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P90/CIP2A Regulates Lung Cancer Cell Proliferation And Cell Apoptosis Via The AKT Signaling Pathway

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Dedication

This work is dedicated to my mother and father, Mrs. Suping Qiao and Mr. Tianyou Lei.

They always believe in me and encourage me to reach my goal.

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by

NINGJING LEI, B.S.

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Abstract

p90/CIP2A is a novel characterized endogenous protein phosphatase 2A (PP2A) inhibitor, which was originally identified as a tumor-associated antigen (TAA) in gastric and liver cancer patients. Many studies have documented that p90/CIP2A plays critical roles in c-Myc stability through post-translational modification. However other functions of this protein in cancer progression are still largely unknown. Recently, we found that p90/CIP2A was overexpressed in human lung cancer specimens as compared to normal lung tissues. And the elevated expression of p90/CIP2A in lung cancer cells led to the increased cell proliferation and decreased cell apoptosis. Human lung cancer is currently the leading cause for cancer-related deaths in both men and women in the United States, which is difficult to diagnose at early stage due to few symptoms felt by the patients. Our results indicate that p90/CIP2A should be closely related to human lung cancer. Therefore, it's of great importance to understand the molecular mechanism of how p90/CIP2A is evolved in lung cancer progression for future biomarker discovery and therapeutic design.

In our laboratory, the combinatorial use of proteomic, bioinformatics and metabolomics approaches implied that p90/CIP2A might promote cell proliferation through the AKT signaling pathway. In this work, we are aiming to address the relationship between p90/CIP2A and AKT phosphorylation in response to growth factor stimulation, the resulted biological consequences and the role of this interaction in chemoresistance in human lung cancer. We hypothesize that p90/CIP2A promotes the aggressiveness of cancer phenotype through the AKT signaling pathway and our results will enable us to propose a novel mechanism by which p90/CIP2A utilizes to promote cancer progression.

Our data showed that upon epidermal growth factor (EGF) stimulation, the phosphorylation of AKT was dramatically enhanced in the presence of p90/CIP2A by decreasing AKT-associated PP2A phosphatase activity. Interestingly, the decreased cell proliferation in p90/CIP2A knockdown cells can be rescued by the introduction of constitutively activate AKT while the increased cell proliferation in p90/CIP2A overexpressed cells can be reverted through expressing dominant negative AKT. As we know, AKT integrates upstream signals and transmits to downstream targets through phosphorylation. To investigate the underlying mechanism of p90/CIP2A-AKT-mediated cell proliferation, we probed the phosphorylation of AKT substrates with phospho-AKT substrates antibody that recognized a consensus motif (RXXpS/T). We found that the phosphorylation of only a subset of AKT substrates was altered in p90/CIP2A depleted cells. Co-immunoprecipitation (Co-IP) and mass spectrometry was then applied to identify the AKT substrates specifically targeted by p90/CIP2A. Among the identified substrates, several are ER-related proteins and regulate cell apoptosis. We further explored that p90/CIP2A could protect cells from ER stress induced cell apoptosis. In addition, we also found that the phosphorylation of mTOR, which is a direct target of AKT and also an important component of mTORC1 and mTORC2 complexes for cancer cell growth, was correlated with the expression level of p90/CIP2A. Furthermore, depletion of p90/CIP2A sensitized lung cancer cells to rapamycin treatment. We also detected several anti-cancer drugs and found that p90/CIP2A might be evolved in cell chemoresistance. Taken together, our results strongly suggest that p90/CIP2A serves as a positive regulator of the AKT signaling pathway to promote cell proliferation and inhibit cell apoptosis. Preliminary results also suggest that p90/CIP2A might be involved in mTOR-mediated protein synthesis pathway as well as cancer cell chemoresistance.

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Chapter 1: Introduction

1.1. Human lung cancer

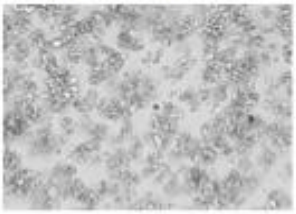
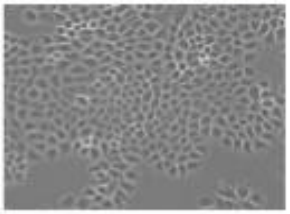
Cancer is a heterogeneous disease manifest in many forms, which is related to unregulated cell growth. Lung cancer is characterized by uncontrolled cell growth in one or both of lungs, usually in the cells lining air passage. It is by far the leading cause of cancer-related death in both men and women in the United States [1]. The estimated new cases and deaths for lung cancer in the United States will be 224, 210 and 159, 260 respectively in the year 2014 [2]. Based on the relative size of cancer cells as examined under a microscope, lung cancer can be classified as non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) [3]. SCLC is less common compared to NSCLC, which accounts for about 15% of all the lung cancer cases. SCLC cells secrete hormones, and usually take place in the primary and secondary bronchi. NSCLC, including adenocarcinoma, squamous cell lung carcinoma and large cell lung carcinoma, accounts for the rest 85% of the lung cancers diagnosed, and the five-year survival rate is as low as 17% [3].

The etiological factors for lung cancer are complex and it is believed that in lung cancer, like in other cancer types, tumor suppressor gene inhibition and oncogene activation play important roles [4]. The etiological factor to small cell lung cancer has been largely attributed to smoking; however the cause of non-small cell lung cancer has been poorly defined. While the exact molecular mechanisms about lung cancer are still under investigation, the current understanding on lung cancer showed that the genetic mutations contribute to the oncogenesis and cancer development. Mutated oncogenes like epidermal growth factor receptor (EGFR), c-

Myc, KRAS, anaplastic lymphoma kinase (ALK), and phosphatidylinositol 3-kinases (PI3K) have been found to contribute to the formation of NSCLC [5-8] (**Table 1**). Thus, the investigation on mutations in lung cancer could enhance the understanding of carcinogenesis as well as identification of potential drug targets.

In our laboratory, we've been working on functions of p90/CIP2A in human lung cancer for several years, mainly focusing on non-small cell lung cancer (NSCLC). We found that p90/CIP2A overexpressed in lung cancer specimens as well as cell lines, and the knockdown of p90/CIP2A via specific shRNA decreased cell proliferation *in vitro*. Our previous study implemented two-dimensional electrophoresis (2DE)-based proteomic approach to examine lung cancer cell line H1299 with and without p90/CIP2A knockdown. Differentially expressed proteins were found by this method and were categorized into functional groups as metabolism (25%), transcriptional and translational control (23%), and signaling pathway and protein degradation (20%) [unpublished data]. The phenotypic analysis of p90/CIP2A in lung cancer cell revealed the role of p90/CIP2A in cancer progression by showing the decreased cell proliferation, clonogenic ability and anchorage-dependent cell growth along with the knockdown of p90/CIP2A. Based on our previous studies, we think CIP2A is closely related to human lung cancer, and play important roles in the process of carcinogenesis.

Table 1: Comparison of SCLC and NSCLC [1, 2]

	Small cell lung cancer	V. S.	Non-small cell lung cancer
Cell Size			
Five-year survival rate	6%		17%
Causes	Smoking		Asbestos, radon and secondhand smoke
Aggressiveness	Very aggressive at early stage		Aggressive
Genetic lesions	p53 and MET		p53, EGFR, Her2, MET, LKB1 and PI3KCA
Treatments	Surgery, radiotherapy and chemotherapy		Surgery, radiotherapy and chemotherapy

1.2. Identification of p90 as a tumor-associated antigen (TAA)

Initially identified as a novel tumor-associated antigen (TAA) in gastric and liver cancer, the protein was named as p90 due to its molecular weight around 90 kDa [9]. The high frequency of autoantibodies to p90 detected in the sera of cancer patients made it a promising candidate for biomarker development when it was firstly identified. p90 is also known as an oncofetal protein, since the expression of this protein is found during embryonic development but disappears from all tissues soon after birth and frequently re-express in and contributory to malignancy. Other

studies by examining tissue specimen of different cancer types revealed that p90 was overexpressed in various cancer tissues but not in adjacent normal tissue [9]. The expression of p90 was also detected in various types of cancer, from solid tumors to hematological malignancies, like breast cancer, gastric cancer, head and neck cancer, lung cancer and leukemia [9-12]. However, since sequence analysis of p90 revealed no significant homology to any protein with known function, further cellular functions of this protein were largely unknown by the time it was identified.

It was described previously that autoantibodies play critical roles in tumorigenesis as reporters recognizing aberrant cellular processes [5]. As a newly identified tumor-associated antigen (TTA), p90/CIP2A can be used as a potential cancer biomarker in TAA-autoantibody system to indicate disease prognosis. Data from our lab as well as others implied that p90/CIP2A is overexpressed in many types of cancer, in both serum samples and tissue samples (**Table 2**). The high expression level of p90/CIP2A in cancer patients suggested that this protein can be sensed by the patients' immune system, and thus can be used as a potential biomarker in cancer detection.

The function of p90 was not discovered until 2007, which revealed that p90 was a novel intracellular PP2A inhibitor and could extend the half-life of c-Myc as well as promote cancer cell proliferation through the inhibition of c-Myc associated PP2A phosphatase activity [13]. The study in search of the interaction partners of PP2A Aα in Hela cells identified p90 was a bona fide PP2A interacting molecule. Therefore, the protein was designated the name as Cancerous inhibitor of PP2A (CIP2A) in that study [13].

Table 2: p90/CIP2A expression in different cancer types (serum and tissues samples).

Cancer Type	Anti-p90 Expression in Patients' serum samples	Percentage	Ref.
Lung cancer	37/182	20.3%	[unpublished data]
HCC	21/160	13.1%	[14]
Gastric Cancer	3/91	3.3%	[14]
Esophageal Cancer	1/20	5%	[14]

Cancer Type	Anti-p90 Expression in Patients' tissue samples	Percentage	Ref.
Breast Cancer	448/1,280	35%	[15]
Osteosarcoma	39/51	76.5%	[16]
Bladder Cancer	18/43	42%	[17]
Ovarian Cancer	100/152	65.79%	[18]
Astrocytoma	75/135	55.6%	[19]

1.3. Protein Phosphatase 2A (PP2A) and its inhibitor CIP2A

Protein phosphatase 2A (PP2A) is a well-known tumor suppressor functions by targeting cancer-related molecules or signaling pathways like AKT, mitogen-activated protein kinase (MAPK), and beta-catenin [20]. It is a large family of heterotrimeric serine-threonine phosphatases, which accounts for the majority of serine-threonine phosphatase activity in

eukaryotic cells [21]. The PP2A holoenzyme consists of three subunits. The A subunit provides a scaffold structure that allows the binding of C subunit and B subunit, and regulates the substrates specificity and the localization of PP2A complex [22, 23]. B subunit is a regulatory subunit, which confers substrate selectivity, and C subunit is a catalytic subunit [24, 25]. In mammalian cells, there are two forms of A subunit (Aα and Aβ), two forms of C subunit (Cα and Cβ) and four families of B subunits B/B55/PR55/PPP2R2 [26-28], B′/B56/PR61/PPP2R5 [29-31], B″/PR72/PPP2R3 [32, 33] and B‴/PR93/SG2NA/PR110/Striatin [34]. The variability in PP2A holoenzyme composition results in a huge diverse enzyme with a vast array of substrate specificities, which can partly explain the multiple functions of PP2A. Aberrant expression, mutation and deletion of PP2A subunits are associated with certain cellular processes that lead to tumor formation (**Figure 1**).

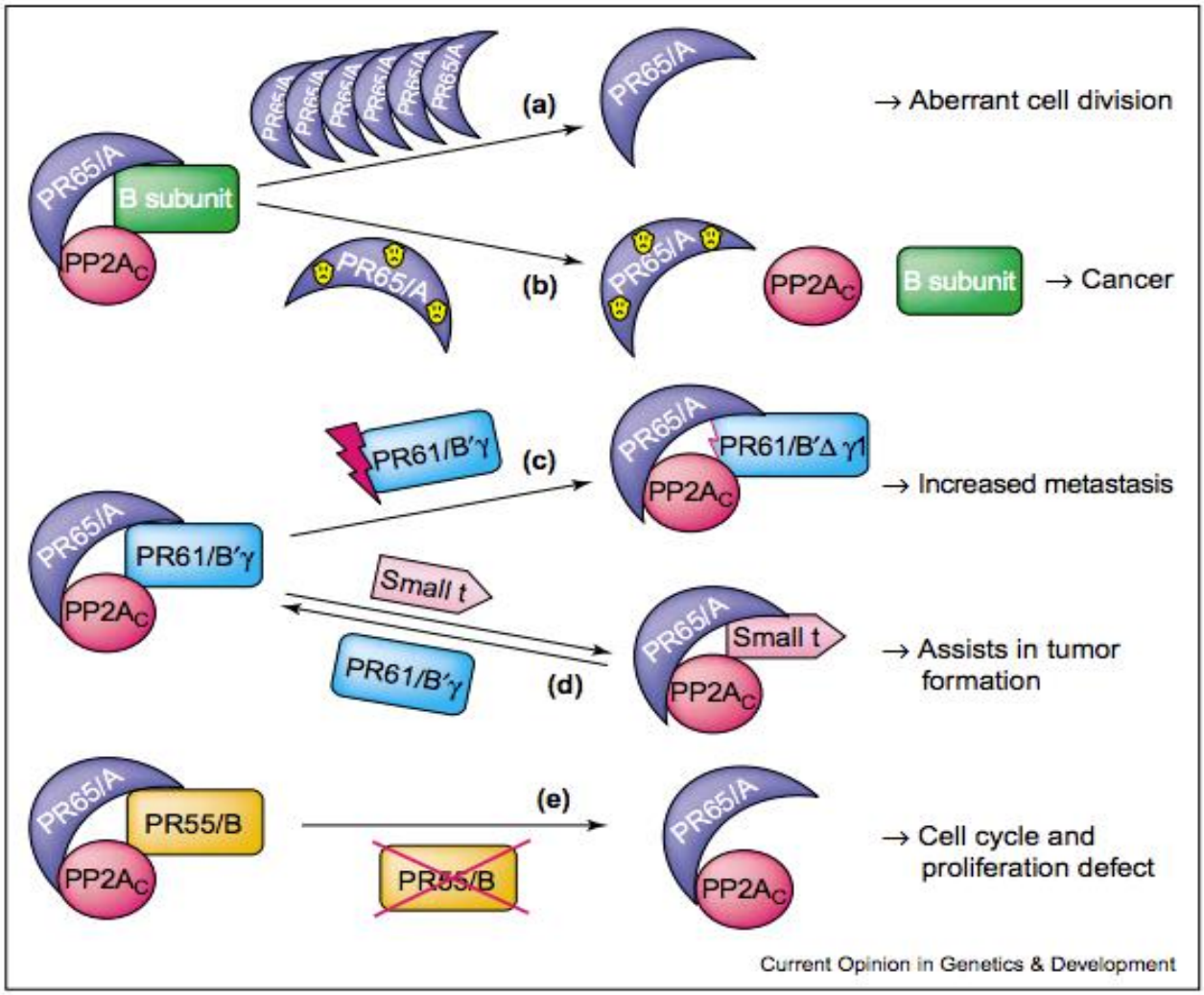


Figure 1: Structure of PP2A holoenzyme and aberrant growth cause by PP2A. (a) overexpression of PR65/A causes multinucleated cells. (b) Mutations in PR65/A, characterized in several human cancers. (c) Truncation of PR61/B alters PP2A activity towards paxillin and Mdm2, leading to radio-resistance and increased metastasis, respectively. (d) By replacing PR61/B with ST, the normal function of the trimer in the Ras-Myc pathway is subverted and leads to cancerous growth. (e) Deletion of PR55/B in yeast and Drosophila leads to cell cycle defects and enhanced MAP kinase signaling. (Janssens V.et al. Curr Opin Genet Dev, 2005)

The tumor suppressor activity of PP2A was initially identified by the discovery of okaic acid, which could promote tumor formation in a mouse model by inhibiting PP2A phosphatase activity at low concentration [20]. Accordingly, it was believed that inactivation of PP2A contributed to cell transformation by reversing certain phosphorylation events. The opinion was further validated by the discovery that small T (ST) antigen expressed by two high-risk viruses, SV40 and polymer virus, which could target PP2A to disrupt the structure of the holoenzyme and facilitate the conversion of normal cell to cancerous cell. For example, the introduction of mutations to ST abolishing the interaction between ST and PP2A would cause the failure of cellular transformation [20]. Furthermore, it has been demonstrated that activation of Ras and telomerase, along with inactivation of the tumor suppressor proteins p53 and retinoblastoma protein (Rb) can immortalize a variety of human cell types and subsequently transform to a tumorigenic state only by inhibiting protein phosphatase 2A (PP2A) [35, 36].

Many cancer-related molecules or pathways like the phosphatidylinositol 3-kinase-AKT (PI3K-AKT), mitogen-activated protein kinase (MARK) and Wnt-beta-catenin pathways are the targets of PP2A. Mutations of the A and B subunits have been frequently observed in cancer. The gain-of-function by exogenous overexpressing the subunits can revert the tumorigenic phenotype. Therefore, recent studies have been focusing on how these three subunits contribute to tumor suppression. In general, the genetic or epigenetic changes of PP2A complexes in human cancer remain to be classified, as well as its impact on cancer signaling and therapeutic responses to targeted therapy.

Some studies have been done to elucidate the roles of PP2A in various aspects of malignant transformation. Inhibition of PP2A phosphatase activity has been found to be a prerequisite for cellular transformation in epithelial cells. The mechanisms involved in the

inactivation of PP2A phosphatase activity include genetic mutations, epigenetic silencing or inhibition. Missense or deletion mutations of the A and B subunits were frequently observed in lung cancer, breast cancer and colon cancer, but mutations of the C subunit are rare. The tyrosine phosphorylation of PP2A can at least in part determine the level of its activity, which is controlled by okadaic acid-sensitive phosphatases and protein-tyrosine kinases [20]. The endogenous PP2A inhibitor was not identified until the discovery of I1PP2 and I2PP2A from bovine kidney cell lysates [37], which are heat-stable and inhibit PP2A phosphatase in a non-competitive manner.

