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Functional Characterization of p90/CIP2A in Human Lung Cancer

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Abstract

p90/CIP2A is a recently characterized oncoprotein which promotes cancer cell proliferation through the inhibition of c-myc-associated PP2A phosphatase activity, from which it derived the name as cancerous inhibitor of PP2A (CIP2A). Although p90/CIP2A has been found to be overexpressed in various cancer types, from solid tumors to hematological malignancies, the function of this protein is still limited to its ability to regulate the stability of c-myc. The recent study showing p90/CIP2A is able to regulate the phosphorylation of protein kinase B (PKB) in response to the treatment of chemotherapy drugs implied p90/CIP2A may have more functions. In order to investigate the function of p90/CIP2A in cancer progression, we examined the expression level of p90/CIP2A in 72 lung cancer tissue specimen and 63 normal human lung tissues by immunohistochemistry, where results demonstrated that p90/CIP2A is significantly overexpressed in lung cancer (84.7% in lung cancer v.s. 11.1% in normal lung tissue). In addition, we also found that p90/CIP2A is overexpressed in primary lung cancer cell lines. Functional analysis of the p90/CIP2A in lung cancer cell lines, either by knock-down or overexpression, confirmed that p90/CIP2A is important for the cell proliferation.

To characterize the function of p90/CIP2A in tumor formation, we used three approaches to interrogate the role of p90/CIP2A in lung cancer. First, we used phosphoproteome array to compare the phosphorylation status of 72 key signaling molecules, and found that c-Jun N-terminal kinase (JNK) was the molecules most significantly altered by the change of p90/CIP2A, which implied a possible target utilized by p90/CIP2A to promote cell proliferation. Second, proteomic approach has been used to identify the proteome change in response to the expression level change of p90/CIP2A. We identified 49 differentially expressed protein spots on 2D-gel in which 47 proteins are identified by mass spectrometry. These proteins belong to different functional groups. A majority of the proteins are associated with metabolism, signal transduction and transcription and translational control. Furthermore, among these 47 differentially expressed proteins, 30 proteins have been well documented to be

associated with cancer, of which 12 proteins are up-regulated and 18 proteins are down-regulated after knock-down of p90/CIP2A. To investigate whether these proteins are associated with any known cancer-related pathway, differentially expressed proteins are searched against the database using Pathway Studio bioinformatic software. The predicted network showed that four possible transcription factors (c-myc, ESR-1, ETS-1 and CREB) may be involved in the regulation of the differentially expressed proteins. Treatment with serum induced less phosphorylation of CREB in cells transduced with p90/CIP2A shRNA than the cells tranduced with control shRNA. However, treatment with KCl and forskolin did not have a significant difference between these two groups. Furthermore, we found that the phosphorylation of the CREB upstream kinase AKT is also down-regulated, while other kinases p38 and p90RSK is unaffected. This preliminary data implied that p90/CIP2A may target AKT-CREB pathway to promote cell proliferation in a c-myc independent manner. At last, we applied gas chromatography-mass spectrometry (GC/MS)-based metabolomic approach to identify the differential metabolites in response to either the loss or overexpression of p90/CIP2A. Seventy-six metabolites were identified, most of which were catergorized to nucleic acid metabolism, carbohydrate metabolism and fatty acid metabolism. By using Significant Analysis of Microarray (SAM) and independent component analysis (ICA), we identified three metabolite: D-glucose, L-proline and 1,2,3-propanoic acid, which can be the biomarkers to differentiate the overexpression of p90/CIP2A to the loss of p90/CIP2A. Furthermore, we found that the depletion of p90/CIP2A would cause the accumulation of glucose inside the cell, while the overexpression would promote glucose utilization. The quantification of glucose in a dynamic way showed that p90/CIP2A does not have an effect on glucose uptake, instead, it regulates the glucose metabolism through the regulation of hexokinase activity. Our data strongly suggested that the role of p90/CIP2A in promoting cell proliferation is not only replied on c-myc, but also in a c-myc-independent manner.

