pathways regulating the alternative splicing of caspase 9 and Bcl-x in NSCLC were examined. The molecular mechanisms regulating the alternative splicing of Bcl-x were thoroughly discussed in chapter 5. Therefore, this chapter will focus on discussing the molecular mechanisms regulating the alternative splicing of caspase 9 and their relevance to our current understanding of NSCLC.

The presented study demonstrates several important and novel findings and begins to clarify the complex mechanism regulating the alternative splicing of caspase 9 as well as impacts the mounting data as to clinical relevance. As to the latter, the study begins by demonstrating that the alternative splicing of caspase 9 is dysregulated in a large majority of NSCLC cell lines and tumors, including adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. More importantly, the presented study also demonstrates the intriguing finding that a key oncogenic factor in NSCLC, EGFR overexpression/mutation, regulates this splicing mechanism. The significance of this finding stems from the

knowledge that EGFR mutation occurs in a large percentage of NSCLCs, roughly 10%. Furthermore, EGFR mutation is also considered an early event in the development of NSCLC, since the mutation is also found in normal epithelial cells prior to metaplasia and adenoma formation, enhancing the hypothesis that the alternative splicing of caspase 9 plays a role in early events leading to the formation of NSCLC. Importantly, overexpressed EGFR, along with its ligands, occur in approximately 70% of NSCLCs. In this regard, this study also demonstrates that the PI3-Kinase/Akt pathway is downstream of the EGFR mutation/overexpression and upstream of the alternative splicing of caspase 9. Again, this makes logical sense in relation to cellular transformation as this pathway is found constitutively active in approximately 58% of NSCLC cell lines and tumors[69,70]. This pathway is also linked to constitutive EGFR activity, NF-κB activation, and the ability of oncogenic ras to transform various cell types. More importantly in a therapeutic sense, Akt activity correlates with patient survival after treatment with agents targeting EGFR (e.g. gefitinib). Furthermore, Akt inhibitors have been shown to circumvent the resistance of cells to gefitinib acquired from the $T^{790}M$ EGFR mutation. Thus, the Akt pathway activated by the EGFR receptor is extremely important in a therapeutic sense, and this study further shows that this activation of Akt is distinctly different from the Akt pathway activated by oncogenic Ras.

Clinical relevance is further enhanced by our findings with the EGFR inhibitor, erlotinib (e.g. Tarceva™). The treatment of NSCLC cells with this compound increased the caspase 9a/9b mRNA ratio to a more normal presentation. More importantly, genetic manipulation of the alternative splicing of caspase 9 had profound effects on the sensitivity

of NSCLC cells to this chemotherapy. Ectopic expression of caspase 9b essentially produced NSCLC cells completely resistant to erlotinib at concentrations 4-times the IC_{50} as compared to control cells. As erlotinib has shown promise in clinical trials by significantly improving the survival rate of NSCLC patients, examining the alternative splicing of caspase 9 may have future prognostic value for a subset of patients.

A role for EGF/PI3-Kinase/Akt signaling in regulating the alternative splicing of caspase 9 in a pro-oncogenic manner is also interesting due to the links between Akt signaling and the modulation of RNA *trans*-factors of the SR protein family. For example, a collaboratory study with Cooper and co-workers demonstrated that Akt2 regulated the alternative splicing of protein kinase CβII pre-mRNA via phosphorylation of the SR protein family member, SRp40[142]. Furthermore, a recent publication by Blaustein and co-workers demonstrated that Akt could phosphorylate SRp30a directly in cells, and the serines 199, 201, 227, and 234 were among those identified[141]. Based on the aforementioned reports, the direct phosphorylation of SRp30a by Akt could occur either in the nucleus or in the cytoplasm. For example, Akt has been known to translocate to the nucleus upon activation by certain stimuli where the enzyme could phosphorylate the RNA *trans*-factor. Conversely, SRp30a is localized almost exclusively in the nucleus upon phosphorylation due to shuttling, and thus activated Akt may also phosphorylate the RNA *trans*-factor in the cytoplasm and induce the translocation of SRp30a to the nucleus, compete with dephosphorylated SRp30a, and repress the inclusion of the exon 3,4,5,6 cassette of caspase 9 pre-mRNA. Cooper and co-workers have in recent years proposed an analogous mechanism as to the former presenting strong data that Akt2 translocates to the