Juttila et al identified p90 as a novel intracellular PP2A inhibitor in search of the interaction partners of PP2A Aα in Hela cells and designated the name as Cancerous inhibitor of PP2A (CIP2A) [13]. The interaction and co-localization of p90/CIP2A with PP2A implied the role of this protein in cancer progression might go through PP2A [13]. The study using p90/CIP2A knockdown and microarray analysis found the change of c-Myc target genes, implying the regulation of c-Myc activity by p90/CIP2A. Now, it is very clear that the underlying mechanism of this regulation is based on the ability of p90/CIP2A to inhibit the c-Myc-associated PP2A phosphotase activity.

The tumorigenic ability of p90/CIP2A was also demonstrated in several ways in that study. Overexpression of p90/CIP2A was observed in many different cancer types. The knockdown of p90/CIP2A in Hela cell resulted in decreased cell proliferation, clonogenic ability and anchorage-independent growth. Furthermore, injection of p90/CIP2A knock down cells into nude mice would decrease the *in vivo* tumor formation. This protein could also substitute ST for cell transformation in the cell line, which had been immortalized by co-expression of hTRET, LT and H-ras [13]. Studies in recent years also suggest that p90/CIP2A expression may be linked

with human breast cancer aggressivity and p90/CIP2A is overexpressed in invasive human mammary carcinomas. Additional functions of p90/CIP2A in cancer progression are still under investigation.

1.4. Functional study of p90/CIPA and MYC

One of the well-studied mechanisms for the tumor-promoting function of p90/CIP2A is its ability to inhibit c-Myc-associated phosphatase activity and thus extend the half-life of c-Myc [13]. c-Myc is a very important transcription factor in both normal cell and malignant cell growth [38]. It is subject to several different mechanisms of both transcriptional and post-transcriptional regulation, undertaking a diverse group of cellular functions, including cell cycle regulation, cell proliferation, cell growth, cell differentiation and metabolism [38].

The stability of c-Myc is regulated by two phosphorylation events [39]. The phosphorylation of Serine 62 by extracellular signal-regulated kinase (ERK) can increase its stability and function while the dephosphorylation at this site by PP2A will target the phosphorylation at Threonine 58 by glycogen synthase kinase 3-beta (GSK 3-β), which is a critical step for c-Myc proteolytic degradation and ubiquitination. The inhibition of PP2A activity by CIP2A will induce c-Myc serine 62 (S62) phosphorylation and leads to c-Myc protein stabilization (**Figure 2**). In addition, depletion of p90/CIP2A resulted in a clear down-regulation of c-Myc protein expression. c-Myc mRNA expression is not significantly altered by p90/CIP2A depletion, implying that p90/CIP2A regulates c-Myc protein levels post-transcriptionally [13].

Based on several studies, p90/CIP2A has been shown to cooperate with other oncoproteins, such as Ras and MYC in regulating malignant growth and transformation [40]. It's of great importance to identify p90/CIP2A as a MYC protein stabilizer for understanding how

MYC activity is regulated in malignant as well as non-malignant cellular processes. Due to its critical roles in tumorigenesis, MYC is considered as a potential target for cancer therapy. Thus, inhibition of p90/CIP2A might provide a therapeutic opportunity that would eliminate the dephosphorylation of MYC.

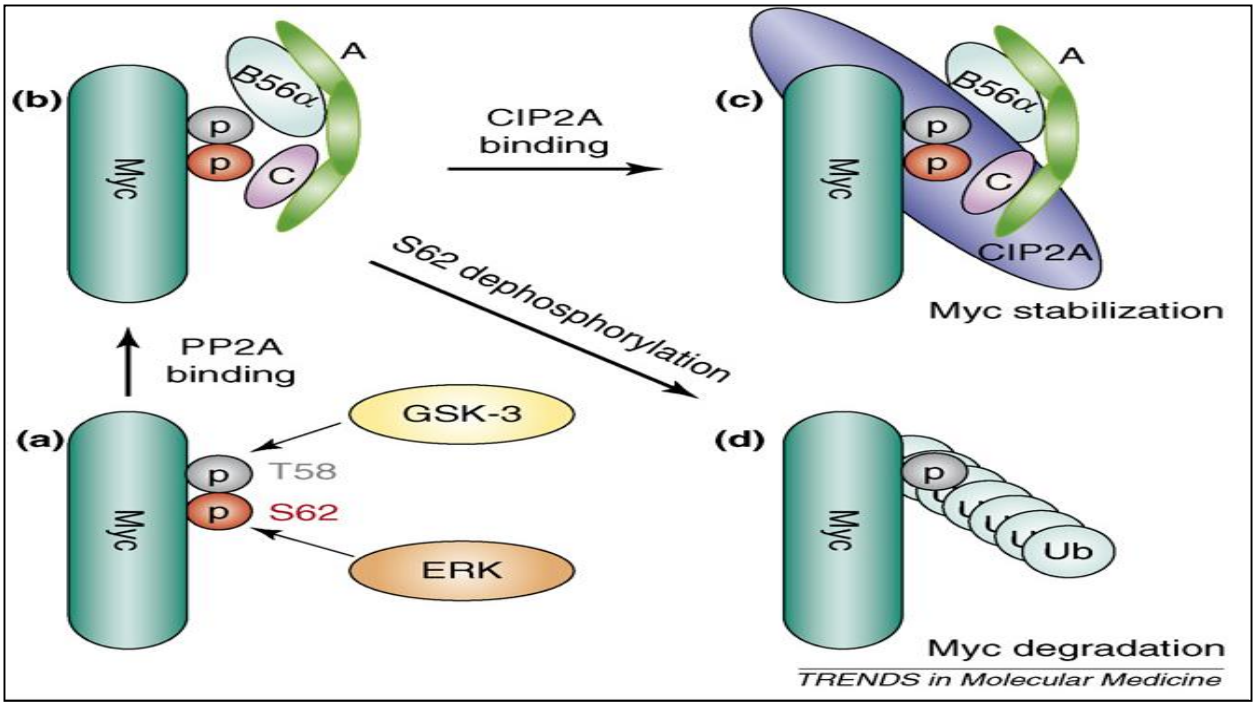


Figure 2: Proposed Model for p90/CIP2A stabilizing c-myc. Phosphorylation of threonine 58 by GSK-3 is required for MYC degradation, whereas Ras-mediated activation of the ERK/MAPK pathway induces MYC phosphorylation at serine 62 (S62). PP2A complexes containing B56a regulate the phosphorylation status of S62. CIP2A directly interacts with Myc and inhibits PP2A-mediated S62 dephosphorylation. Inhibition of S62 dephosphorylation results in Myc protein stabilization. In the absence of CIP2A, PP2A dephosphorylates S62, which targets Myc phosphorylated at T58 for ubiquitination and for proteolytic degradation. (Juttila et al. Trends in molecular medicine, 2009)

1.5. Background information on the AKT signaling pathway

Cancer metabolic signaling networks are considered to be one of the hallmarks of cancer [41]. A variety of evidence has shown that almost every oncogene has downstream effectors to be associated with cancer metabolism, such as c-Myc, Ras, Src and AKT. The mutation of these oncogenes can stimulate the anabolism in transformed cells and constitute major metabolic pathways in tumor cells [41].

AKT, also known as protein kinase B (PKB), is a positive regulator of cell proliferation, cell growth, cell survival, metabolism and angiogenesis [42-44]. AKT acts by integrating different signals from upstream pathways [45], which makes it an attractive anticancer drug target. AKT can be activated through different mechanisms including the mutated PI3K, growth factor stimulation, cytokine stimulation or integrin stimulation [46-48]. Activation of PI3K will lead to the activation of downstream molecule AKT, which further phosphorylates a wide variety of downstream substrates and affects cell activity. The activation of AKT is frequently deregulated in cancer, involving the phosphorylation of two residues: threonine 308 (Thr308) in the activation loop of the kinase by phosphoinositide-dependent kinase 1 (PDK1) and serine 473 (Ser473) in the hydrophobic motif by the mTORC2 complex [49-51].

More and more studies have shown that AKT has a variety of functions in cell behavior. AKT activation is able to stimulate cell growth by increasing enzymatic activity of glycolytic enzymes like hexokinases and phospho-fructose [52]. AKT can also increase gene expression associated to nutrient transporters like amino acid transporter and glucose transporter [53]. Besides, AKT can regulate other proliferation promoting molecules like mammalian target of rapamycin (mTOR). It has been drawn attention that activation of AKT has potential roles in intracellular signaling pathways to prevent apoptosis and promote cell survival. AKT can be

activated through recruitment to the cellular membrane by PI-3 kinase lipid products in response to mitogens and survival factors, while aberrant activation of AKT has been observed in a variety of human cancers through diverse mutations [54]. AKT regulates cellular processes through phosphorylation and more than two hundred substrates have been identified. Interestingly, AKT is not able to phosphorylate all the substrates at one time; instead it shows substrate specificity [55]. Therefore, one central unresolved question regarding the role of AKT during carcinogenesis is how AKT regulates substrate specificity [55].

In NSCLC, AKT phosphorylation is observed at a frequency between 50 - 70%, showing high frequent event of activation of PI3K/AKT signaling in this malignancy. Phosphorylated AKT, the active form AKT, is found in most of NSCLC cell lines and confers the cell survival and resistance to chemotherapy and radiation therapy [50]. In NSCLC, the study of AKT activity is attractive for predictive, prognostic and pharmacodynamics purposes [56].

Previous studies also implicate a strong connection between PP2A and the PI3K/AKT pathway. In one study, genetic substitution experiments showed that either activated PI3K or a combination of the activated PI3K effectors AKT and Rac1 induced human cell transformation in place of ST expression, and blocking PI3K function inhibited ST-mediated transformation [57]. Other evidences also implicate that activation of the AKT pathway in human cell transformation is induced by PP2A dysfunction. AKT is known as one target of PP2A, while PP2A complexes may act downstream of AKT in the mTOR signaling pathway [58].

A recent report showed that p90/CIP2A could protect the hepatocellular carcinoma (HCC) cell lines from bortezomib-induced apoptosis by inhibiting phospho-AKT-associated PP2A phosphatase activity [59], which drew us great interests in new investigating direction of this protein. The knockdown of p90/CIP2A via siRNA was found to sensitize HCC cell lines to

bortezomib treatment [60]. This study showing p90/CIP2A could regulate the phosphorylation of AKT in response to the treatment of chemotherapeutic drugs suggested that this protein might have more functions in tumorigenesis. However, whether the interplay between p90/CIP2A and phosphor-AKT plays a role in cancer progression, and how p90/CIP2A facilitates AKT-mediated resistance to chemotherapy, remains unresolved.

1.6. mTOR and rapamycin: cancer chemotherapy

The mTOR pathway is a key component of the PI3K/AKT/mTOR pathway that can be found in many cancer cells. The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase of the phosphatidylinositol-3-OH kinase (PI3K)-related family, which plays a role as a master regulator of cellular growth and metabolism, and is subjected to the negative regulation of PP2A [61]. It functions in two different complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is activated by insulin and other growth factors through the PI3K/AKT signaling pathway, promoting messenger RNA translation and protein synthesis through at least two of its substrates: ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [61]. And mTORC1 is inhibited by rapamycin through the binding of FK506-binding protein FKBP12. FKBP12 could interact physically with the complex and decrease its activity [61] (**Figure 3**). Since mTORC1 is a downstream target of AKT when cells are stimulated with growth factors, it is highly possible that p90/CIP2A may have regulation on this protein and may lead to the resistance of the cancer cell to the rapamycin treatment.

The PI3K/AKT/mTOR pathway is an intracellular signaling pathway important in many types of cancer, including the non-small cell lung cancer [42, 44, 45]. This pathway is activated

by numerous inhibitors of kinases in cancer, such as growth factor tyrosine kinase, insulin, nutrient and oxygen [51-53]. It comprises the central axis of a complex signaling network to play an important role in controlling cell growth, proliferation, metabolism, survival, and tumor angiogenesis [62]. The over-activation of this pathway reduces apoptosis and promotes proliferation in many cancers, which allows cancer drugs to be designed to inhibit the signaling sequence at some points.

Rapamycin, an mTOR kinase inhibitor, has sporadic anticancer activity through inhibiting the mTORC1 activity. It suppresses tumor cell growth by arresting cells in G1 phase or potentially inducing apoptosis of cells [63]. However, rapamycin and its analogues have limited success, and the clinical outcomes are always unpredictable due to rapamycin resistance. One of the mechanisms of rapamycin resistance is the feedback loop triggered by rapamycin itself, which inhibits mTOR activity but increases AKT phosphorylation by up-regulating PI3K and mTORC2 activity [64]. Therefore, decreasing AKT phosphorylation and rapamycin treatment should have synergetic effects on sensitizing cells to rapamycin. Since p90/CIP2A is a factor that modulates AKT phosphorylation, it is highly possible that down–regulation of p90/CIP2A coupled with rapamycin treatment would significantly decrease cell survival. In addition, the sensitivity of rapamycins is affected by genetic mutations, e.g. mutations of mTOR or FKBP12 prevent rapamycin from binding to mTOR, conferring rapamycin resistance [65]. It has been reported that p53 [66], PTEN/AKT [67], PP2A-related phosphatases [68], and mTOR related proteins, including S6KA and 4E-BP1 [68] are also associated with rapamycin sensitivity.

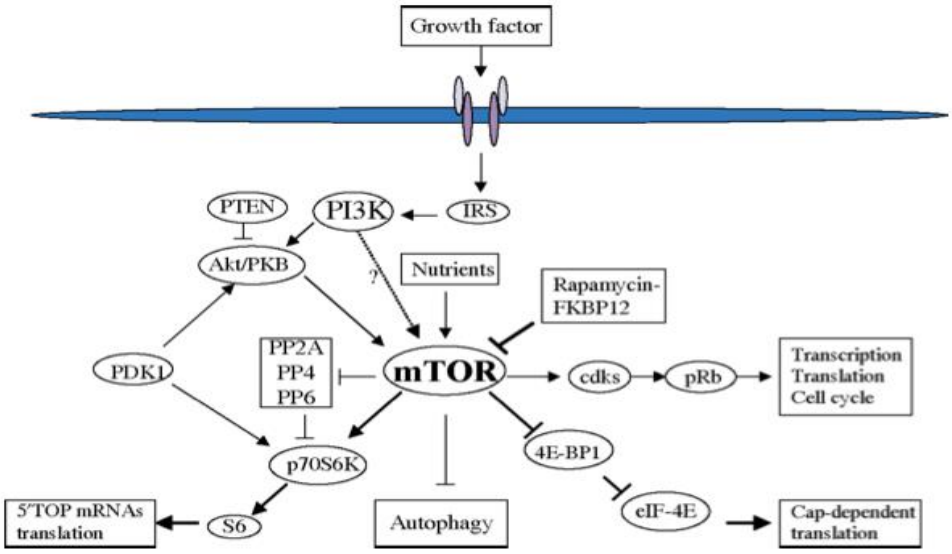


Figure 3: Schematic flowchart shows mTOR signaling pathway. PI3K and AKT lie upstream of mTOR, while S6K1 and 4E-BP1 are two downstream effectors of mTOR. Several cellular events controlled by mTOR are also shown here. **(***Shile Huang, Reter J. Houghton. Drug Resistance Updates, 2001*)

Chapter 2: Hypothesis and Aims

2.1. Hypothesis

p90/CIP2A is an endogenous oncofetal protein inhibiting c-Myc-associated PP2A phosphatase activity via an unknown mechanism. The observation that depletion of p90/CIP2A did not induce a global change of PP2A phosphatase activity implied that p90/CIP2A might recognize specific PP2A targets and target particular signaling pathways. Based on the recent finding that p90/CIP2A could promote cell survival by up-regulating AKT phosphorylation under genotoxic agent like bortezomib, it indicated the possible association between p90/CIP2A and AKT in cancer progression. Being a central regulator in cell growth and cell proliferation, AKT phosphorylates hundreds of substrates and leads to the activation of various cellular pathways under different stimulation; however the mechanism of how the substrates are specifically selected is still unclear. Our previous work found that p90/CIP2A was able to regulate the phosphorylation AKT to promote cell proliferation. We also found that p90/CIP2A could target specific AKT substrates and affect cell apoptosis. **Therefore, we hypothesize that p90/CIP2A may mediate lung cancer cell proliferation and cell apoptosis through the AKT signaling pathway.**

2.2. Significance

p90/CIP2A has been found to extend the half-life of gene regulatory protein c-Myc and promote cell survival via regulating AKT phosphorylation. This protein is also regarded as a tumor-associated antigen (TAA) since its initial identification from gastric and live patients.

Currently many research groups have been working on its functions from different aspects and well demonstrated that CIP2A is highly associated with cancer progression (**Figure 4**).

The observation on the association between p90/CIP2A and AKT phosphorylation at residue serine 473 (S473), a site indicative for the kinase activation and enzyme activity, in our lab and other labs suggested the role of p90/CIP2A in regulating AKT phosphorylation. Deregulated pathways are common events in cancer progressions. Key signaling molecules are increased to promote tumor growth or downregulated to reduce the constraint of cell proliferation. Our previous results found several signaling molecules were altered with the loss of p90/CIP2A. Using comparative proteomics, we found that p90/CIP2A was able to regulate the metabolism, translational and transcriptional control, and signaling transduction of cancer cells. In this study, we are trying to define novel p90/CIP2A targets by proteomic-based systematic screening. The candidates obtained from our study will expand the understanding on protein-substrate interaction and therefore direct the cancer drug design. At the same time, we will focus on the study of the mechanism of AKT phosphorylation to further investigate how p90/CIP2A is involved in the PI3K/AKT/mTOR pathway, which is critical to cancer progression.