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Chapter 1: General introduction

1.1 Lung cancer

Lung cancer is a type of cancer which usually forms in the result of uncontrolled cell growth in the tissue of the lung. Lung cancer has been recognized as one of the most common causes of cancer-related death in both men and women throughout the world [1]. It is the second leading cancer "killer" in the United States, accounting for more deaths than breast cancer, prostate cancer and colon cancer combined [1]. According to the latest data from American Cancer Society, there would be about 222,520 new cases of lung cancer (both small cell and non-small cell) in the United States in 2010 and about 157, 300 people would die from it the same year [1]. The average lifetime chance that a man will develop lung cancer is 1 in 13. For a woman it is 1 in 16. About 4 out of 10 people with lung cancer will still be living one year after finding out they have lung cancer[1].

According to the histological type, lung cancer can be classified into small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). Compared to NSCLC, SCLC is less common and accounts for 15~20% of all the lung cancer cases. Most of SCLC cases occur in the larger airways including primary and secondary brochi [2]. SCLC cells are fast-growing cells and thus become quite large under microscope [2]. The SCLC cells normally contain dense neurosecretory granules containing hormones like neuroendorcrine, which give this tumor an endocrine/paraneoplastic syndrome association [3]. SCLC is sensitive to the treatment of chemotherapy and radiation, it always have metastasized at presentation and therefore carries a worse prognosis [2] . This type of lung cancer is strongly associated with smoking [4]. The stage of SCLC can be dichotomously divided into limited and extensive stage disease.

The NSCLC has three main types: squamous cell lung carcinoma, adenocarcinoma and large cell lung carcinoma. These three types of cancer are grouped together simply because they behave similarity; share similar prognosis and management [2]. Squamous cell lung carcinoma, which usually starts near a central bronchus, account for 25% of lung cancers [4, 5]. A hollow cavity and associated necrosis are commonly found at the center of the tumor [6]. Well-differentiated squamous cell lung cancers often grow more slowly than other cancer types [7].

Adenocarcinoma accounts for 40% of non-small cell lung cancers [6]. It usually originates in peripheral lung tissue. Most cases of adenocarcinoma are associated with smoking; however, among people who have never smoked ("never-smokers"), adenocarcinoma is the most common form of lung cancer [6]. Lung cancers are highly heterogeneous malignancies were containing more than one subtype is very common [3].

1.2 Pathogenesis of lung cancer

Although the oncogenesis of lung cancer is still poorly understood, like many other cancers, it is believed that lung cancer is initiated by activation of oncogenes and inactivation of tumor suppressor genes [8].

K-ras (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) is a protein with GTPase function that belongs to the Ras gene family. It is an early player for signal transduction by acting as an on/off molecular switch [9]. Upon growth factor activation (e.g. EGF), wild type K-ras can bind to GTP and convert it to GDP through its intrinsic enzyme activity [10, 11]. After that, the K-ras will be turned off and leave the substrate. Unfortunately, the mutation of this gene, for example the K-ras G12D mutant gene, can generate constitutively active form of K-ras, which will keep converting GTP to GDP[11]. Thus, this K-ras mutation results in the over propagation of downstream pathways such as c-Raf and

PI3K pathway leading to tumor growth[9]. The mutations of this gene are found in 10–30% of lung adenocarcinomas [10] .