nucleus in response to insulin or IGF-1 to phosphorylate the RNA *trans*-factor, SRp40, which is closely-related to SRp30a[142]. Thus, we propose that EGF induces the activation and translocation of the predominantly expressed Akt isoform to the nucleus/nuclear membrane, which phosphorylates SRp30a at specific regulatory residues leading to the exclusion of the exon 3,4,5,6 cassette from caspase 9 mRNA (Figure 6-1).

A hyper-phosphorylated SRp30a affecting the inclusion/exclusion of exons is in line with reports in the literature on the functionality of this RNA *trans*-factor. For example, Manley and co-workers demonstrated that phosphorylation of SRp30a did not affect the ability of the RNA *trans*-factor to bind to a specific RNA *cis*-element, but hyper-phosphorylation did inhibit the ability of SRp30a to recruit other RNA *trans*-factors required for the role of this protein in modulating alternative splicing cascades[133]. Thus, hyper-phosphorylation of SRp30a by Akt would likely inhibit the ability of this RNA *trans*-factor to recruit other spliceosomal components and enhance the inclusion of the exon 3,4,5,6 cassette of caspase 9. This study completely supports these early findings of Manley and co-workers and suggests that a hyperphosphorlated SRp30a on serines 199, 201, 227, and 234, and possibly other unidentified sites by Akt, is incompetent as an activator of the inclusion of the exon 3,4,5,6 cassette of caspase 9.