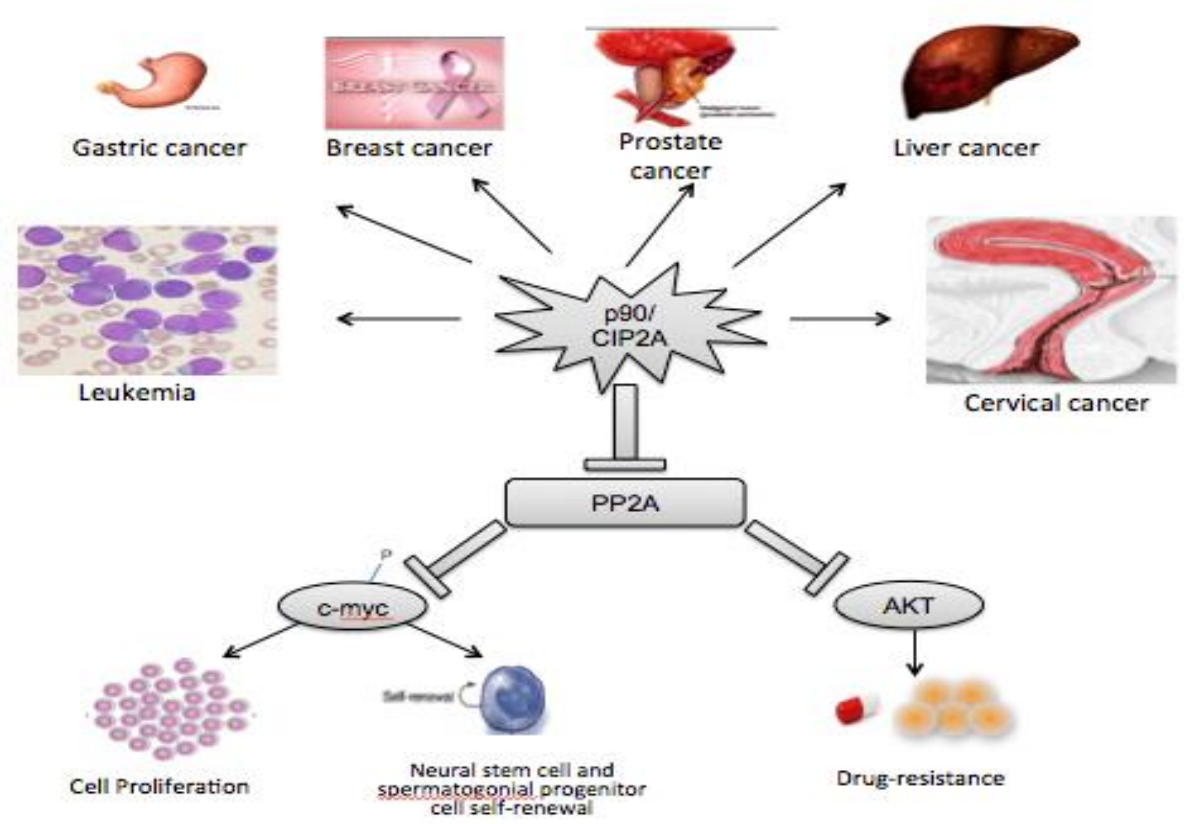


Figure 4: Schematic summary of p90/CIP2A functions. p90/CIP2A is overexpressed in various types of tumors, from solid tumors to hematological malignancies. It is able to inhibit PP2A phosphatase activity and promote c-myc stability, leading to the increased cell proliferation and neural stem cell renewal. It can also enhance AKT phosphorylation and confer anti-cancer drug resistance.

2.3. Specific aims

In this study, we will investigate the interrelationship between p90/CIP2A and the AKT signaling pathway in lung cancer cells and biological consequences resulted from this interaction. In addition, we will also explore the mechanism of p90/CIP2A-regulated AKT

signaling network in cancer progression and chemoresistance. To achieve this goal, we have the following specific aims:

Specific aim 1: **To determine the relationship between the expression level of p90/CIP2A and the phosphorylation of AKT in NSCLC cell lines.**

In this specific aim, we will use phospho-AKT antibody to detect AKT phosphorylation at S473 in several NSCLC cell lines with either p90/CIP2A depletion or p90/CIP2A overexpression. This study will help us to establish whether AKT phosphorylation is correlated with the expression level of p90/CIP2A in lung cancer cells and also provide the essential information for the subsequent study on how p90/CIP2A acts as a regulator in the AKT signaling pathway. We will also detect whether p90/CIP2A affects AKT-associated PP2A phosphatase activity to further explore the mechanism.

Specific aim 2: **Identification of the AKT substrates specifically targeted by p90/CIP2A using an immunoproteomic approach.**

In this specific aim, we will identify downstream substrates of AKT targeted by p90/CIP2A. Based on our hypothesis, p90/CIP2A modulates AKT phosphorylation through specific substrates. Co-immunoprecipitation (Co-IP) will be used to pull down the AKT substrates and proteomic methods will be used to identify the differentially expressed substrates in p90/CIP2A deficient cells. The data obtained from this specific aim can help us to build the network about how p90/CIP2A participates in this signaling pathway. We will further

investigate the association between p90/CIP2A and the identified substrates. We will specifically address the regulation of p90/CIP2A in the mTOR pathway by evaluating whether p90/CIP2A could affect the phosphorylation of mTOR and whether p90/CIP2A could affect cell sensitivity to rapamycin treatment. This data will establish a model by which p90/CIP2A is using to promote cancer progression through the AKT signaling pathway and an alternate way to potentiate chemoresistance in cancer cells.

Specific aim 3: **To determine the role of p90/CIP2A via AKT regulation in NSCLC.**

In this specific aim, we will determine whether p90/CIP2A-regulated AKT phosphorylation can potentiate cell activities in lung cancer cells, such as cell proliferation and cell apoptosis. We will first examine whether p90/CIP2A affects cell proliferation and whether the regulation is attributed to the AKT pathway. Based on the characteristics of AKT substrates identified, we will explore whether p90/CIP2A is involved in other cell activities, like cell apoptosis. The data acquired will allow us to report mechanism on functions of p90/CIP2A in cancer progression, which will facilitate the future use of it as a biomarker in immunodiagnosis and therapeutic design.

Chapter 3: Materials and Methods

Material. Mammalian target of rapamycin (mTOR) inhibitor rapamycin, Thapsigargin (TG) and dithiothreitol (DTT) were purchased from Sigma Chemical Company (St. Louis, MO). Antibodies against p90/CIP2A and beta-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against GPR78, phospho-AKT (Ser473), AKT, phospho-mTOR (Ser2448), mTOR, phospho-p70S6 kinase (Thr389), phospho-4EBP1 were purchased from Cell Signaling Technology (Beverly, MA).

Cell culture. Three lung cancer cell lines, NCI-H838, NCI-H1299 and NCI-H460 were purchased from American Type Culture Collection (ATCC, Manassas, VA). All cell lines were cultured in RPMI-1640 medium (Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Life Technologies) at 37^{o}C with 5% CO_2.

Transfection of p90/CIP2A overexpression. Transient transfection of pcDNA3.1 control or pcDNA3.1 containing p90/CIP2A cDNA (general gift from Dr. Westermarck, Finland) into H1299 lung cancer cell line was performed with Lipofectamine LTX (Life Technologies) according to manufacturer's protocol.

Knockdown of p90/CIP2A by lentivirus encoding p90/CIP2A short hairpin RNA. Five p90/CIP2A short hairpin RNAs, ligated in pLKO.1 vector, were obtained from Open Biosystems (Huntsville, AL). The knockdown efficiency of these shRNAs was evaluated in H1299 cells by western blot analysis and two of them showing the higher knockdown efficiency were chosen to produce lentivirus. Lentivirus was produced by co-transfection of pLKO.1 control (ligated with

scramble sequence) or other pLKO.1-derived vector with pMD2.G (Addgene, Cambridge, MA) and pCMV-VSVG (Addgene) into HEK293T packaging cell lines. The supernatants containing lentivirus of HEK293T were harvested at 36 hours and 72 hours post transfection. Supernatants were pooled, centrifuged to remove cells and then filtered through a 0.45 μm low protein-binding filter. Cells were plated in monolayer at different densities and infected with lentivirus constructs using 8 ng/mL polybrene (Sigma, St. Louis, MO). The stable cell lines were selected in the presence of 1 μg/mL puromycin (Sigma, St. Louis, MO) for two weeks.

Cell proliferation assay. To compare cell proliferation in cells with p90/CIP2A transfection, the MTT assay was performed. The transfected cells and control cells were plated quadruplicate in 96-well microplate at a density of 3×10^3/100 μL medium for each well. After 3-day culture, 15 μL of the dye solution was added to each well and incubated at 37°C for another 4 hour. At the end of the incubation, 100 μL stop solution was added to each well and colorimetric absorbance was read in the SpectraMax Plus (Molecular Devices) at 570 nm. Cell free medium was used as mock control.

PP2A phosphatase assay. Measurement of PP2A phosphatase activity was performed by using the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit (Life Technologies). Cell lysates were prepared by using low detergent buffer (1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM PMSF, and complete protease inhibitor cocktail). Total of 50 μL cell lysates were incubated with 1 X PP2A phosphatase reaction buffer for 30 minutes at 37 °C. Fluorescence intensity was measured using excitation at 355 nm and emission at 485 nm. The fluorescence intensity was normalized to the expression level of PP2A catalytic domain.

AKT substrates assay. The commercially available anti-phospho-AKT substrates antibody developed by the company of *Cell Signaling Technology* was used to perform immunoprecipitation. The antibody is able to recognize the motif of RXXS*/T* (* indicates the phosphorylation site). Immunoprecipitation was performed on the group of cells transfected with vector control or on cells transiently transfected with p90/CIP2A. Immunoprecipitates were eluted with low pH buffer, precipitated with acetone, and resolved by SDS-PAGE gel. Differentially phosphorylated proteins would be excised and analyzed with liquid-chromatography mass spectrometry (LC-MS).

Flow cytometric analysis. Equal number of cells transfected with either vector control or p90/CIP2A were seeded at a density of 1×10^5 per well in six-well plates. After 24 hours, cells were treated with 1mM DTT. Samples were analyzed using the EPICS XL Flow Cytometer (Coulter, Miami, FL) and the extent of cell death was determined by evaluating the percentage of cells with DNA content <2N.

Western blot analysis. Cells were plated in 6-well tissue culture plates at 80% confluence and incubated overnight. Cell lysates were obtained using cold radioimmunoprecipitation assay buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 10% glycerol, 1% NP40, 0.5% sodium deoxycholate]. Twenty micrograms of protein mixture were separated on 10% SDS-PAGE gels and wet transferred to nitrocellulose membrane (GE Healthcare Life Sciences) and then blocked for 1 hour at room temperature in TBS-T buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat milk or BSA. Membranes were then incubated

overnight at 4 ℃ or 1 hour at room temperature with the respective primary antibodies: anti-CIP2A (1:500), and anti-actin (1:1,000), (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-mTOR (1:1,000), mTOR (1:1,000), phospho-AKT (S473) (1:1,000), (Cell Signaling Technology, Danvers, MA). Anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology) were used to visualize the stained bands with an enhanced chemilluminescence visualization kit (Santa Cruz Biotechnology). X-ray films were developed and results were visualized.

MALDI-MS Analysis. As previously described, 0.5 μL of the sample solution, along with equivalent matrix solution (α-cyano-4-hydroxycinnamic acid), was mixed and applied onto the MALDI-TOF target for MALDI-TOF/MS analysis. MALDI-TOF spectra were calibrated using trypsin autodigestion peptide signals and matrix ion signals. MALDI analysis was performed using a fuzzy logic feedback control system (Reflex III MALDI-TOF system Bruker, Karlsruhe, Germany) equipped with delayed ion extraction. Peptide masses were searched against the Swiss-Prot database using the MS-Fit program (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) or the NCBI database using the MASCOT program (http://www.matrixscience.com) against *Homo sapiens* database in NCBI.

Statistical Analysis. Data are expressed as mean +/- standard error of the mean. Statistical analysis was performed using Student's test for normally distributed data. Correlations were assessed using the Pearson correlation coefficient test. The p values < 0.05 were considered statistically significant.

Chapter 4:

p90/CIP2A is associated with AKT phosphorylation in human lung cancer

4.1. Overview

p90/CIP2A is an intracellular endogenous protein phosphatase 2A (PP2A) inhibitor with oncogenic activities. We previously found that p90/CIP2A was overexpressed in human lung cancer specimens as compared to normal lung tissues. The observation on the association between p90/CIP2A and AKT phosphorylation at residue serine 473 suggested the role of p90/CIP2A in regulating AKT phosphorylation. In addition, the depletion of p90/CIP2A did not induce a global change of AKT phosphatase activity, which implied that p90/CIP2A might recognize specific AKT targets and play certain roles in this signaling pathway. In this study, we are trying to define the association between p90/CIP2A and the AKT signaling pathway in human lung cancer. The results obtained from our study will expand the understanding on protein-substrate interaction and therefore direct the cancer drug design. In addition, the study on the mechanism of AKT phosphorylation will expand the understanding on how p90/CIP2A is involved in the PI3K/AKT/mTOR pathway.

4.2. Rationale, experimental design and alternative approach

***Specific aim 1*: To determine the relationship between the expression level of p90/CIP2A and the phosphorylation of AKT in NSCLC cell lines and tissues.**

4.2.1. Rationale:

The purpose of this aim is to address the relevance of p90/CIP2A expression and AKT phosphorylation in NSCLC cell lines and the biological consequences. Although our initial studies demonstrated that the decreased expression of p90/CIP2A would cause the hypophosphorylation of AKT, we were not able to make conclusions that this observation could be applicable in other cases. Since different cancer cells have various genetic backgrounds, it may cause context-based observations. In this case, we analyze such possible correlation in several lung cancer cell lines, including p90/CIP2A overexpression cells and p90/CIP2A-depleted cells. Furthermore, whether p90/CIP2A regulated AKT phosphorylation is a direct or indirect effect is still unknown. Actually, several upstream kinases like PI3K and mTORC2 are also the targets of PP2A. Therefore, identification of the kinases targeted by p90/CIP2A in AKT signaling pathway will be helpful in understanding the p90/CIP2A-related pathogenesis in lung cancer. The results from this specific aim can help us make evidence-based conclusions about the correlation between p90/CIP2A expression and AKT phosphorylation, as well as direct our further study on resulted biological consequences.

4.2.2. Experimental Design & Methods:

Our previous study investigated the endogenous expression of p90/CIP2A in different NSCLC cell lines, and we constructed stable transfection of p90/CIP2A in three of them: NCI-H460, NCI-H1299 and NCI-H838. The H1299 and H838 cell lines were used to generate cell lines stably expressing p90/CIP2A shRNA by lentiviral-mediated transduction. The H460 cell line, which expressed the least amount of endogenous p90/CIP2A expression, was transfected

with p90/CIP2A plasmid for overexpression. To validate successful transfection, western blot analysis would be used.

The correlation between p90/CIP2A and phospho-AKT was evaluated in established NSCLC cell lines by western blot analysis. Before cell lysis, cells would be starved for 24 hours and stimulated with 10 ng/mL epidermal growth factor (EGF) for 30 minutes. Expression of p90/CIP2A and phosphorylation of AKT would be examined by mouse anti-p90/CIP2A and rabbit anti-phospho-AKT, respectively. The expression level of either of p90/CIP2A or phospho-AKT was normalized to actin and AKT expression by using densitometry. Spearman correlation coefficient was used to examine the correlation based on the ratios of p90/CIP2A to actin and phospho-AKT to actin. The expression level of AKT remained the same.

We next examined whether the association between p90/CIP2A and AKT phosphorylation affected PP2A phosphatase activity. H838 cells were transiently knockdown by lentiviral shRNA. For phosphatase assays, p90/CIP2A knockdown cells or control cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 7.5% Glycerol, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM Na3VO4, Complete Protease Inhibitor), cleared from debris by centrifugation, and incubated with AKT beads. The beads were analyzed for PP2A activity using Serine /Threonine Phosphatase Assay kit according to the manufacturer's specification. Fluorescence was measured using a fluorescence microplate reader at 570 nm.

All values from in vitro assays were expressed as mean ± SD or SEM of at least three independent experiments or replicates. P values were calculated with the two-tailed Student's t test. A p value <0.05 is considered statistically significant.

4.2.3. Potential problems & alternative approaches:

The correlation of p90/CIP2A and ATK might have similar problems with undetected correlation since cancer cell lines have distinct genetic backgrounds. Other factors may have similar effects on the regulation of the phosphorylation of AKT. In such a case, the loss-of-function by shRNA or gain-of-function by overexpression will be applied to investigate whether the forced protein change will affect the phosphorylation status of AKT.

4.3. Results

4.3.1. p90/CIP2A is associated with AKT phosphorylation

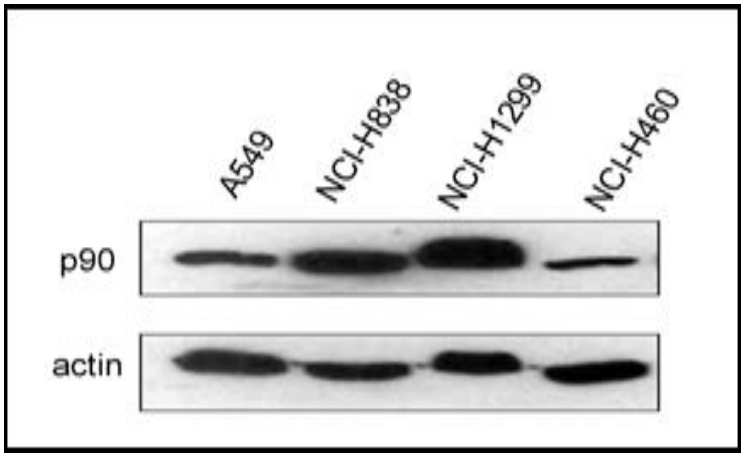
We first examined the expression of p90/CIP2A in four different lung cancer cell lines, A549, NCI-H838 (H838), NCI-H1299 (H1299) and NCI-H460 (H460). The four non-small cell lung cancer (NSCLC) cell lines expressed apparently different levels of endogenous CIP2A (**Figure 5A**). We generated five cell lines with two wild types (pcDNA3.1 and shRNA control), two deficient p90/CIP2A via stable transfection of two independent shRNAs (CIP2A shRNA1 and CIP2A shRNA2) and overexpressed p90/CIP2A (pcDNA3.1+CIP2A) via transient transfection. The p90/CIP2A expression level in different transfected cell lines were confirmed by western blot analysis (**Figure 5B**).

In response to epidermal growth factor (EGF) stimulation, AKT becomes phosphorylated at residue serine 473, a site indicated in enzymatic activity and substrate specificity. Therefore, we detected AKT phosphorylation in the three transfected NSCLC cell lines with either depleted p90/CIP2A using shRNA or elevated p90/CIP2A expression level via ectopic overexpression. As shown in **Figure 6**, the knockdown of p90/CIP2A in H1299 and H838 cells would cause the

hypophosphorylation of AKT while overexpression of p90/CIP2A in H460 cells caused the hyperphosphorylation of AKT. The total amount of AKT was unaffected by the level of p90/CIP2A. Therefore, p90/CIP2A regulates AKT phosphorylation in lung cancer cells.