EGFR (epidermal growth factor receptor) is a cell-surface protein involved in a wide variety of cell processes such as cell proliferation, apoptosis, angiogenesis and cell invasion by binding to the mitogenic factors like EGF [9]. Upon ligand binding, EGFR is activated to phosphorylate down-stream molecules through its tyrosine kinase activity. Like K-ras, mutations and amplification of this gene are also very common in non-small cell lung cancer [10]. The mutation of this gene is always associated with its tyrosine kinase activity. For example, the mutation of T570 and L858 within the kinase domain is commonly found in non small-cell lung cancer patients [11, 12]. The recently developed EGFR inhibitor drug, Gefitinib (Iressa), has been introduced to treat patients who are carrying EGFR mutation [9].

p53, which is encoded by TP53 gene in human genome, is a frequently mutated gene in lung cancer. The deletion of this gene or mutation at DNA binding domain sites is very common in lung cancer [13]. p53 is a tumor surpressor gene and exert its anti-tumor function by regulating the transcription of a variety of pro-apoptotic genes like p16, p21 and p27, which will induce cell growth arrest, cellular senescence or apoptosis [14]. Of notice, some mutant p53, like p53 R175H and R275H, have dominant effects on the wild type p53. They can also promote cell invasion and metastasis [15].

LKB1 (STKE11) is a kinase which is frequently mutated in non small cell lung cancer [16]. This protein was initially identified as an energy homeostasis regulating kinase [17]. The subsequent studies showed that LKB1 exerts its anti-tumor activity by activating AMPK and AMPK-related kinases to inhibit tumor cell growth when energy and nutrients become scarce. Another important function of LKB1 is in cell polarity. LKB1 is important for epithelial cell polarization and prevention of tumor cell expansion, whereas the loss of this protein in cancer cells will lead to unorganized cell structure and

tumor growth [17]. The mutations of this gene are also found in other cancers like cervical cancer, testicular cancer, breast cancer, pancreatic cancer and skin cancer [18].

Other genes that are often mutated or amplified are c-MET, NKX2-1, LKB1, PIK3CA and BRAF [19]. Several genetic polymorphisms are associated with lung cancer. These include polymorphisms in genes coding for interleukin-1, cytochrom P450, pro-apoptotic proteins like caspase-8, and DNA repair molecules such as XRCC1[13, 20, 21 22] (Table 1).

Table 1. Genetic abnormalities specific in the lung to non-small-cell lung cancer and small-cell lung cancer (Herbst R. et. al *NEJM, 2008*)

Abnormality	Non–Small-Cell Lung Cancer		Small-Cell Lung Cancer
	Squamous-Cell Carcinoma	Adenocarcinoma	
Precursor			
Lesion	Known (dysplasia)	Probable (atypical adenomatous hyperplasia)	Possible (neuroendocrine field)†
Genetic change	*p53* mutation	*KRAS* mutation (atypical adenomatous hyperplasia in smokers), *EGFR* kinase domain mutation (in nonsmokers)	Overexpression of c-MET
Cancer			
KRAS mutation	Very rare	10 to 30%‡	Very rare
BRAF mutation	3%	2%	Very rare
EGFR			
Kinase domain mutation	Very rare	10 to 40%‡	Very rare
Amplification§	30%	15%	Very rare
Variant III mutation	5%¶	Very rare	Very rare
HER2			
Kinase domain mutation	Very rare	4%	Very rare
Amplification	2%	6%	Not known
ALK fusion‖	Very rare	7%	Not known
MET			
Mutation	12%	14%	13%
Amplification	21%	20%	Not known
TITF-1 amplification	15%	15%	Very rare
p53 mutation	60 to 70%	50 to 70%‡	75%
LKB1 mutation	19%	34%	Very rare
PIK3CA			
Mutation	2%	2%	Very rare
Amplification	33%	6%	4%

1.3 Protein phosphatase 2A and cancer

Protein phosphatase 2A (PP2A), a large family of heterotrimeric serine-threonine phosphatases, account for the majority of serine-threonine phosphatase activity in eukaryotic cells [23]. The PP2A holoenzyme consists of a structural A subunit, known as PR65 or PPP2R1, a catalytic C subunit, known as PP2Ac or PPP2C and a regulatory B subunits. The A subunit provides a scaffold that allows the the binding of C subunit and B subunit, which regulate the substrate specificity and the localization of the PP2A complex. In mammalian cells, the A subunit and C subunits are encoded by two distinct genes which produce two isoforms for each unit, Aα and Aβ and Cα and Cβ, respectively. So far, four families of B subunits have been reported: B/B55/PR55/PPP2R2 [24-27], B′/B56/PR61/PPP2R5 [28-30], B″/PR72/PPP2R3 [31-34] and B‴/PR93/SG2NA/PR110/Striatin [35] (Fig. 2).