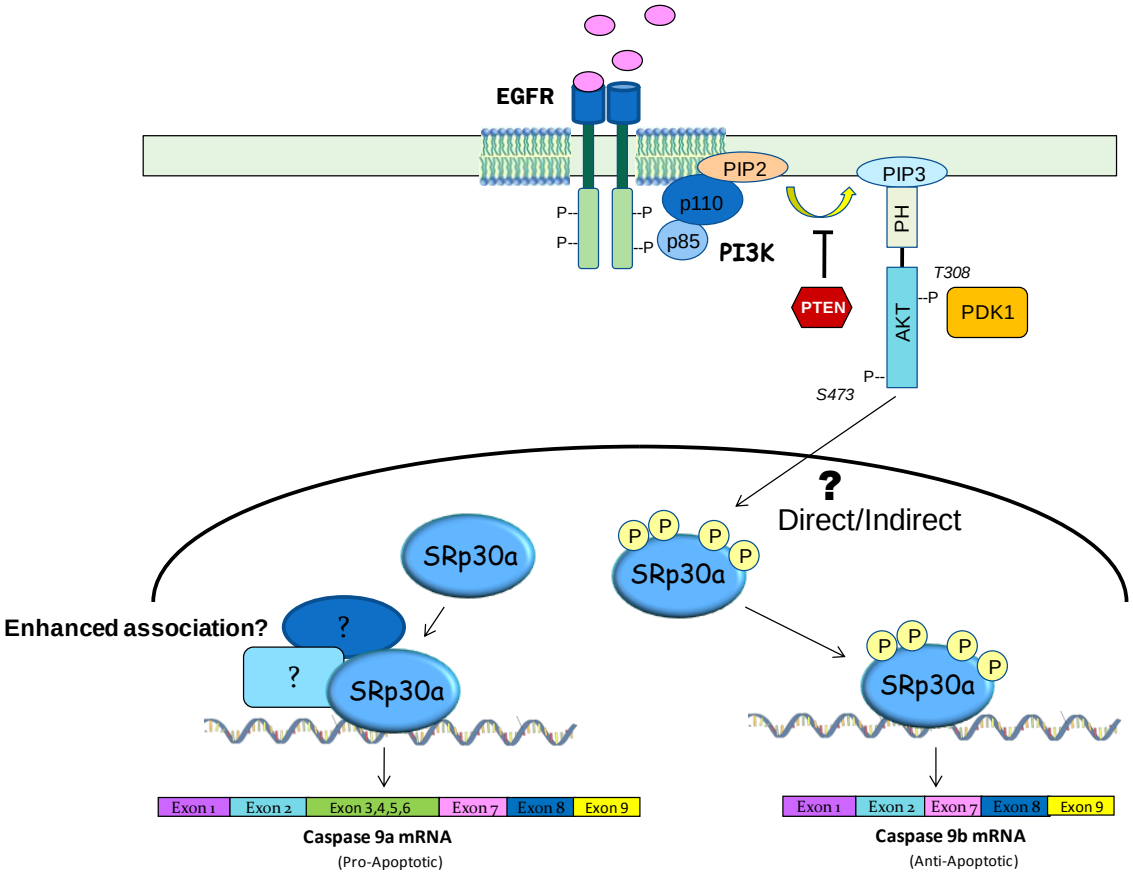

Figure 6-1. The alternative splicing of caspase 9 is regulated by the EGFR/PI3-Kinase/Akt pathway in a phospho-SRp30a-dependent manner. Following EGFR stimulation, PI3Kinase becomes activated and in turn, generates specific inositol-phospholipids that are recognized by Akt. Akt then translocates to the membrane through binding of its PH-domain and becomes activated. Akt is then released to perform both cytosolic and nuclear functions. It is unclear whether Akt modifies SRp30a in the nucleus or the cytoplasm, or if Akt phosphorylates SRp30a directly or indirectly. Phosphorylated SRp30a, specifically on serine residues 199, 201, 227, and 234, then functions to repress the inclusion of the exon 3,4,5,6, cassette of caspase 9, favoring the production of caspase 9b mRNA. We hypothesize when SRp30a is phosphorylated, steric hindrance prevents the association of key binding partners such. However, non-phosphorylated SRp30a is able to associate with key binding partners, allowing it to enhance inclusion of the exon 3,4,5,6, cassette of caspase 9, favoring the production of caspase 9a.

Indeed, a preliminary search of the Splicing Rainbow database[252] predicts multiple RNA *cis*-elements for SRp30a within the exon 3,4,5,6 cassette of caspase 9, possibly explaining the major role that SRp30a plays in mediating the inclusion of this exon cassette. Interestingly, the phospho-state of SRp30a in regulating the inclusion/exclusion of the exon 3,4,5,6 cassette also "fits" well with our previous findings that ceramide induced both the dephosphorylation of SR proteins (including SRp30a) and the inclusion of the caspase 9 exon cassette favoring caspase 9a expression[226]. Furthermore, our laboratory demonstrated that SRp30a was required for ceramide effects on the inclusion of the exon cassette. Thus, a dephosphorylated SRp30a correlates with exon inclusion and enhanced complex formation. Based on our previous results and results presented in this study, this mechanism of SRp30a phospho-status and the alternative splicing of caspase 9 may be a key distal point by which ceramide acts as a tumor suppressing/cell senescence agent. Ceramide signaling and the PI3-Kinase/AKT pathway are well established to antagonize each other at various points, but this study suggests that the phospho-state of SRp30a and the alternative splicing of caspase 9 may well be a key point and a cellular biostat for whether a cell undergoes apoptosis/senescence or is transformed to a malignant phenotype.