To investigate whether the down-regulated AKT phosphorylation is due to the elevated PP2A phosphatase activity, we analyzed the AKT-associated PP2A phosphatase activity by immunoprecipitation and commercial assay kit (**Figure 7**). The knockdown of p90/CIP2A in H838 cells increased the AKT-associated PP2A phosphatase activity, while the overexpression of p90/CIP2A in H460 cells decreased the AKT-associated PP2A phosphatase activity. This result indicated that p90/CIP2A associated with AKT phosphorylation would be related with PP2A phosphatase activity.

(A)



(B)

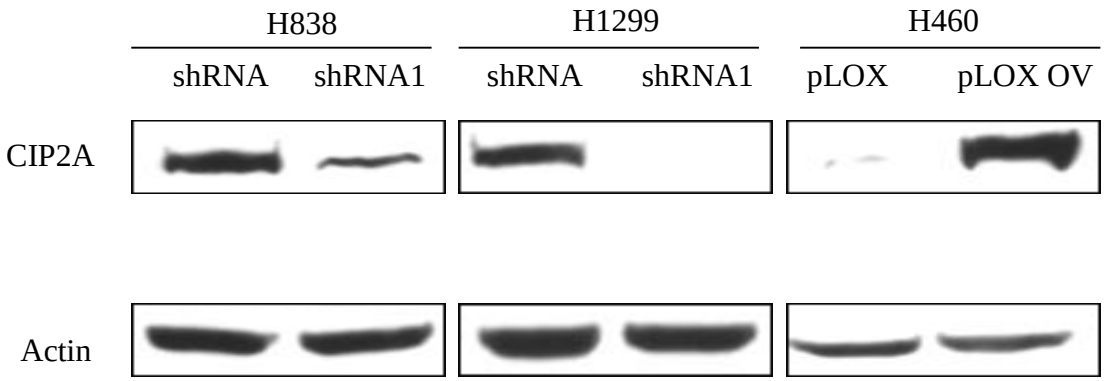


Figure 5: Expression and transfection of p90/CIP2A in NSCLC cell lines. (A) p90/CIP2A expression in several NSCLC cell lines, including A549, H838, H1299 and H460, was examined by western blot analysis using anti-p90/CIP2A antibody. (B) Cells transfected with shRNA control or shRNA p90/CIP2A, pLOX control or pLOX-p90/CIP2A in three NSCLC cell lines (H838, H1299 and H460), and the expression level was evaluated using western blot analysis. Actin was used to normalize the expression level.

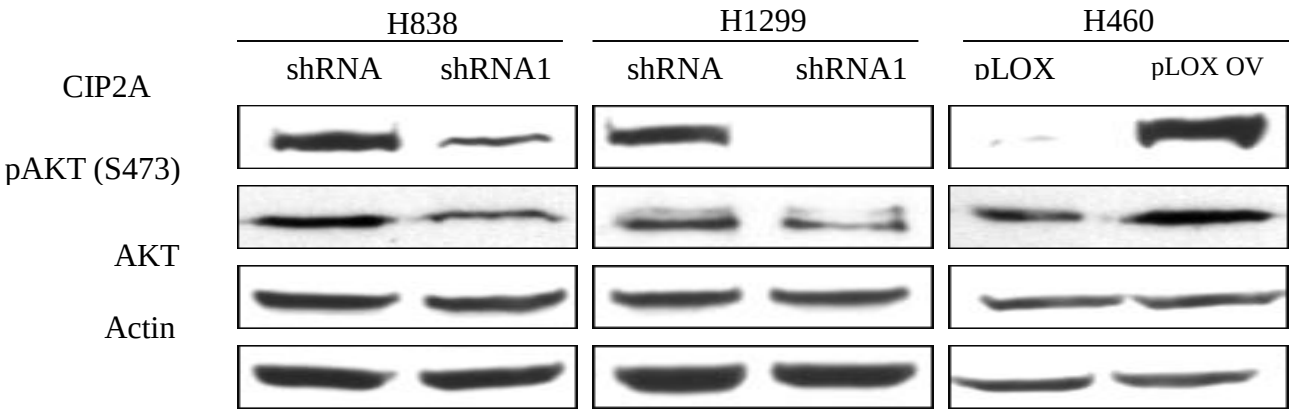


Figure 6: p90/CIP2A regulates AKT phosphorylation in lung cancer cells. Phosphorylation of AKT between cells transfected with shRNA control or shRNA p90/CIP2A, pLOX control or pLOX-p90/CIP2A in three NSCLC cell lines (H838, H1299 and H460) was evaluated. Equal number of cells were starved for 24 hour and then stimulated with 100 ng/mL EGF for 30 minutes. Cells were harvested and lysed in 1X Laemmli sample buffer, resolved on 10% SDS-PAGE gel, transferred to nitrocellouse membrane and probed with rabbit anti-phospho-AKT (1:1000). AKT expression was detected as normalization. The expression level to actin ratio was analyzed with densitometry.

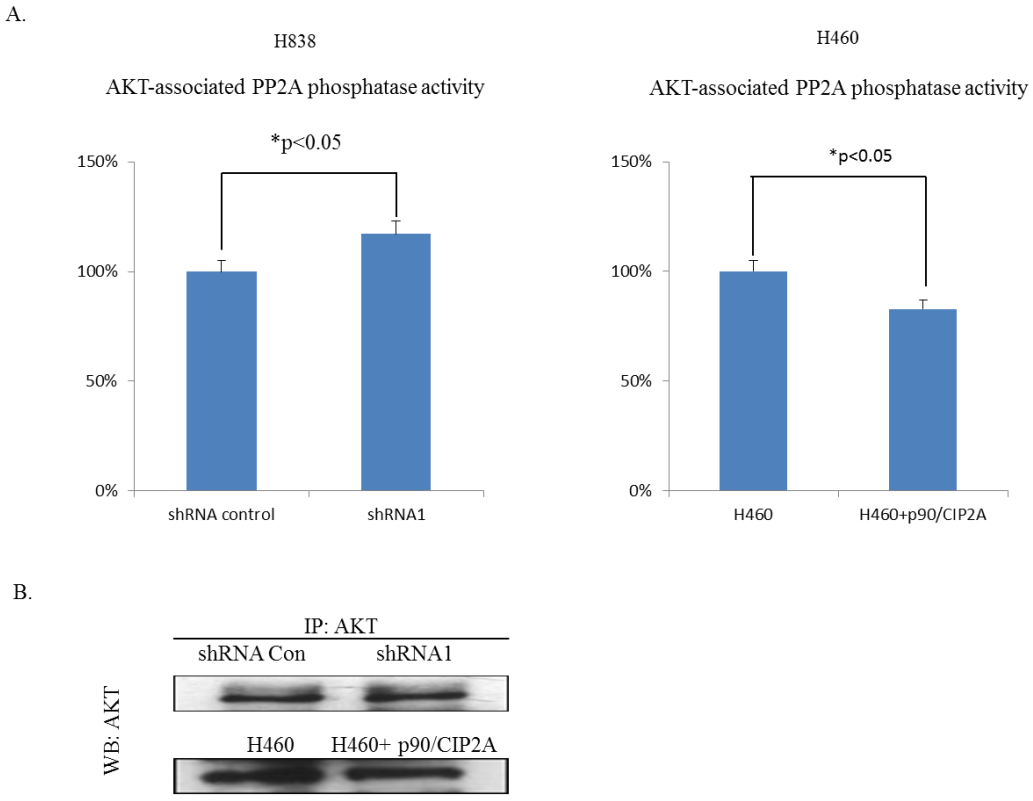


Figure 7: p90/CIP2A modulates AKT-associated PP2A phosphatase activity. (A) AKT-associated PP2A phosphatase activity was evaluated in two lung cancer cell lines: H838 and H460, with either p90/CIP2A depletion (left panel) or overexpression (right panel). (B) Western blot result showed the amount of AKT in the 5% of the immunopreicpitates in both of H838 and H460 cells, which was used to normalize the PP2A phosphatase activity.

Chapter 5:

p90/CIP2A targets specific AKT downstream substrates

5.1. Overview

Activated AKT regulates cellular processes through phosphorylation and more than two hundred substrates have been identified. Interestingly, AKT is not able to phosphorylate all the substrates at the same time; instead it shows substrate specificity. p90/CIP2A has been found to be associated with AKT phosphorylation. We found that the phosphorylation of AKT was dramatically enhanced in the presence of p90/CIP2A by decreasing AKT-associated PP2A phosphatase activity. In addition, we also found that the phosphorylation of only a subset of AKT substrates was altered in p90/CIP2A-depleted cells, which showed high specificity. In this study, we are aiming to explore how p90/CIP2A is involved in regulating AKT phosphorylation by targeting specific AKT downstream substrates.

5.2. Rationale, experimental design and alternative approach

***Specific aim 2*: To determine the AKT substrates targeted by p90/CIP2A using immunoproteomic approach.**

5.2.1. Rationale:

In this specific aim, we are trying to identify the specific AKT targets that can be recognized by p90/CIP2A using an immunoproteomic approach. Our preliminary data indicated

that over 90% knockdown of p90/CIP2A caused only partial loss of AKT phosphorylation, suggesting that p90/CIP2A might targets specific substrates of AKT to affect AKT phosphorylation. The most efficient way to answer this question is to identify AKT substrates that are differential expressed in response to the altered expression of p90/CIP2A. Since many stimuli can activate AKT, we choose EGF as cell stimulation to mimic the constitutive activation of EGF receptor (EGFR). The mutations of EGFR have been found in 30% of NSCLC patients, and these mutations can increase the cell proliferation and invasiveness of NSCLC. Using this approach, we can specifically examine the role of p90/CIP2A in EGFR-AKT-mediated cell activity.

Our data showed that upon EGF stimulation, the phosphorylation of AKT was dramatically enhanced in the presence of p90/CIP2A by decreasing AKT-associated PP2A phosphatase activity. As we know, AKT integrates upstream signals and transmits to downstream targets through phosphorylation. To investigate the underlying mechanism of p90/CIP2A-AKT association, we probed the phosphorylation of AKT substrates with phospho-AKT substrates antibody that recognizes a consensus motif (RXXpS/T). We found that the phosphorylation of only a subset of AKT substrates was altered in p90/CIP2A depleted cells. Co-immunoprecipitation (Co-IP) was then applied followed by mass spectrometry (MS) to identify the AKT substrates targeted by p90/CIP2A and twelve proteins were identified. In addition, we also found that the phosphorylation of mTOR, which is a direct target of AKT and also an important component of mTORC1 and mTORC2 complexes for cancer cell growth, was correlated with the expression level of p90/CIP2A.

5.2.2. Experimental Design & Methods:

AKT belongs to the arginine (Arg)-directed kinases, whose substrates share a common motif as the Arg is at the -3 position relative to the phophosrylated serine (Ser) or threonine (Thr) residues. The commercially available anti-phospho-AKT substrates antibody developed by the company of *Cell Signaling Technology* was used to perform immunoprecipitation in our study, which could recognize the motif of RXXS*/T* (* indicates the phosphorylation site). Since our preliminary data clearly showed that p90/CIP2A could target certain AKT substrates in the H1299 cell line, we will continue to use this established model to analyze the role of p90/CIP2A in the AKT signaling network. Immunoprecipitation will be performed on the group of cells transfected with vector control or transiently knockdown with p90/CIP2A. Immunoprecipitates will be eluted with low pH buffer, precipitated with acetone, and resolved by SDS-PAGE gel. Differentially phosphorylated proteins will be excised and analyzed by liquid-chromatography mass spectrometry (LC-MS) to identify them.

To validate whether the identified proteins are AKT substrates, several methods can be used. First, these proteins will be searched against AKT substrates databases *(http://www.cellsignal.com/reference/pathway/akt_substrates.html).* Second, we will confirm the phosphorylation events by using a commercially available phospho-specific antibody. However, if phospho-antibodies are not available, the phospho-tag SDS-PAGE gel can also be used to validate the phosphorylation change by visualizing the mobility shift. Furthermore, the phosphorylation change of all the differentially phosphorylated substrates will be examined in the presence of PI3K inhibitor, LY294002, which can abolish the EGFR-induced AKT phosphorylation.

Evaluation of the phosphorylation of mTOR will be performed in knockdown of p90/CIP2A in H838 cell line and overexpression of p90/CIP2A in H460 cell line. Cells will be starved for 24 hours and stimulated with EGF for 30 minutes. Phosphorylation of mTOR will be probed with anti-phospho-mTOR (S2441) by western blot analysis. To further confirm the regulation of p90/CIP2A on the mTOR pathway, the phosphorylation of mTOR downstream targets, p70RSK and 4EBP1, will also be detected in the western blot analysis. A major function of mTOR pathway is to promote protein synthesis in response to growth factors stimulation. Therefore, it is highly possible that p90/CIP2A may cause the global change of proteins synthesis, which can be measured by non-radioactive protein synthesis assay kit.

5.2.3. Potential problems & alternative approaches:

Although the antibody we use can target a majority of the substrates being phosphorylated by AKT, there are still some targets that do not contain such motifs, but can still be phosphorylated. Therefore, if the differentially phosphorylated substrates are not informative enough to understand the role of p90/CIP2A in the AKT signaling network, a complementary method of using the commercially available phosphor-AKT substrates antibody array should be used.

In addition, the sample preparation for SDS-PAGE after protein elution may cause the loss of samples and the low abundance of some proteins may not be visualized in SDS-PAGE gel. These methods will therefore decrease the resolution of discovery of the p90/CIP2A targeted AKT substrates. As an alternative approach, cells can be labeled with isotope during cell culture, immunoprecipitated with phospho-AKT substrates, and eluted and analyzed with LC-MS directly, which may improve the resolution of the discovery of the substrates.

5.3. Results

5.3.1. p90/CIP2A modulates AKT downstream substrates specificity by regulation of AKT phosphorylation

AKT is a central regulator that phosphorylates hundreds of substrates and participates in a wide variety of biological processes. The ability of p90/CIP2A in regulating the phosphorylation of AKT indicating that p90/CIP2A may regulate specific AKT substrates recognition and activation.

We probed the phosphorylation of AKT substrates using commercial available antibody, which recognizes a canonical AKT phosphorylation motif. For a better comparison of the substrates whose phosphorylation was affected by p90/CIP2A, H1299 cells were transfected with either p90/CIP2A depletion or p90/CIP2A overexpression. Upon EGF treatment, the intensity of several bands showed differential expression between p90/CIP2A depletion and p90/CIP2A overexpression cells. The presence of several bands that showed no change among these groups implied that p90/CIP2A might regulate the phosphorylation of specific AKT substrates rather than causing global effects (**Figure 8**).

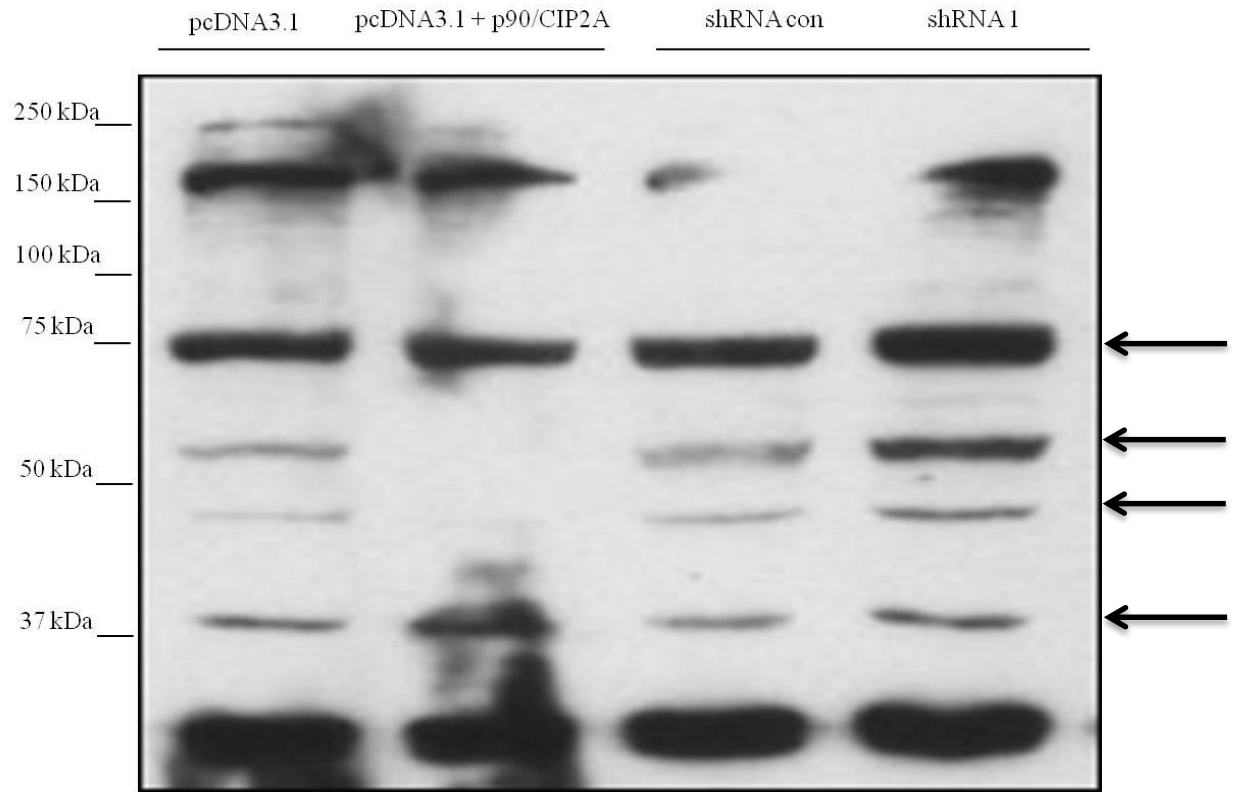


Figure 8: p90/CIP2A regulates phosphorylation of AKT substrates specificity. Four transfected cell lines with either p90/CIP2A overexpression (and control) or knockdown (and control) were treated with 100 ng/mL EGF for 30 min to stimulate phosphorylation. Cells were harvested and lysed in 1X Laemmli sample buffer, resolved on 10% SDS-PAGE gel, transferred to nitrocellouse membrane and probed with rabbit anti-phospho-(Ser/Thr) AKT substrates (1:1000). Secondary antibody (goat anti-rabbit 1:5000) was used to visualize the positive bands. Arrows indicated the differentially phosphorylated AKT substrates.