The tumor suppressor activity of PP2A was firstly resulted from the discovery of okadaic acid, a selective but not specific PP2A inhibitor (inhibit PP2A at picomolar concentration and inhibit both of PP2A and PP1 at higher concentration), which can promote tumor formation in mouse model [36]. Therefore, it is believed that inhibition of PP2A is critical for human cell transformation which validated by the fact that small T antigens from two high risk viruses, SV40 and polyoma can target PP2A and this is essential for the cell transformation. When the mutations were introduced to abolish the interaction between ST and PP2A, the normal cell cannot be transformed [36].

Since the PP2A holoenzyme is a combination of A, B and C subunits, recent studies have been focusing on how the different subunits contribute to tumor suppression. A pioneering study by knocking-down of the B56 family, Chen et al. have developed an assay system by using the non-tumorigenic HEK-TRV cell line which has been immortalized with hTERT, large T antigen and H-ras. The advantage of this system is based on the fact that the inhibition of PP2A in this cell line can result in cell transformation. They found that the down-regulation of B56 γ in this cell line led to cell transformation, which is

mimicking the expression of small T- antigen [37]. Although A alpha and A beta have low frequency of somatic mutation in cancers, the loss of either of these two genes will enhance the cell transformation [36-38]. The mutations of these two genes can abolish the PP2A complex due to the inability to form the A-C heterodimer [39]. Although the mechanism of how PP2A exerts its tumor suppressor function is poorly understood, the A alpha subunits can form complex with B56 gamma to control the phosphorylation of AKT [36, 40, 41]. The depletion of either A alpha or B56 gamma will induce the hyperphosphorylation of AKT, indicating the necessary role of active AKT in cell transformation [40, 41]. In addition, the PP2A A alpha-B56 gamma complex can also regulate the stability of p53, another important tumor suppressor [42]. MDM2 is an E3 ligase for p53 by phosphorylating p53 at Thr55, which is a signal for proteasome-mediated protein degradation. The dephosphorylation of p53 at Thr55 by PP2A is essential for its anti-tumor activity [42]. The mutation of A beta subunits was recently found to exert its tumor suppressor function by regulating the phosphorylation of Ral A which is an important oncoprotein at the downstream molecule of the Ras signaling pathway [43]. The mutation of A beta was found to be missing in lung cancer specimen and several lung cancer cell lines [37]. The introduction of mutant A beta into immortalized cell line will result in cell transformation. The C subunits do not have tumor suppressor activity [44]. The B56 family subunits can regulate the stability of beta-catenin and B56 alpha can regulate the stability of c-myc by dephosphorylating c-myc at Serine 62, which is phosphorylated by ERK and is essential for its stability[45][46]. The desphorylation at this site will result in the subsequent phosphorylation of c-myc by GSK-3β [46]. These two events will result in the proteosome-mediate c-myc degradation [47].