How caspase 9b is acting to drive tumorigenic capacity is more of an enigma. Whereas the role of caspase 9b in circumventing the p53 knockdown requirement is obvious from the work of Lowe and co-workers, how caspase 9b is important in the development of anchorage-independent growth is completely unclear. As we have demonstrated, stable or transient removal of caspase 9b using shRNA blocked the ability of E746-A750 del EGFR-expressing HBEC-3KT cells to induce anchorage-independent

growth. This can likely not be attributed to the blockade of an initiator caspase such as caspase 9a, and suggests a role in cell signaling for caspase 9b. The possibility that caspase 9b acts as a signaling molecule has been described in the literature by Latchman, D.S. and co-workers[170]. Specifically, this laboratory group showed that ectopic expression of caspase 9b induced the activation of NF-κB irrespective of caspase activation as a pan-caspase inhibitor could not elicit the same effect[170]. Furthermore, this group demonstrated that only the CARD domain was required for this effect. Activation of NF-κB by caspase 9b expression "fits" well with cooperation with *k-Ras* mutations for the induction of cellular transformation based on the findings of Baldwin and co-workers. This laboratory group showed in several different ways that NF-kB activation enhances the ability of oncogenic Ras to induce cellular transformation[253,254]. Coupled with the knowledge that EGFR overexpression/mutation leads to both cooperation with oncogenic ras in cellular transformation and NF-κB activation, a role for caspase 9b in these pathways important for oncogenesis is logical. Furthermore, increased expression of caspase 9b is conferred by EGFR signaling. Thus, caspase 9b may act as a scaffolding protein to elicit downstream signaling events and having a role outside the simple inactivation of caspase 9a. This possibility is far from inconceivable as initiator pro-caspases such as caspase 8 and 2 are suggested to play roles in the recruitment cell survival factors such as PI3-Kinase subunits[255-257]. Although these caspases have roles in extrinsic pathways of programmed cell death, caspase 9b may be playing an analogous role in survival signaling to block the intrinsic pathway of programmed cell death. This would suggest that the alternative

splicing of both caspase 8 and 2 (both of which have dominant-negative splice variants) may play roles in cell survival signaling as well.

In conclusion, the presented study reports several major findings taking a comprehensive approach. First, the dysregulation of the alternative splicing of caspase 9 toward a pro-survival phenotype was confirmed in a large sample size of non-small cell lung cancers as well as individual cell lines. Second, a survival/mitogenic pathway involving PI3-Kinase and Akt was shown to be upstream in regulating this distal splicing mechanism. Third, EGFR overexpression/mutation affected the alternative splicing of caspase 9 to produce caspase 9b via this survival pathway. Lastly, the phospho-state of SRp30a was shown to regulate this distal mechanism via Akt signaling. Therefore, the presented study demonstrates a novel and key distal mechanism in NSCLC and provides a slew of new target mechanisms for the development of therapeutics to combat one of the deadliest cancers in the world.

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PERSONAL INFORMATION

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EDUCATION

December, 2009	**Ph.D. in Biochemistry & Molecular Biology** Dissertation: The Regulation of Alternative Splicing by Oncogenic Signaling Pathways. Cumulative GPA: 4.0 *Virginia Commonwealth University, Richmond, Virginia* School of Medicine, Department of Biochemistry & Molecular Biology
August, 2003	**B.S. in Biology** *James Madison University, Harrisonburg, Virginia* Department of Biology

PROFESSIONAL & TEACHING EXPERIENCE

2004-2009 **Graduate Student**
Virginia Commonwealth University, Richmond, Virginia
Department of Biochemistry & Molecular Biology

- Conduct scientific research for the Department of Biochemistry, School of Medicine by performing laboratory experiments related to biochemistry, molecular biology, and cell biology.
- Collect and analyze data from the following Molecular biology techniques: Plasmid propagation and cloning, PCR, RNA and DNA isolation, gel electrophoresis, siRNA transfections, fluorescent imaging, cell sorting, and confocal microscopy.
- Collect and analyze data from the following Biochemical techniques: Western immunoblotting, protein quantification and purification, and protein assays. Experience in Mammalian tissue culture of human immortalized epithelial cells, primary keratinocytes, Non-small cell lung cancer cell lines, and HeLa cells.
- Maintain accurate and timely laboratory records and experimental protocols.