5.3.2. Identification of p90/CIP2A-targeted AKT substrates by Co-IP and LC-MS

To identify the AKT substrates targeted by p90/CIP2A, we firstly used the same antibody for Co-immunoprecipitatation (Co-IP) to establish the AKT substrates profile. A total of 14 bands had been detected and 12 of them were successfully identified by liquid chromatography-mass spectrometry (LC-MS) (**Figure 9**). A summary of these identified proteins and their functions can be found in **Table 4**. Several proteins like Hsp90 beta, Hsc70, Grp78, SSA1 and peroxiredoxin 1 are stress response proteins and they have been found to be AKT substrates identified by either immunoprecipitation or radioactive labeled methods from previous independent groups. Therefore, the protein substrates identified in our study might be potential targets of p90/CIP2A in the AKT signaling pathway.

To characterize the p90/CIP2A-targeted proteins, we compared the expression profile with the western blot result showed in **Figure 8**. The four differentially expressed proteins were also identified (**Table 3**). Grp78 and SSA1 are two important regulators of ER stress, which will lead to cell apoptosis and are thus suppressed in cancer cells. The overexpression of p90/CIP2A led to the decreased phosphorylation of these two chaperones suggested that p90/CIP2A might negatively regulate the activity of them and thus relieve the ER stress to protect cell from apoptosis. Therefore, we examined whether p90/CIP2A regulated ER stress-induced cell apoptosis through the AKT signaling pathway in our next specific aim.

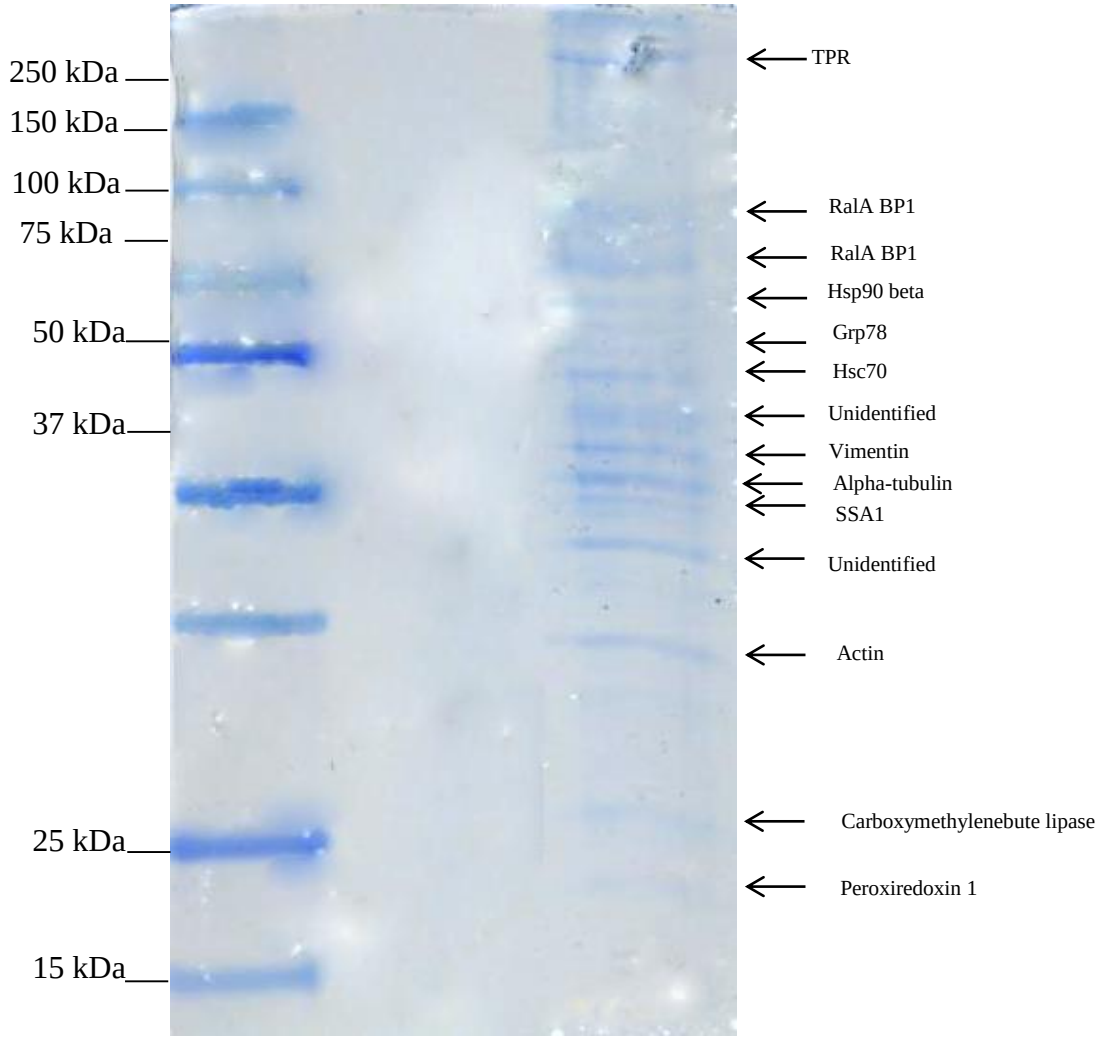
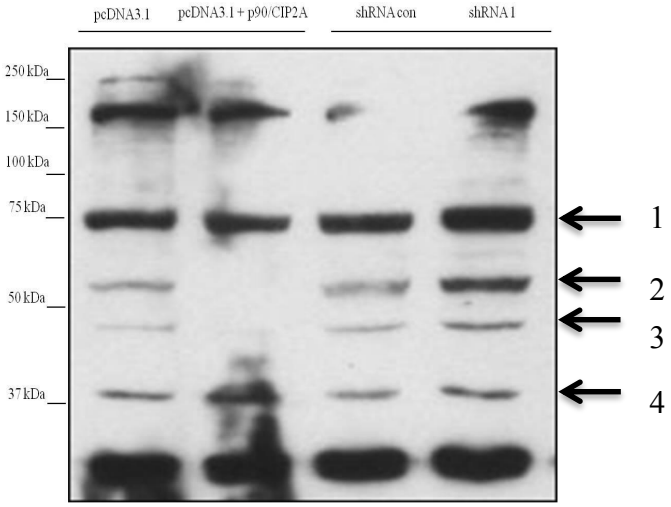


Figure 9: Identification of p90/CIP2A-targeted AKT substrates by Co-immunoprecipitation (Co-IP) and liquid chromatography-mass spectrometry (LC-MS). $1X10^7$ cells were starved overnight and then stimulated with EGF (100 ng/ml) for 30 minutes. Cells were washed with PBS for three times and lyzed with RIPA buffer. Cells lysates were collected and pre-cleared with protein A sepharose beads. The cleared cell lysates were incubated with 100 µL beads-conjungated phospho-AKT substrates antibody overnight at 4 ˚C. The immunoprecipitates were washed three times with RIPA buffer and dissolved with 1X Lammilie buffer. Proteins were resolved on SDS-PAGE gel, and the gel was subsequently stained with Colloidal blue. All detected bands were excised for mass spectrometry analysis.

Table 3: Identified p90/CIP2A-targeted AKT substrates by mass spectrometry



Band No.	Protein name	Score
1	Glucose-regulated protein 78	100
2	Vimentin	170
3	Heat-shock protein SSA1	92
4	Actin	546

Table 4: Summary of all identified AKT substrates targeted by p90/CIP2A

Name	Full Name	Score	Functional Description
TPR	Tetratricopeptide repeat	167	Structure motif, which forms scaffold to mediate protein-protein interactions
RalA BP1	RalA-binding protein 1	361	Mediates ATP-dependent transportation and catalyzes transportation of glutathine
Hsp90 beta	Heat shock protein 90 beta	255	Chaperone protein that facilitates other proteins' folding and stabilization against heat stress
Grp 78	Glucose regulated protein 78	100	HSP70 molecular chaperone that binds newly-synthesized proteins and maintains them stable
Hsc 70	Heat shock cognate protein 70	149	Chaperone protein and a key component of chaperone-mediated autophagy
Vimentin		170	Type 3 IF; marker of EMT; important in supporting and anchoring the position of the organelles in the cytosol
Alpha tubulin		104	Globular protein that make up microtubules
SSA1	Heat shock protein SSA1	92	Important in the transport of polypeptides both across the mitochondrial membranes and into the ER
Actin		546	Globular multi-functional protein that forms microfilaments
Carboxymethlenebutelipase		68	
Peroxiredoxin 1		129	Antioxidant enzyme that reduces hudrogen peroxide and alkyl hydroperoxides; have a proliferative effect

5.3.3. p90/CIP2A regulates mTOR phosphorylation and its downstream effectors.

mTOR is a 289 kDa serine/threonine kinase from mammalian cells, which is a direct target of AKT and also an important component of mTORC1 and mTORC2 complexes for cancer cell growth [69]. mTORC1 is activated by insulin and other growth factors through PI3K and AKT kinase signaling, promoting messenger RNA translation and protein synthesis through at least two of its substrates: ribosomal protein S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) [69].

In this study, we firstly tested whether p90/CIP2A was associated with mTORC1 (**Figure 10**). To further test whether p90/CIP2A would affect the mTOR signaling axis, we measured the phosphorylation of mTOR at Serine 2441 (S2441). The knockdown of p90/CIP2A in H838 decreased the phosphorylation of mTOR while the overexpression caused the increased phosphorylation of mTOR (**Figure 11**). This data suggested that p90/CIP2A would influence the mTOR phosphorylation in lung cancer cells. Since mTOR has two important downstream substrates: eukaryotic initiation factor 4E binding protein 1 (4EBP1) and p70 ribosomal S6 kinase 1 (P70S6K1), we also detected whether p90/CIP2A was associated with any of mTOR downstream effectors. We found that depletion of CIP2A up-regulated phosphorylation of both P70S6K1 and 4EBP1.

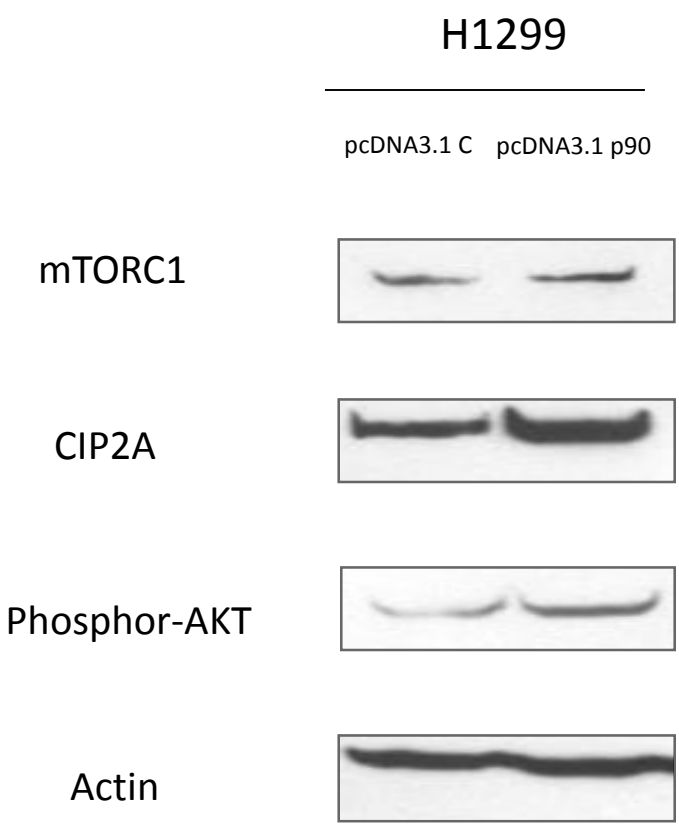


Figure 10: The expression level of p90/CIP2A is associated with mTORC1. H1299 cells were transfected with either pcDNA3.1 control or pcDNA3.1 p90/CIP2A. Western blot analysis showed the expression level of mTORC1, p90/CIP2A, and phosphorylation of AKT. Actin was used for normalization. Overexpression of p90/CIP2A unregulated the expression level of mTORC1 and phosphorylation of AKT.

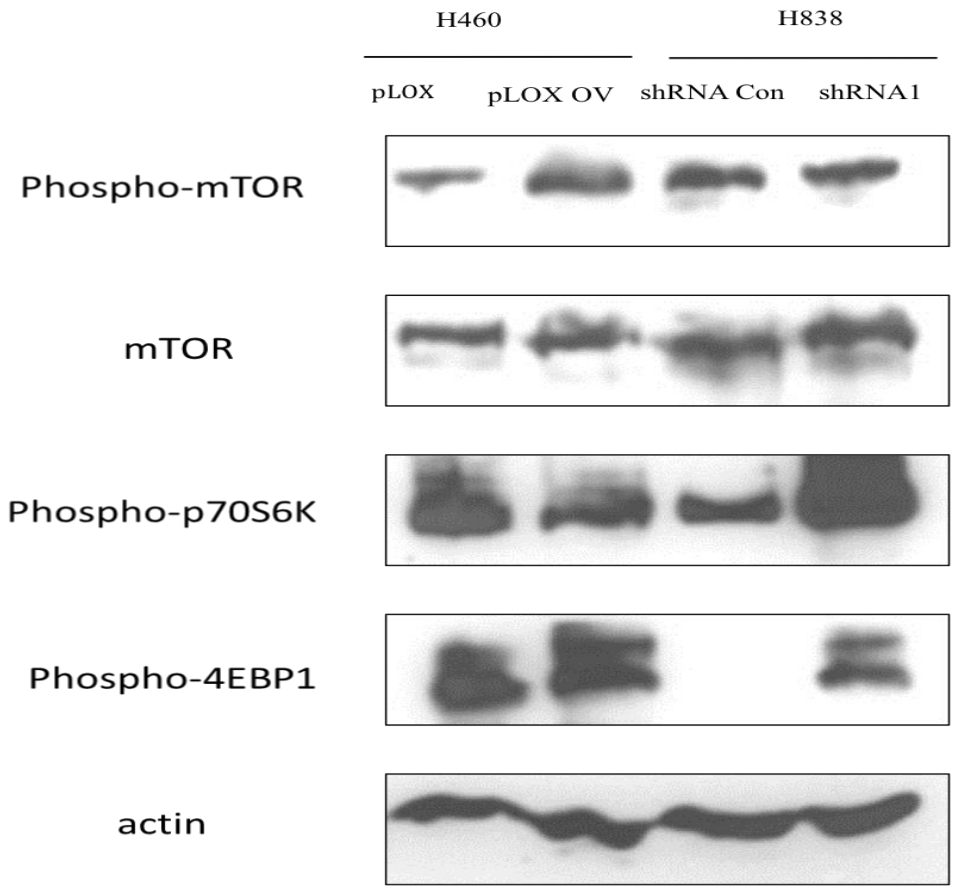


Figure 11: p90/CIP2A modulates mTOR phosphorylation and the expression level of mTOR downstream substrates. mTOR phosphorylation and its downstream effectors (P70S6K and 4EBP1) phosphorylation were analyzed in H838 and H460 cells with either p90/CIP2A depletion or p90/CIP2A overexpression. Cells were starved overnight, and stimulated with EGF (100 ng/ml) for 30 minutes. Cells were harvested and lyzed with 1X Lammali buffer. Proteins were resolved on 10% SDS-PAGE gel. Phosphorylation of mTOR and its substrates was analyzed with rabbit anti-phospho-mTOR (S2441) (1:1000), anti-phospho-p70S6K and anti-phospho-4EBP1. Actin was used for normalization.

Chapter 6:

p90/CIP2A regulates cell activity via AKT signaling pathway

6.1. Overview

Our previous data showed that the elevated expression of p90/CIP2A in lung cancer cells resulted in an increased cell proliferation. Since AKT is an important regulator of cell proliferation, we are trying to define the association between p90/CIP2A and AKT in regulating lung cancer cell proliferation. We also found that p90/CIP2A might recognize specific AKT targets and play certain roles in this signaling pathway. The identified AKT targets that can be specifically recognized by p90/CIP2A in the previous aim play various roles in cell activity as summarized in Table 4. Among the identified proteins, we chose glucose-regulated protein (Grp78) to do further investigation. Grp78 is an important regulator of ER stress, which will lead to apoptosis and is thus suppressed in cancer cells. The overexpression of p90/CIP2A led to the decreased phosphorylation of Grp78 suggested that p90/CIP2A might negatively regulate the activity of Grp78 and thus relieve the ER stress to protect cell from apoptosis. Therefore, the data acquired indicated that p90/CIP2A might regulate certain cell activities through the AKT signaling pathway.

6.2. Rationale, experimental design and alternative approach

***Specific aim 3*: To determine whether p90/CIP2A is involved in certain cell activities in human lung cancer cells.**

6.2.1 Rationale:

The overexpression of p90/CIP2A in cancer cell is associated with increased cell proliferation. However, the mechanism of p90/CIP2A in cancer cell proliferation is still poorly understood. Here, we reported the evidence that p90/CIP2A could regulate AKT phosphorylation under growth factor stimulation and our results showed that p90/CIP2A might promote cell proliferation through the AKT signaling pathway.

In addition, depletion of p90/CIP2A did not induce a global change of AKT phosphatase activity, which implied that it might recognize specific AKT targets and play certain roles in the signaling pathway. Several identified AKT substrates that might be targeted by p90/CIP2A are ER stress related proteins. We also detected whether p90/CIP2A is involved in regulating ER stress related cell apoptosis.

6.2.2 Experimental Design & Methods:

We first examined cell proliferation in NSCLC cells with either p90/CIP2A depletion or overexpression, and we found that cell proliferation was associated with the expression level of p90/CIP2A. To investigate whether AKT is responsible for p90/CIP2A-mediated cell proliferation, we introduced constitutively active AKT into p90/CIP2A-knockdown cell line and dominant negative AKT into p90/CIP2A overexpression cell line for cell proliferation detection.