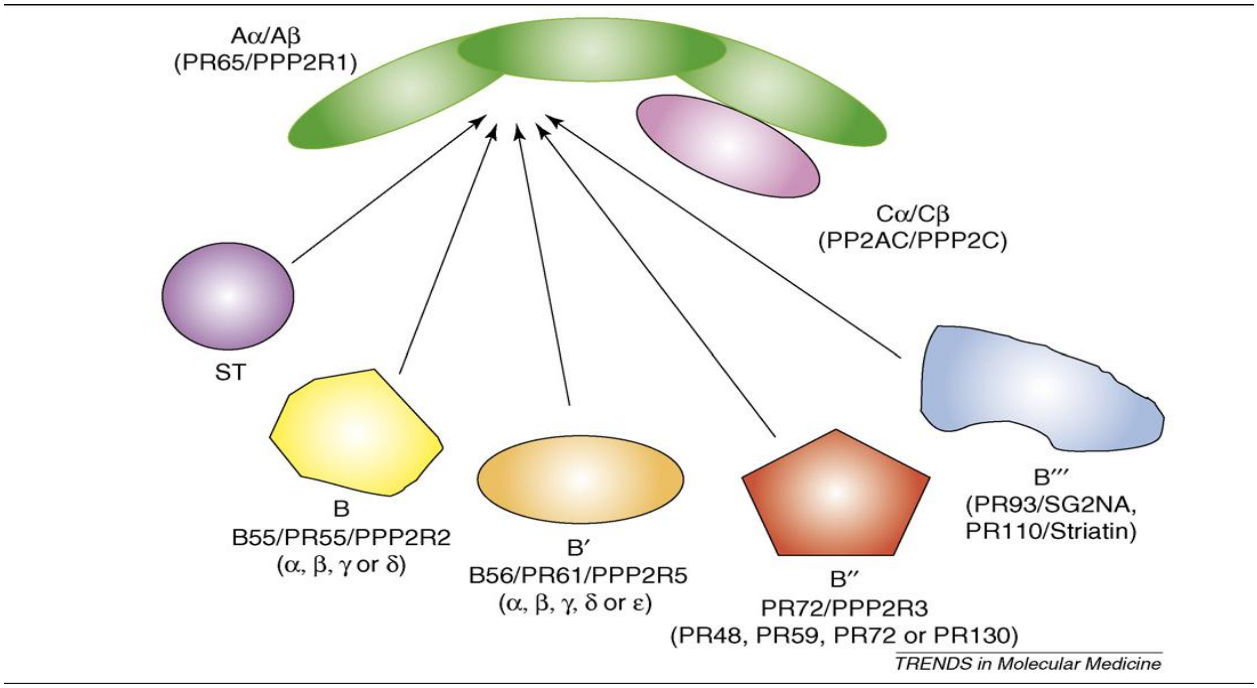


Figure 1. Structure of PP2A holoenzyme. PP2A is a heterotrimeric complex consisting of a scanffold subunit (Aα or Aβ), catalytic subunit (Cα or Cβ) and a regulatory subunit (B, B', B'' or B'''), which regulate the substrate specificity and subcellular localization. (Westermarck J & Hahn WC. *Trends Mol Med* ,2008)

1.4 PP2A inhibitors

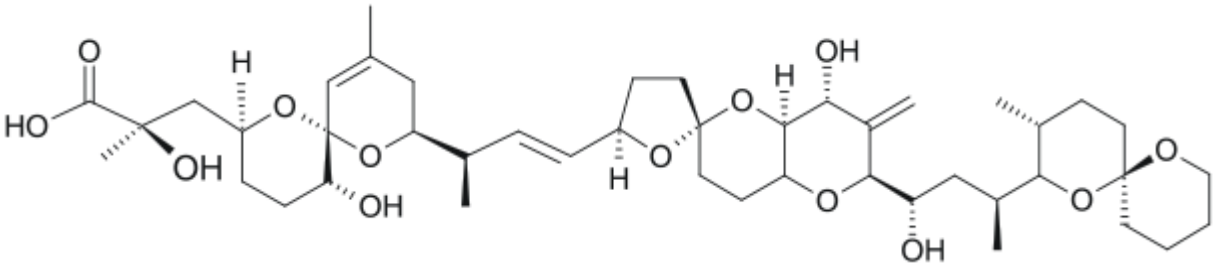
Okadaic acid is the first chemical shown to inhibit PP2A phosphatase activity [36] (Fig. 2A). The essential role of oncoprotein, small T antigen of SV40 virus and polyma virus, in cell transformation is heavily replied on its ability to inhibit PP2A phosphatase activity [48]. Small T antigen inhibits PP2A activity by replacing B subunits and preventing the formation of active PP2A holoenzyme [48]. Unfortunately, no endogenous PP2A inhibitor was identified until the discovery of I1PP2 and I2PP2A from bovine kidney cell lysates [49]. These two inhibitors are heat-stable and inhibit PP2A phosphatase in a non competitive manner [49]. Although the function of both of I1PP2A and I2PP2A is not fully understood, the overexpression of I2PP2A/SET has been observed in several different tumor types [36]. As one of the cancer-related pathways, mitogen activated protein kinase (MAPK) are important targets for PP2A. The inhibition of PP2A by okadaic acid or ectopic expression of small T antigen can promote the activation of MAPK pathway through phorsphorylation regulation. For example, the overexpression of small T antigen or I2PP2A can induce the phosphoryaltion of c-Jun at S63 and T73, which mediate the transcriptional activity of oncoprotein AP-1 [50-52]. However, the question that remains to be answered, is whether I2PP2A can directly or indirectly regulate these phosphorylation events. Besides that, the overexpression of I2PP2A in Hela cell can inhibit death-receptor-mediated apoptosis through activation of MEK-ERK-MAPK pathway [53].