2005-2007 **Teaching Assistant**
Virginia Commonwealth University, Richmond, Virginia
Department of Biochemistry & Molecular Biology

- Teaching assistant for BIOC 503-504/MIC 503-504, *Biochemistry, Cellular and Molecular Biology I, II.*
- Provided tutoring to dental and pharmacy students in basic biochemistry and reviews on current concepts of modern cellular and molecular biology.

2002 **Undergraduate Research Assistant**
James Madison University, Harrisonburg, Virginia
Department of Biology
Provided technical support in maintaining Drosophila stocks and scoring genetic phenotypes

HONORS & AWARDS

2009 **Nominated for membership into Phi Kappa Phi Honor Society**

2000 **Nominated for membership into the National Society of Collegiate Scholars**

CONFERENCES & PRESENTATIONS

January, 2009 **Emerging Tumor Suppressors.**
2009 Keystone Symposia Conference
Taos, New Mexico
Poster Presentation: *The alternative splicing of caspase 9 is modulated by the PI3Kinase/Akt pathway and dependent on the phospho-status of SRp30a.*

November, 2008 **The 43rd Annual Southeastern Regional Lipid Conference.**
Cashiers, North Carolina
Poster Presentation: *The alternative splicing of caspase 9 is modulated by the PI3Kinase/Akt pathway in a pro-survival/oncogenic manner.*

October, 2008 **Daniel T. Watts Research Poster Symposium.**
Virginia Commonwealth University, Richmond, Virginia
Poster Presentation: *The alternative splicing of caspase 9 is modulated by the PI3Kinase/Akt pathway in a pro-survival/oncogenic manner.*

October, 2008 **Massey Cancer Center Research Retreat.**
Virginia Commonwealth University, Richmond, Virginia
Poster Presentation: *The alternative splicing of caspase 9 is modulated by the PI3Kinase/Akt pathway in a pro-survival/oncogenic manner.*

November, 2006 **The 41st Annual Southeastern Regional Lipid Conference.**
Cashiers, North Carolina
Poster Presentation: *The alternative splicing of Bcl-x is regulated by the PI3Kinase/PKCι pathway.*

November, 2006 **Signaling and Metabolism of Lipids Seminar Series.**
Virginia Commonwealth University, Richmond, Virginia
Oral Presentation: *The proto-oncogene AKT2 regulates the alternative splicing of caspase 9 in a pro-survival manner.*

October, 2006 **Daniel T. Watts Research Poster Symposium.**
Virginia Commonwealth University, Richmond, Virginia
Poster Presentation: *Akt2 regulates the alternative splicing of caspase 9 in A549cells in a pro-survival/oncogenic manner.*

March, 2006 **ICAMS Seminar Series**
Virginia Commonwealth University, Richmond, Virginia

Oral Presentation: *Keratinocyte Function: Signaling by Electrospun Extracellular Matrices.*

July, 2005 **Tissue Repair and Regeneration.**
2005 Gordon Research Conference Programs
Colby-Sawyer College, Colby-Sawyer, New Hampshire

PROFESSIONAL MEMBERSHIPS

2000 – Present **National Society of Collegiate Scholars**
James Madison University Chapter, Harrisonburg, Virginia

2009 – Present **Phi Kappa Phi Honor Society**
Virginia Commonwealth University Chapter, Richmond, Virginia

PUBLISHED MANUSCRIPTS AND PAPERS

1. **Jacqueline C. Shultz** and Charles E. Chalfant. Emetine and the alternative splicing of Bcl-x: where to next? *Chem Biol* **14**, 1313-4 (2007).
2. Ayres, C., Bowlin, G.L., Henderson, S.C., Taylor, L., **Shultz, J.**, Alexander, J., Telemeco, T.A., Simpson, D.G., Modulation of anisotropy in electrospun tissue-engineering scaffolds: Analysis of fiber alignment by the fast Fourier transform. *Biomaterials* **27**, 5524-34 (2006).