To detect cell apoptosis activity, we treated cells with DTT, which would cause ER stress induced cell apoptosis. Cell viability was then analyzed by flow cytometric analysis. Among the identified AKT substrates that might be potential targets of p90/CIP2A, we chose GRP78 to further detect whether it might be regulated by p90/CIP2A. To investigate whether p90/CIP2A would affect the sensitivity of cancer cell in resistance to rapamycin, H838 cells transfected with

control shRNA or shRNA1 would be plated sextuplet in 96-well plates. Cells were treated with 100 μM rapamycin and cell proliferation was measured at day 1, day 3, day 5 and day 7 using MTT assay according to the protocol. This study will enable us to reveal more functions of p90/CIP2A in human lung cancer.

6.2.3 Potential problems & alternative approaches:

The correlation between p90/CIP2A and ATK might have certain problems with undetectability due to distinct genetic backgrounds occurred in different cancer cell lines. In addition, other factors may have similar effects on the regulation of the phosphorylation of AKT with p90/CIP2A. In such a case, the loss-of-function by shRNA or gain-of-function by overexpression should be applied to investigate whether the change of phosphorylation status of AKT is caused by p90/CIP2A.

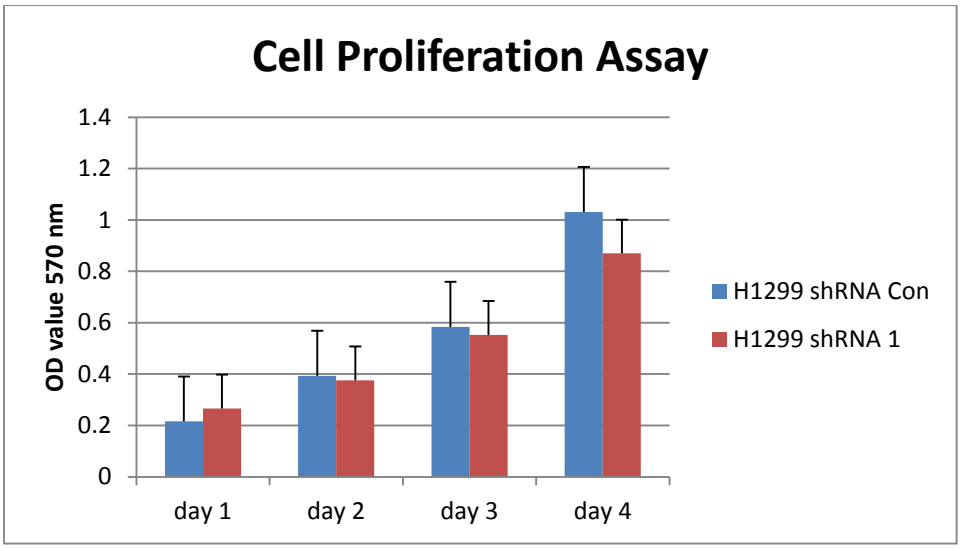
Another possible problem may be raised is whether depletion of p90/CIP2A will sensitize cells to rapamycin treatment. According to our study, p90/CIP2A positively regulates the phosphorylation of AKT that leads to the enhanced performance of mTOR signaling. However, the negative feedback of the down-regulation of p90/CIP2A might not be obvious at the early stage of treatment. To solve this possibility, we will extend the length of drug incubation to more than five days. The depletion of p90/CIP2A may not be able to completely abolish the activation of mTOR that can be countered by the prolonged drug incubation. Furthermore, the consequence of the synergism of the depletion of p90/CIP2A and rapamycin can be measured by flow cytometry to determine the cellular effects like apoptosis or growth arrest.

6.3. Results

6.3.1 p90/CIP2A regulates lung cancer cell proliferation through the AKT signaling pathway.

We detected cell proliferation in the transfected NSCLC cell lines by MTT assay. The knockdown of p90/CIP2A in H1299 cells caused the decreased cell proliferation while overexpression of p90/CIP2A in H460 cells led to the increased cell proliferation (**Figure 12**). These data suggested the function of p90/CIP2A in promoting cancer cell proliferation.

Since AKT is an important regulator in cancer cell proliferation and tumor cell growth. We wanted to test whether p90/CIP2A-mediated cell proliferation could be partly attributed to the AKT phosphorylation. We introduced two AKT mutants in our transfected cell lines. In H1299 cells, the knockdown of p90/CIP2A decreased cell proliferation to 78%, which was consistent with the previous finding. However, the introduction of constitutively active AKT (AKT CA) would rescue the decreased cell proliferation to about 119%. Furthermore, the expression of dominant negative AKT (AKT KD) decreased the cell proliferation imposed by the p90/CIP2A overexpression (123% *v.s.* 81%). These data suggested that p90/CIP2A could promote cell proliferation through the regulation of AKT (**Figure 13**).



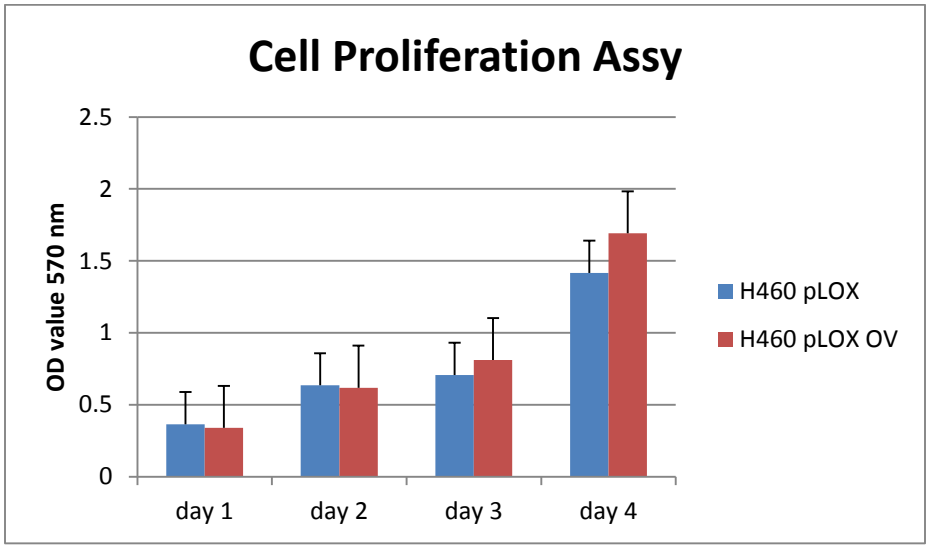


Figure 12: Cell proliferation assay was performed in H1299 cell line transduced with shRNA control or shRNA p90/CIP2A and H460 cell line transduced with pLOX or pLOX-p90/CIP2A. Five thousand cells of each group were plated sextuplet in 96-well plates and grew for indicated time (day1, 2, 3 and 4). Cell proliferation assay was performed by incubation cell culture with MTT for 4 hours and the absorbance was measured at 570 nm in a colorimetric meter. Statistical significance was done with Students' t-test analysis.

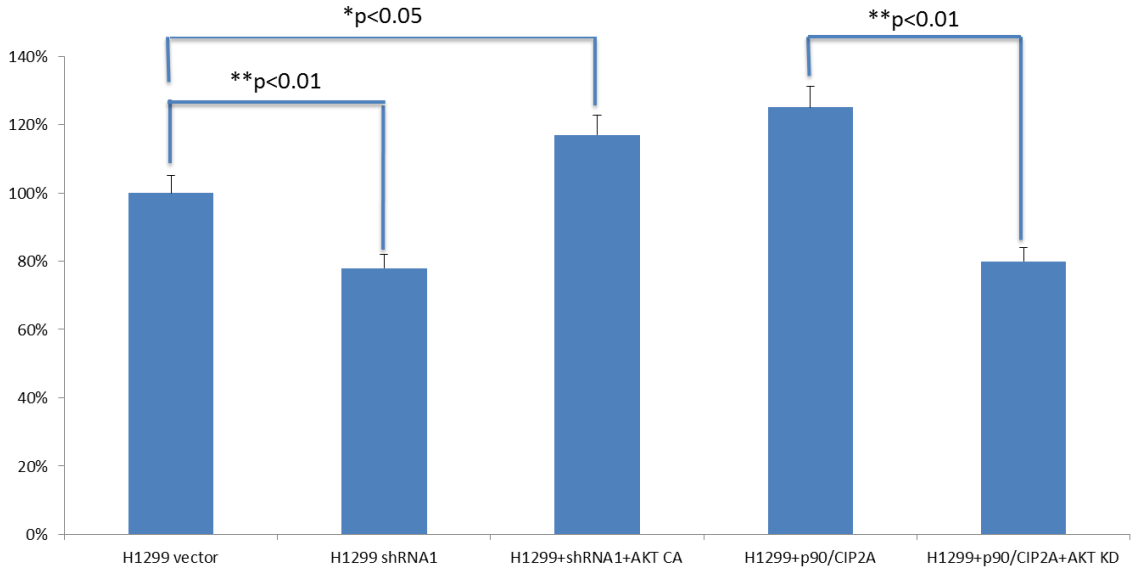


Figure 13: p90/CIP2A promotes cell proliferation through the AKT signaling pathway. Cell proliferation was measured in cell lines transfected or co-transfected with either shRNA control, p90/CIP2A shRNA (shRNA1), shRNA1 and AKT CA, pCDNA3.1+p90/CIP2A or pcDNA3.1-p90/CIP2A and AKT KD. Five thousand transfected cells were plated sextuplet in 96-well plate and cell proliferation was measured 48 hours post-transfection. After 4 hours incubation with MTT, reaction was stopped with stop solution and absorption was measured at 570 nm in a colorimetric meter. Statistical significance was done with Students' t-test analysis.

5.3.4. p90/CIP2A reduces GRP78 accumulation and protects cancer cell from ER stress-induced cell death

PI3K/AKT inhibition is found to promote endoplasmic reticulum (ER) stress induced apoptosis in a glucose-regulated protein 78 (GRP78)-dependent manner [70]. It has been shown that PI3K/AKT activity would decrease with long-term exposure to ER stress stimuli [71]. ER is the organelle where secreted and membrane-bound proteins are synthesizing and folding. When ER function is impaired, the unfolded or misfolded proteins accumulate in the ER, leading to unfolded protein responses (UPRs) [72, 73]. The activation of UPR is believed to promote cell survival [74]. GRP78 is an UPR target gene, which is a well-characterized ER chaperone proteins. GRP78 induction is critical in protecting the cells against ER stress-induced apoptosis [75].

Our previous result showed that p90/CIP2A negatively regulated GRP78 phosphorylation (**Figure 8**). In this study, we further detected that the expression level of p90/CIP2A in association with GRP78. We found that depletion of p90/CIP2A down regulated the expression level of GRP78 while overexpression of p90/CIP2A increased the expression level of GRP78 (**Figure 14**). In addition, the overexpression of p90/CIP2A would protect cancer cells from ER-stress induced cell apoptosis (**Figure 15**). We also detected whether upon ER stress, the expression of p90/CIP2A would be impaired. Our data showed that incubation of H838 cells with ER stress inducers dithiothreitol (DTT) and thapsigargin (TG) markedly reduced the expression level of p90/CIP2A (**Figure 16**). Based on our data, we think it's highly possible that p90/CIP2A is associated with GRP78 and is involved in the process of ER stress induced cell apoptosis.

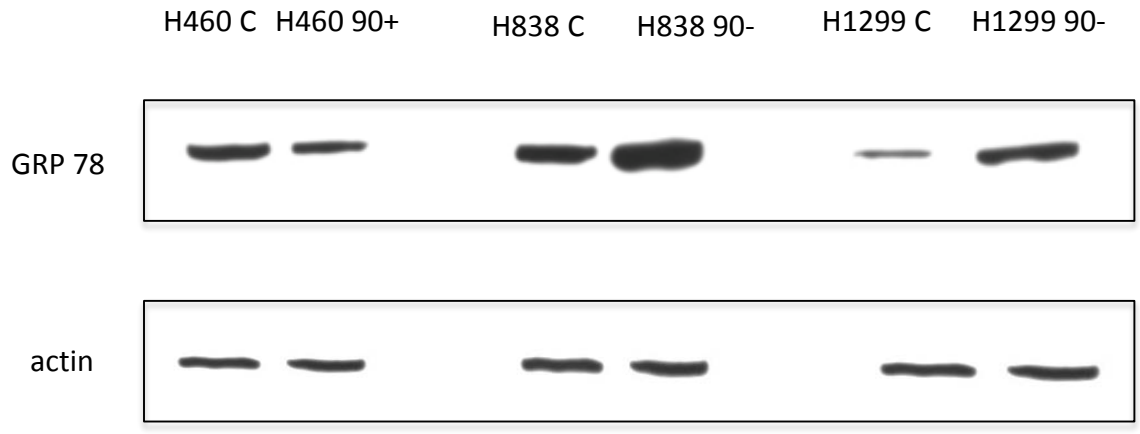
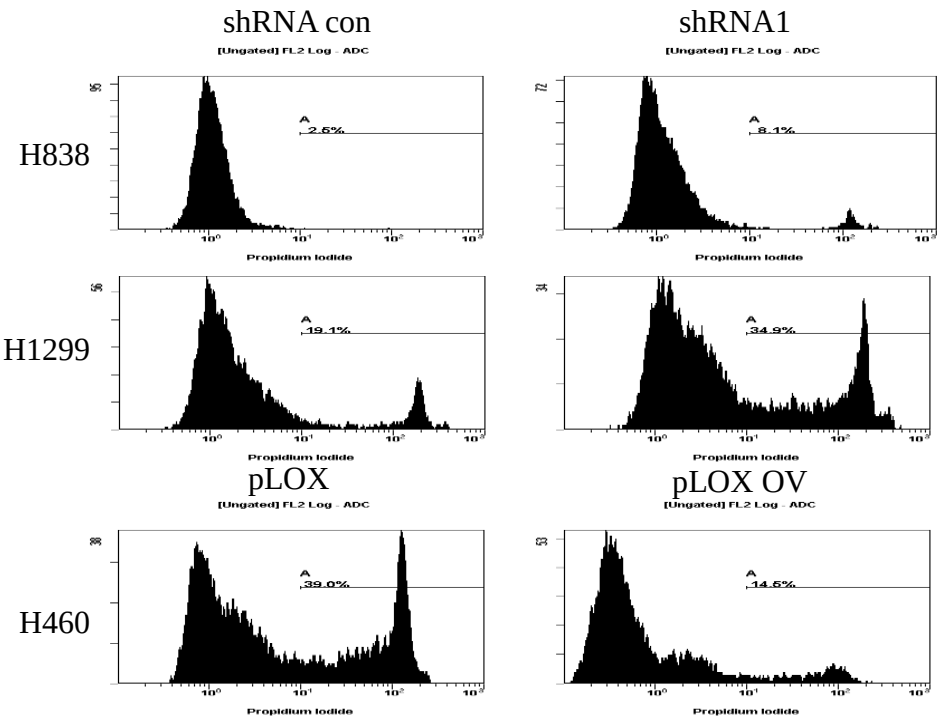


Figure 14: p90/CIP2A regulates AKT substrate GRP78 specificity. Expression of GRP78 between cells transduced with shRNA control or shRNA p90/CIP2A, pLOX or pLOX-p90/CIP2A was evaluated in three cell lines (H838, H1299 and H460). Cells were harvested and lysed in 1X Laemmli sample buffer, resolved on 10% SDS-PAGE gel, transferred to nitrocellouse membrane and probed with rabbit anti-GRP78 (1:1000). p90/CIP2A to actin ratio was analyzed with densitometry as a normalization.



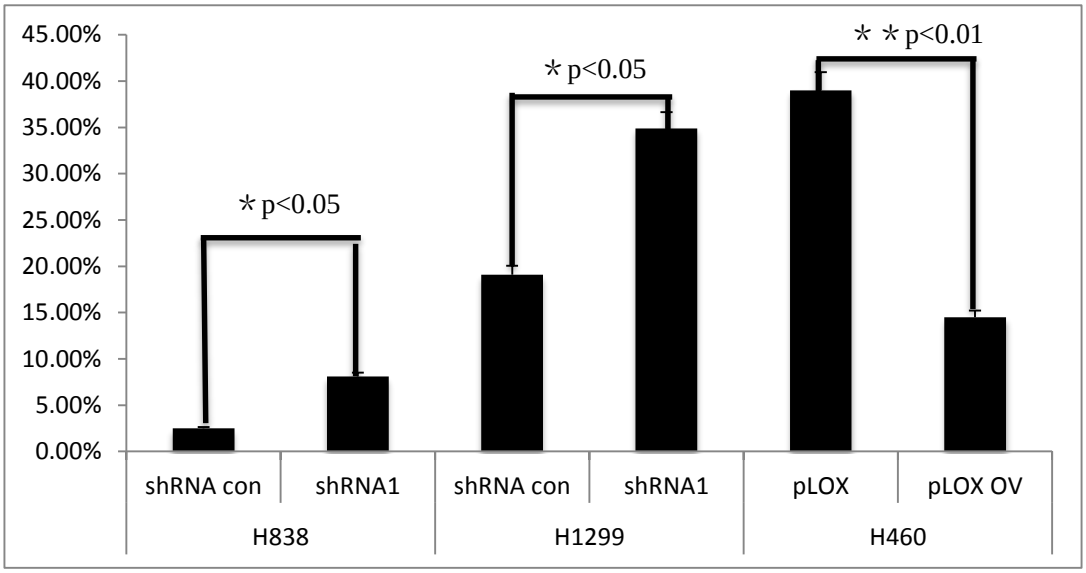


Figure 15: p90/CIP2A protects cancer cell from ER stress-induced cell death. Cells transduced with shRNA control or shRNA p90/CIP2A, pLOX or pLOX-p90/CIP2A were seeded at a density of 1×10^5 per well in six-well plates. After 24 hours, cells were treated with 1mM DTT. Samples were analyzed using the EPICS XL Flow Cytometer. The absence of p90/CIP2A in knockdown

cells reduced the number of dead cells and the overexpression of p90/CIP2A increased cell death.