In addition to its function on PP2A phosphatase activity inhibition, I2PP2A/SET can also form fusion proteins with Nup214, and SET-Nup214, which are overexpressed in chronic myelogenous leukemia (CML) [54]. In this study, it is also found that the overexpression of this fusion protein is essential in CML for the oncogenic activity of BCR-ABL kinase. Downregulation of this fusion protein via sequence-specific siRNA can reduce the stability of BCR-ABL kinase by promoting its proteolytic degradation. In addition to that, several signaling pathways involved in CML pathogenesis got interrupted. Interestingly, the overexpression of I2PP2A/SET can also be induced by the fusion protein

BCR-ABL kinase since the inhibition of BCR-ABL by Imatinib can markedly inhibit I2PP2A/SET expression [36, 54] (Fig 2B).

A recent study showed that the re-activation of PP2A by forced expression of PP2A C subunit or by Imatinib can result in the inhibition of c-myc expression by dephosphorylation of STAT5, ERK, AKT, BAD, Jak2 and pRB. It has been shown that the proteolytic degradation of BCR-ABL induced by the loss of I2PP2A/SET was highly dependent on tyrosine phosphatase SHP-1. Although the underlying mechanism for the SHP-1 inactivation by overexpression of I2PP2A/SET remains unknown, it is possible that the PP2A may be able to directly regulate the phosphorylation status of SHP-1. The overexpression of I2PP2A/SET can greatly increase the colony-forming growth in soft agar and cell proliferation. These resuls showed that I2PP2A/SET can promote CML tumorigenesis by increasing the stability of the oncogene BCR-ABL through inhibition of PP2A phosphatase activity [54].

A.