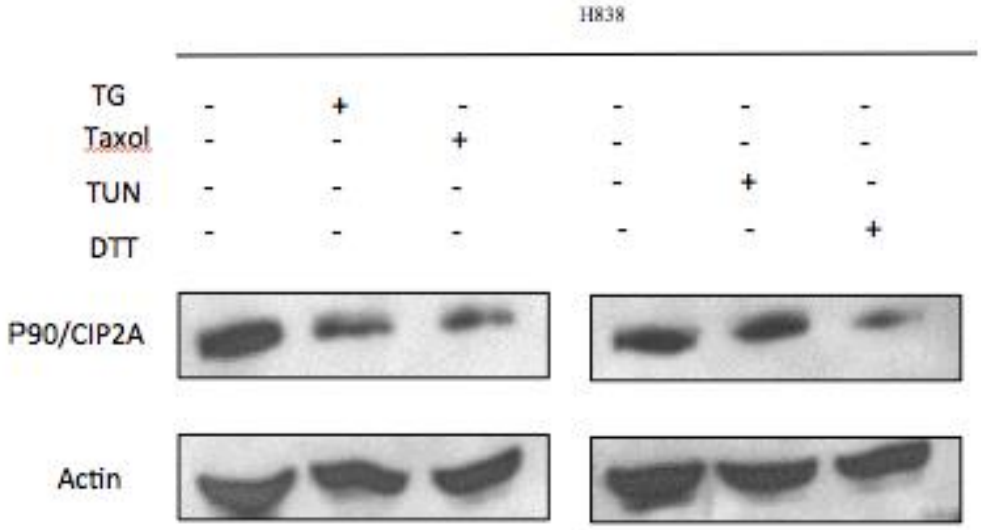


Figure 16: p90/CIP2A is involved in ER stress-induced cell apoptosis. Two ER stress inducers dithiothreitol (DTT) (2.5 mM) and thapsigargin (TG) (1 μM) were used to treat H838 cells. Tunicamycin (TUN) was used as a control. Cells were harvested and lysed in 1X Laemmli sample buffer, resolved on 10% SDS-PAGE gel, transferred to nitrocellouse membrane and probed with mouse anti-p90/CIP2A (1:2000) antibody. Actin was used to normalize the expression level.

5.3.5. Depletion of p90/CIP2A sensitizes cancer cell to rapamycin-mediated cell growth.

Rapamycins belong to a novel family of anticancer agents, including rapamycin and its derivatives. They represent anticancer action by inhibiting the function of mTOR that associates with mitogen stimulation to protein synthesis and cell cycle progression. Rapamycins are able to suppress tumor cell growth by arresting cells in G1 phase, leading to apoptosis of cells [76].

In this study, we designed experiments to determine whether p90/CIP2A can potentiate cancer cell for rapamycin resistance. To investigate whether p90/CIP2A would affect the sensitivity of cancer cell in resistance to rapamycin, H838 cells transduced with control shRNA or shRNA1 will be plated sextuplet in 96-well plates. Cells are treated with 100 μM rapamycin and cell proliferation will be measured at day 1, day 3, day 5 and day 7 using MTT assay according to the protocol. The result showed that depletion of p90/CIP2A coupled with rapamycin treatment would significantly decrease cell growth (**Figure 17**). It allows us to report part of the mechanism on p90/CIP2A in regulation of cancer cell chemoresistance via the AKT/mTOR signaling pathway.

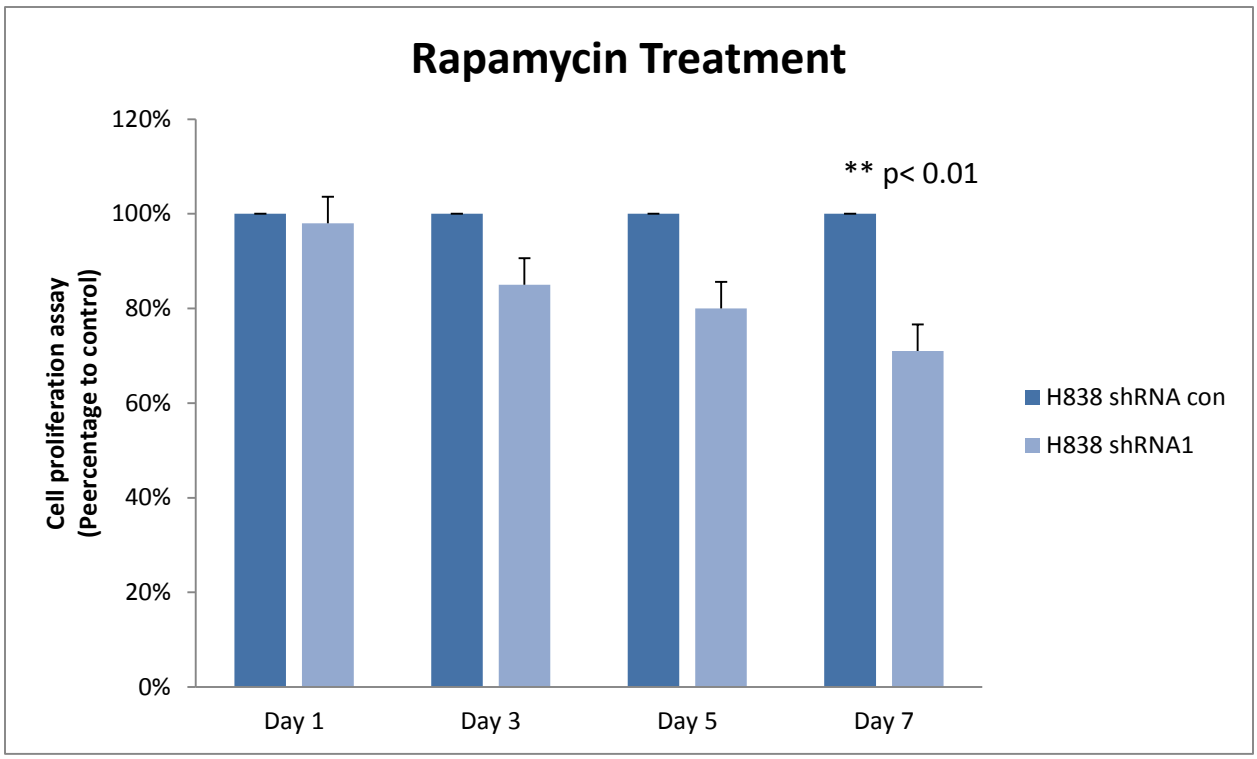


Figure 17: Absence of p90/CIP2A affects cellular response to rapamycin. Five thousand H838 cells transduced with either shRNA or shRNA1 were plated sextuplet in 96-well plates and were treated with rapamycin (100 nM) for indicated time (day 1, 3, 5 and 7). Cell proliferation assay was performed by incubation cell culture with MTT for 4 hours and the absorbance was measured at 570 nm in a colorimetric meter. Statistical significance was done with Students' t-test analysis

Chapter 7: Discussion and Future Direction

7.1. Discussion

Lung cancer causes most cancer-related deaths in both men and women in the United States [4]. Early diagnosis of lung cancer is technically difficult, because there are few noticeable symptoms felt by the patients at the onset of the disease [4] . Although early diagnosis cannot guarantee recovery, appropriate treatment can be given to prevent the advancement of this disease [9]. Therefore, understanding the mechanisms underlying lung cancer tumorigenesis can be of great importance for the development of anti-cancer drugs and therapeutic design.

Cancerous inhibitor of PP2A (CIP2A) was originally identified as a tumor-associated antigen in gastric and liver cancer, which was named as p90 due to its molecular weight around 90 kDa [13]. Previous studies have indicated that p90/CIP2A is an oncofetal protein to promote cancer cell proliferation through inhibition of c-Myc associated PP2A phosphatase activity [77]. The expression of this protein is tightly regulated during development but often re-express in lung cancer tissues. Studies in our laboratory have also demonstrated that autoantibodies against p90/CIP2A appears in high frequency in sera from lung cancer patients. The expression of this protein also has high frequency in lung cancer tissues. The overexpression of p90/CIP2A in cancer patients can elicit immune response in prostate cancer, gastric cancer and lung cancer as far as being tested [9]. Taken together, our previous studies can give strong support to the clinical use of this protein as biomarker in lung cancer detection.

Functional studies of p90/CIP2A in lung cancer have not been fully understood and its clinical relevance is not yet been established. Our previous work found that p90/CIP2A was able

to regulate the phosphorylation of CREB and hexokinase via AKT to promote cell proliferation in lung cancer cells. In this study, we are trying to define novel p90/CIP2A targets by proteomic-based systematic screening. The candidates obtained from our study will expand the understanding on protein-substrate interaction and therefore direct the cancer drug design. At the same time, we work on the mechanism of AKT phosphorylation to further investigate how p90/CIP2A is involved in the PI3K/AKT/mTOR pathway, which is critical to cancer progression.

In this study, we found the possible interaction between p90/CIP2A and the AKT signaling pathway, which could help to reveal the mechanism for p90/CIP2A-promoted cancer progression. We firstly attempted to address the relationship between p90/CIP2A and AKT phosphorylation in human lung cancer cells. The positive correlation between p90/CIP2A and AKT phosphorylation in our results clearly demonstrated the possible regulation of AKT by p90/CIP2A through growth factor stimulation. This is not astonishing since the previous studies from others have shown the role of p90/CIP2A in promoting cell survival through down-regulating AKT-associated PP2A phosphatase activity. Our studies not only confirmed the regulation under different treatments but also pointed an alternative way by which p90/CIP2A to promote cancer progression in human lung cancer. It was also demonstrated that the altered cell proliferation occurred by the introduction of mutant AKT (AKT CA and AKT DN), suggesting that p90/CIP2A regulates cell proliferation can be partly attributed to AKT phosphorylation.

The observation that depletion of p90/CIP2A did not induce a global change of AKT phosphatase activity implied that it might recognize specific AKT targets and play specific roles in this signaling pathway. In this case, understanding of how p90/CIP2A recognizes and interacts with its targets is of great importance. Being a central regulator in cell growth and cell

proliferation, AKT phosphorylates hundreds of substrates and leads to the activation of various cellular pathways under different stimulation; however the mechanism of how the substrates are specifically selected is still unclear. We used the commercially available anti-phospho-AKT substrates antibody to perform immunoprecipitation and constructed a protein file by liquid-chromatography mass spectrometry (LC-MS). In addition, to explore whether these differentially phosphorylated proteins are associated with certain cancerous phenotypes, we use certain bioinformatics software (for example, Ingenuity Pathway Analysis, IPA) to analyze the biological backgrounds of these proteins and link them to known signaling pathways as well as cell processes, which may help us to gain more information of how p90/CIP2A contributes to AKT-induced pathogenesis.

In our study, we identified twelve AKT substrates as the potential targets of p90/CIP2A, which could be used to further study roles of p90/CIP2A in the AKT signaling pathway. Among the four differentially expressed proteins as shown in Figure 8, two of them glucose regulated protein (Grp78) and heat shock protein SSA1 are stress response proteins, whose activity are up-regulated in most cancer types to protect ER stress-induced cell death [77]. The phosphorylation of Grp78 and SSA1 are inversely correlated with the expression level of p90/CIP2A that pose an interesting question about the relationship between p90/CIP2A and ER stress. Although the functional relevance of phosphorylated Grp78 and SSA1 to ER stress is not identified yet, it seems that the increased expression of p90/CIP2A will relieve the ER stress through the regulation of the post-translational modification of these two chaperones in our preliminary data. Another two cytoskeleton proteins, actin and vimentin, were also identified. Several studies have proven that AKT is able to phosphorylate actin and reorganize cytoskeleton to regulate cell motility [78, 79]. Our previous data does not actually find the difference of cell migration and

invasion between p90/CIP2A wild type cells and p90/CIP2A depletion cells (data not shown). Therefore, it is possible that p90/CIP2A may influence other cellular process through the post-translational modification of actin. Furthermore, among the twelve identified AKT substrates, no canonical AKT substrates have been detected, e.g. glycogen synthetize kinase 3β, BAD and Foxo3 [45]. To gain further understanding on this issue, protein chips including all known AKT substrates should be used.

We also analyzed the association between p90/CIP2A and GRP78, to investigate whether p90/CIP2A played a role in ER stress-induced cell apoptosis. It was previously demonstrated that the AKT pathway played a critical role in regulation cell survival under ER stress conditions [78, 79]. GRP78 is essential in protecting cells from apoptosis during ER stress [75]. Here, we found that p90/CIP2A reduced GRP78 accumulation in lung cancer cells and protected cells from ER stress-induced apoptosis. Our results suggest that p90/CIP2A also play a role in lung cancer cell apoptosis, which may be go through GRP78 regulation.

In addition, we also showed that p90/CIP2A regulated the phosphorylation status of mTOR, the target of rapamycin and also affected the sensitivity of cancer cells to the rapamycin treatment; however how this process is actually executed is not determined yet. Resistance to rapamycin of cancer cell is the major concern for the clinical use of this drug to treat cancer [80]. mTOR is the major component of the two complexes, mammalian target of rapamycin complex 1 and mammalian target of rapamycin complex 2 (mTORC1 and mTORC2). mTORC1 is a direct target of AKT while mTORC2 is the upstream kinase of AKT [81]. mTORC1 is composed of mTOR, MLST8 and PRAS40, two of which mTOR and PRAS40 are the targets of AKT. Therefore, p90/CIP2A-mediated rapamycin sensitivity may be dependent on the regulation of mTOR, PRAS40 or both, which need further studies to confirm. In addition, p90/CIP2A also

affects the phosphorylation of the two downstream targets of mTOR (S6K1 and 4EBP1). The activation of these effectors is related to protein synthesis, cell metabolism and cell growth. It's important to explore the roles of p90/CIP2A in regulating these cell activities in our future study.

Taken together, our studies not only establish the correlation between p90/CIP2A and AKT phosphorylation but also find novel functions of p90/CIP2A in promoting the AKT signaling cascade. Although more studies are still needed to increase the resolution for the identification of p90/CIP2A-targeted AKT substrates as well as the role of p90/CIP2A in cell activity, based on our preliminary data, we think p90/CIP2A is a promising target for future drug design since it is an effective regulator to restrain cancer cell growth. The temporal expression of p90/CIP2A will enable researchers to design specific drugs to target p90/CIP2A, which may result in fewer side effects. In addition, restoration of PP2A phosphatase activity is another therapeutic strategy in cancer treatment. Therefore, repressing the expression of p90/CIP2A may be an indirect way to reverse tumorigenic phenotypes and to treat cancer patients more efficiently.

7.2. Proposed Model

Based on our results, we proposed the following models that summarized functions of p90/CIP2A in the AKT/mTOR signaling pathway:

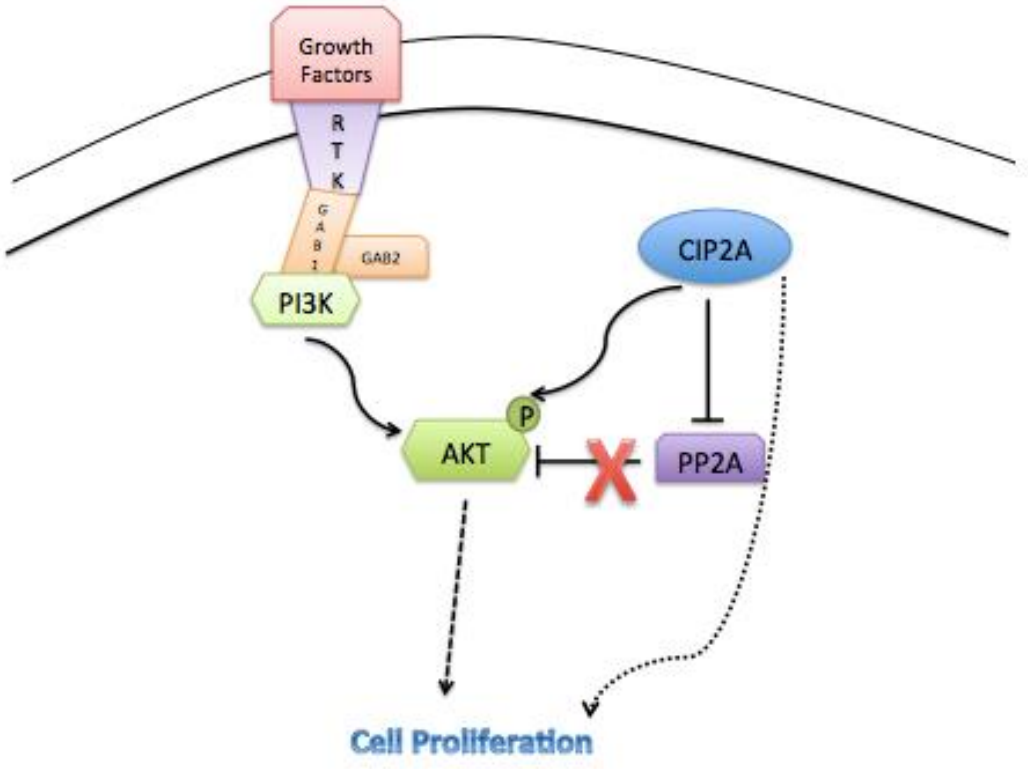


Figure 18 (Proposed Model 1): p90/CIP2A regulates cell proliferation via the AKT signaling pathway. Binding to a ligand (e.g. growth factor) to a receptor tyrosine kinase (RTK) in the cell membrane would active PI3K and cause the phosphorylation of AKT. Phosphorylated AKT regulates various cell activities, including cell proliferation. The expression of p90/CIP2A is found to be associated with AKT phosphorylation and regulate cell proliferation. The effect of p90/CIP2A on cell proliferation might be partly related to AKT regulation. p90/CIP2A is also an inhibitor of PP2A, while PP2A inhibits AKT activation. In the presence of p90/CIP2A, AKT phosphorylation is upregulated by decreasing PP2A associated phosphatase activity.

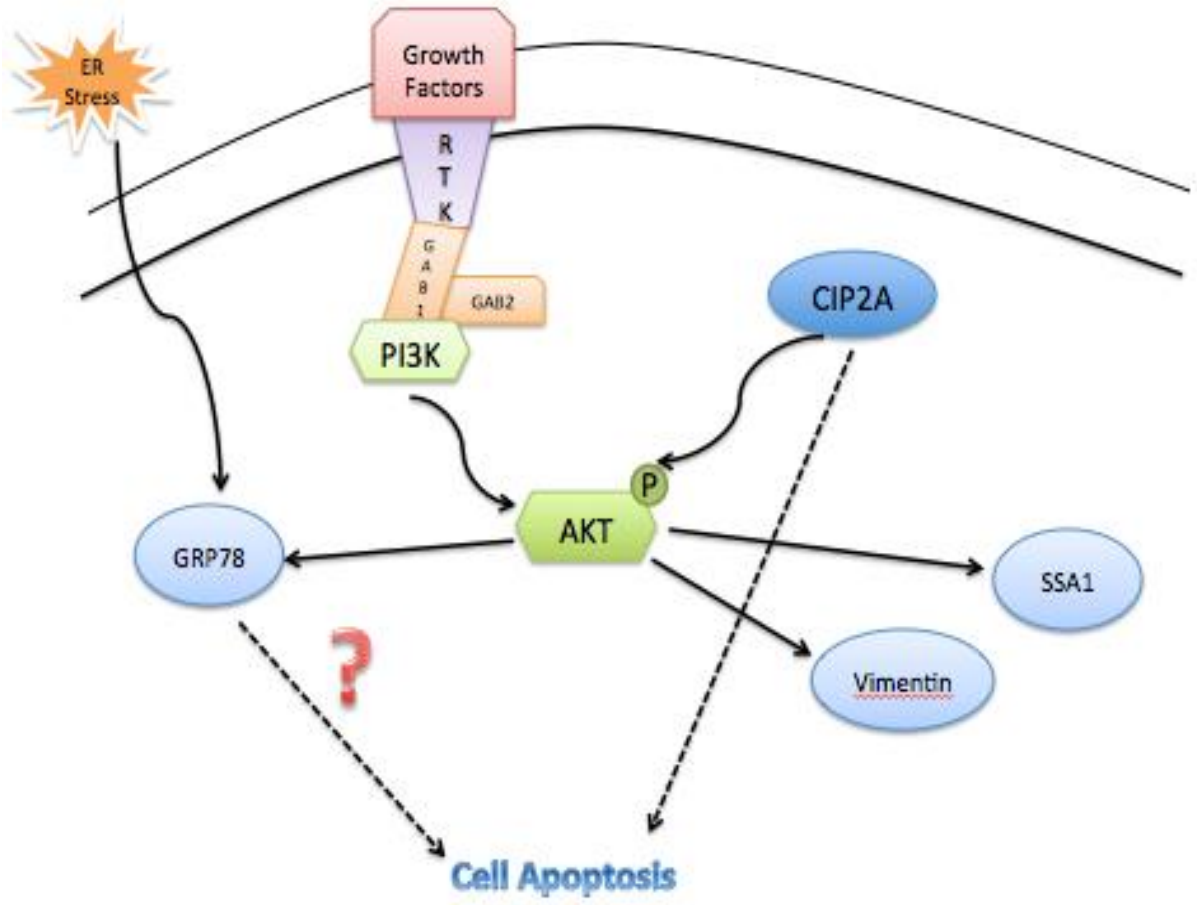


Figure 19 (Proposed Model 2): p90/CIP2A regulates cell apoptosis via the AKT signaling pathway. Upon growth factor stimulation, AKT becomes phosphorylated. AKT exerts its effect by phosphorylating a variety of downstream substrates. p90/CIP2A regulates AKT phosphorylation by targeting specific substrates, such as GRP78, SSA1 and vimentin. GRP78 is an ER stress related protein, leading to cell apoptosis. p90/CIP2A is associated with ER stress induced cell apoptosis and related to the phosphorylation and expression of GRP78.

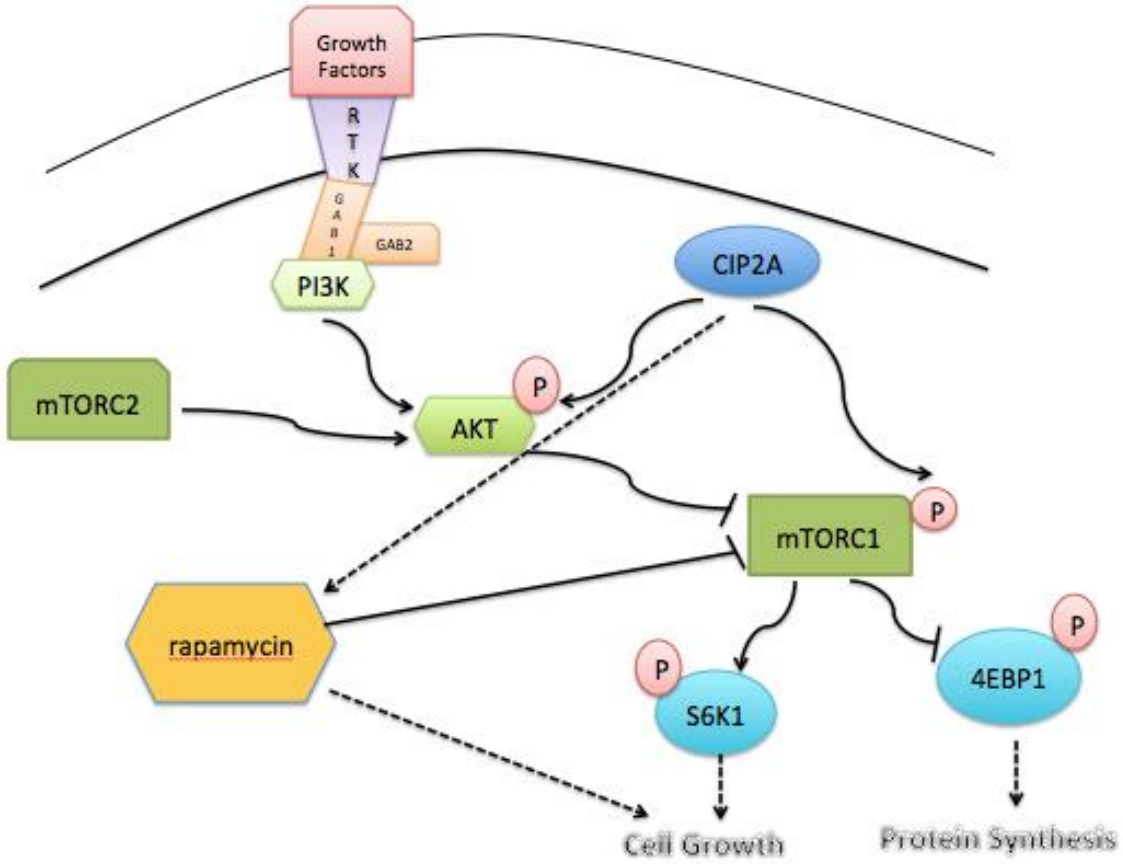


Figure 20 (Proposed Model 3): p90/CIP2A regulates cell growth and protein synthesis via the AKT/mTOR signaling pathway. AKT regulates cell activity in response to growth factor stimulation by phosphorylating a variety of downstream factors, including mTORC. Its downstream effectors further affect cell growth and protein synthesis. Activation of mTOR results in the phosphorylation of ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein (elF4EBP1). p90/CIP2A is related to phosphorylation of mTOR and its downstream effectors. In addition, p90/ CIP2A alters cancer cell response to rapamycin treatment, which might be involved in cancer cell chemoresistance.

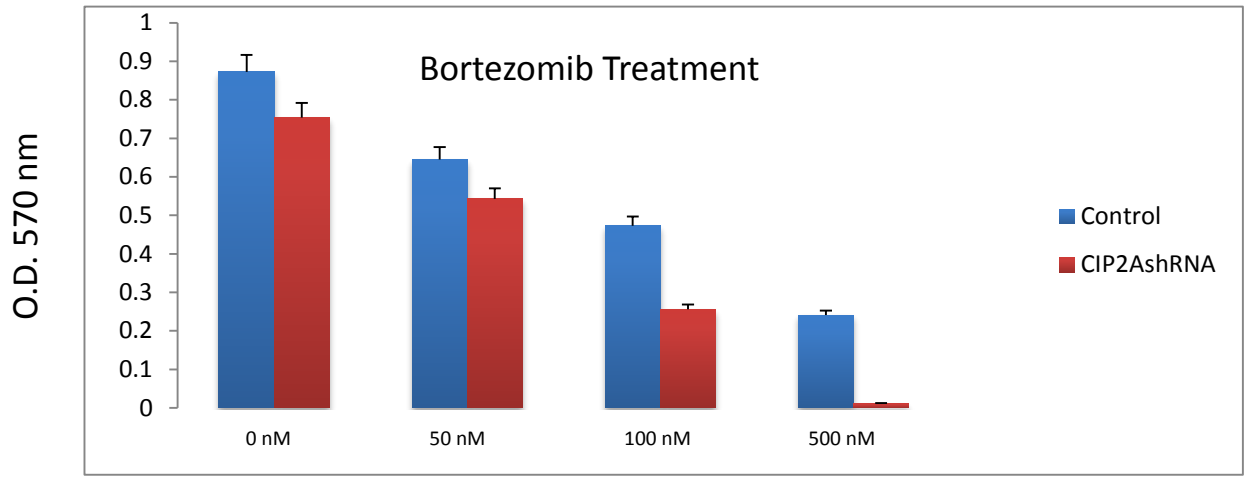
7.3. Future Direction

Functional studies on p90/CIP2A suggest that it plays critical roles in maintaining tumor progression by inhibiting PP2A phosphatase activity. The re-expression of PP2A subunits can reverse the cancer phenotype *in vitro* and thus has clues on the anti-cancer drug design. p90/CIP2A is considered to be a promising target for future drug design in several recent studies since it is an effective regulator to restrain cancer cell growth. The temporal expression of p90/CIP2A will enable researchers to design specific drugs to target this protein, which may result in fewer side effects. In addition, restoration of PP2A phosphatase activity is another therapeutic strategy in cancer treatment. Accordingly, repressing the expression of p90/CIP2A may be an indirect way to reverse tumorigenic phenotypes and to treat cancer patients more efficiently. Thus, p90/CIP2A can be a valuable target for the development of PP2A re-activation drugs. We propose that p90/CIP2A can be a potential biomarker to be incorporated into existing biomarkers for lung cancer diagnosis as well as a promising target for anti-cancer drug development.

Recent studies suggest that p90/CIP2A is able to regulate the phosphorylation of AKT in response to the treatment of chemotherapeutic drugs. AKT acts by integrating different signals from upstream pathways [45], which makes it an attractive anticancer drug target. p90/CIP2A might play important roles in tumorigenesis by regulating cell chemoresistance to anti-cancer drugs. In the future study, we are aiming to investigate how p90/CIP2A facilitates AKT-mediated resistance to chemotherapy. In this case, we could extend the previous finding that p90/CIP2A plays a role in chemoresistance to bortezomib in HCC [59]. And we will also work on the mechanism of how p90/CIP2A facilitates the AKT-mediated chemoresistance by analyzing the phosphorylation of substrates identified previously in our study.

It was previously reported that epidermal growth factor receptor (EGFR) mutations have been associated with sensitivity to several anti-cancer drugs. In addition, EGFR overexpression has been observed in about 40% to 80% of NSCLC patients, thus some anti-EGFR agents have been developed and studied in treating NSCLC [82, 83]. In the future study, we should first examine whether p90/CIP2A is associated with EGFR mutations, in order to further detect response to the combination of EGFR inhibitors with standard chemotherapy in lung cancer patients.

We already assessed the anti-proliferative activity in the panel of both wild type and p90/CIP2A-mutant NSCLC cell lines. Differences in sensitivity to drug treatment between two groups of cells were found (**Figure 21**). To elucidate the role of p90/CIP2A in AKT-mediated resistance to anti-cancer drugs in NSCLC, we will perform a series of experiments including 1) to determine the IC50 of NSCLC cell lines to different anti-tumor drugs (e.g. cisplatin, paclitaxel and docetaxel); 2) to determine whether the knockdown of p90/CIP2A would affect the IC50 to those drugs; 3) to determine whether increased drug sensitivity of the cells depleted with p90/CIP2A can be rescued by the ectopic expression of constitutively active AKT or by a clinically identified PI3K mutant; and 4) to determine whether p90/CIP2A exerts any substrates specificity on AKT-mediated phosphorylation of apoptosis-associated proteins.



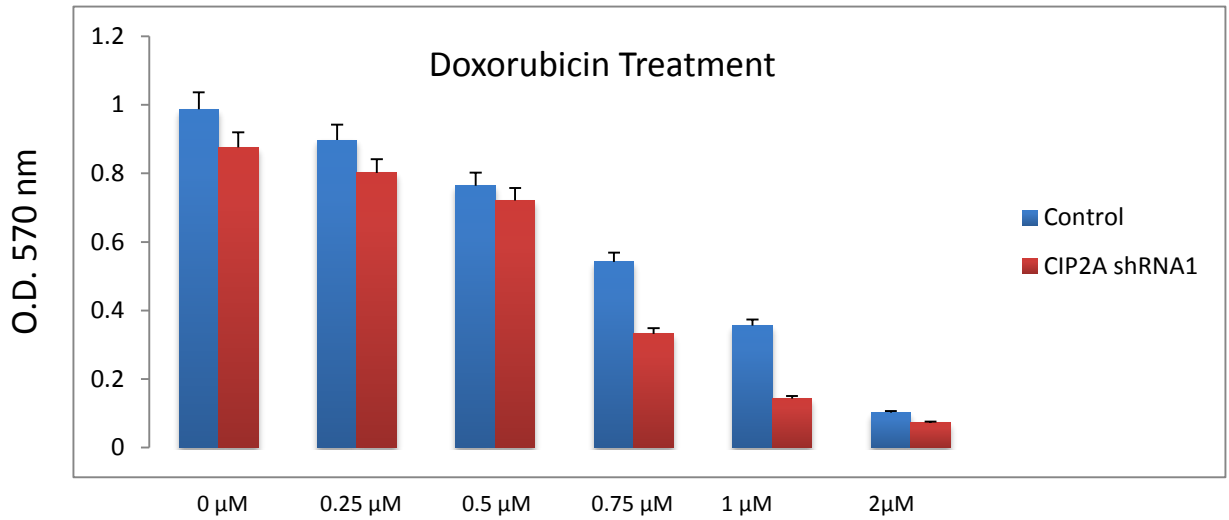


Figure 21: Depletion of p90/CIP2A in H838 cell line sensitizes cancer cell to drug treatment. Equal numbers of cells with either shRNA control or shRNA1 were plated sextuplet in 96-well plates and were treated with bortezomib or doxorubicin with the indicated concentration for 24 hours. Cell survival assay was performed by incubation cell culture with MTT for 4 hours and the absorbance was measured at 570 nm in a colorimetric meter.

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Appendix

List of abbreviations

CIP2A	Cancerous inhibitor of PP2A
PP2A	Protein phosphatase 2A
NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
EGF	Epidermal growth factor
EFGR	Epidermal growth factor receptor
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PTEN	Phosphatase and tensin homolog
mTOR	Mammalian target of rapamycin
MAPK	Mitogen-activated protein kinases
ERK	Extracellular signal-regulated kinases
GSK 3	Glycogen synthase kinase 3
4E-BP1	Eukaryotic initiation factor 4E binding protein 1
GRP 78	Glucose-regulated protein 78
SDS-PAGE	Sodium docecyle sulfate-polyacrlamide gel eletrophoresis
HRP	Hrseradish peroxidase
IP	Immunoprecipitation
MS	Mass spectrophotometer
OD	Optical density
PBS	Phosphate-buffered saline
TG	Thapsigargin
DTT	Dithiothreitol

List of Publications and Manuscripts

1. ***Lei N***, Peng B, Zhang JY. CIP2A regulates cell proliferation in human lung cancer via AKT signaling pathway. Oncology Reports. 2014, Aug; 32:1689-1694
2. Dai L*, ***Lei N****, Liu M, Zhang JY. Autoantibodies to TAA as biomarkers in HCC. Exp Hematol Oncol. 2013 May 20; 2(1): 15. (*First authors)
3. Peng B, ***Lei N***, Chan E, Zhang JY. CIP2A regulates cancer metabolism and CREB phosphorylation in non-small cell lung cancer. Molecular BioSystems. 2014, DOI: 10.1039/C4MB00513A
4. Jacquez P, ***Lei N***, Weigt D, Xiao C, Sun J. Expression and purification of the functional ectodomain of human ATR2. Protein Expr Purif. 2014 Mar; 95: 149-55
5. ***Lei N,*** Peng B, Li J, Zhang JY. CIP2A affects lung cancer cell apoptosis via AKT signaling pathway and targets specific AKT substrates. (Submitted)
6. ***Lei N,*** Peng B, Zhang JY. p90/CIP2A: a cancerous inhibitor of protein phosphatase 2A (PP2A) as a potential biomarker and promising target in cancer chemotherapy. (Ready for submission)
7. Liu X, Peng B, Li Y, ***Lei N***, Li W, Zhang JY. p90/CIP2A mediates breast cancer cell proliferation and apoptosis. Mol Bio Rep. 2014 Aug 3.
8. Ren P, Ye H, Dai L, Liu M, Liu X, Chai Y, Shao Q, Li Y, ***Lei N***, et al. Peroxiredoxin 1 is a tumor-associated antigen in esophageal squamous cell carcinoma. Oncol Rep. 2013 Nov;30(5):2297-303.
9. Shao Q, Ren P, Li Y, Peng B, Dai L, ***Lei N***, et al. Autoantibodies against GRP78 as serological diagnostic biomarkers in HCC. Int J Oncol. 2012 Sep; 41(3): 1061-7.
10. Li J, Dai L, ***Lei N***, Casiano C, Zhang JY. Identification and characterization of Ral A as biomarkers in human prostate cancer. (Ready for submission)

Curriculum Vita

Ningjing Lei was born on July 17th, 1988 in Zhengzhou, Henan Province, China. She received her bachelor degree in the University of Zhengzhou, China in 2010. She was majored in Biotechnology, and she also got a second degree in Management. After graduation, she came to the United States for her doctoral training in the Department of Biological Sciences at the University of Texas at El Paso (UTEP) in August 2010. She has been working at Dr. Jianying Zhang's lab and her research project focuses on functional study of p90/CIP2A in the AKT signaling pathway.

She attended two international meetings organized by American Association for Cancer Research (AACR): 104th AACR Annual Meeting in Washington D.C., 2013 and 105th AACR Annual Meeting in San Diego, 2014. She gave poster presentations on both of these meetings and received travel awards from the Department of Biological Sciences and College of Science at UTEP.

She has published two first-author manuscripts and several co-authors papers. She also has another two fist-author manuscripts in preparation. After graduation, she will apply for a postdoc position to continue the research in cancer study. Her long-term goal is to become an independent researcher to study biological sciences.

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