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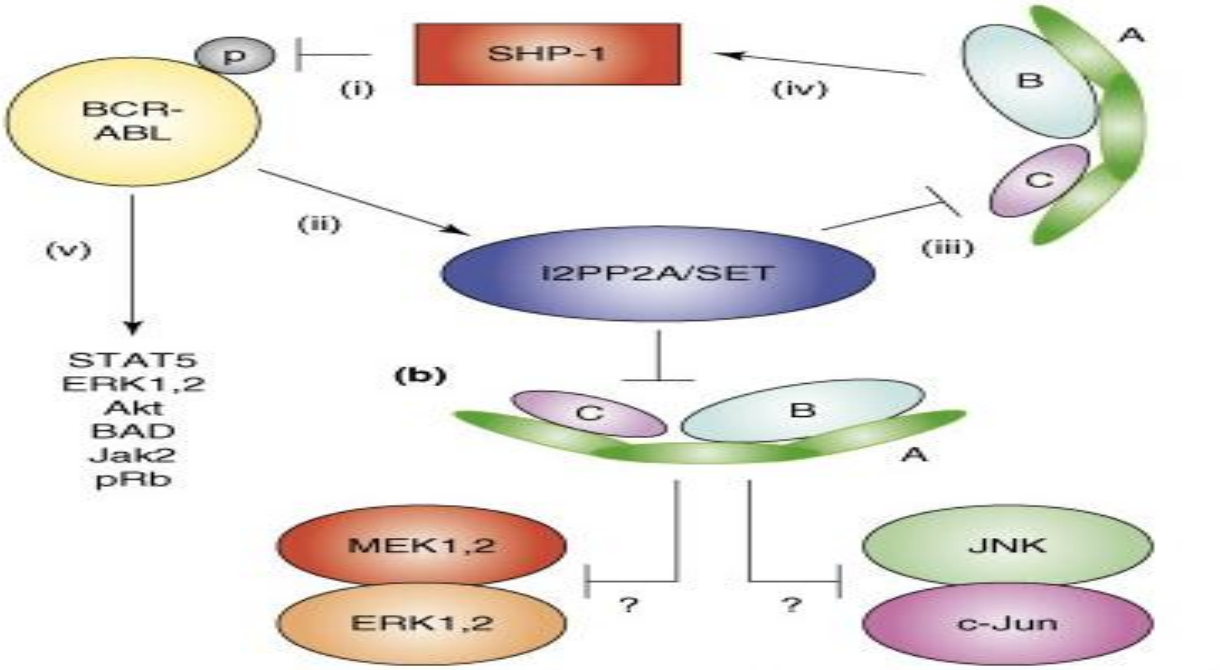


Figure 2. (A) Chemical structure of PP1 and PP2A inhibitor (Wikipeida, the free encyclopedia). (B) Signaling pathway targeted by PP2A inhibitor I2PP2A. Oncoprotein BCR-Abl is negatively regulated by SHP-1 phosphatase, which is positively regulated PP2A phosphatase. The BCR-Abl can stimulate the expression of I2PP2A/SET, which in turn inhibit PP2A phosphatase activity and thus increase the BCR-abl activity. Although I2PP2A can increase the phosphorylation of MEK1/2 and JNK-c-Jun, the direct targets of I2PP2A are unknown. (Westermarck J & Hahn WC. *Trends Mol Med* ,2008) .

1.5 90-kD tumor associated autoantigen or cancerous inhibitor of PP2A (p90/CIP2A)

c-myc is an important transcriptional factor in both normal cell and malignant cell growth [55]. This protein is subject to tight regulation through several different mechanisms of both transcriptional and post-transcriptional [55]. Although the overexpression of this protein in tumors always resulted from gene amplification or translocation, some evidence showed the mRNA level is not correlated with protein level, which implied the post-transcriptional level is also of great importantce in regulating its stability [56-60]. In fact, the c-myc protein has a half-life with only 30 mins under normal conditions [61]. The stability of this protein is regulated by two phosphorylation events [47]. The phosphorylation of Serine 62 by ERK can increase its stability and function. The dephosphorylation at this site by PP2A will result in the phosphorylation at T58 by GSK 3beta, which is a critical step for c-myc proteolytic degradation. The mutation of Serine 62 to Alanine will result in the instability of c-myc, but the mutation of T58 to A will result in the accumulation of c-myc and can enhance cell transformation [47].

p90/CIP2A is a novel PP2A inhibitor which can extend the half life of c-myc [62] (Fig. 3). This protein was initially identified as a tumor-associated autoantigen during the screening of biomarker in gastric cancer. This protein was originally named p90 autoantigen due to its migration band at approximately 90kD on SDS-PAGE gel [63]. The function of this protein at that time was unknown. Recently, this protein was found to be co-precipitated with PP2A A alpha subunit. The global microarray analysis revealed it can regulate the stability of the oncoprotein c-myc. The underlying mechanism of this regulation is based on the ability of p90/CIP2A to inhibit the phosphatase activity of PP2A. Therefore, they proposed to call cancerous inhibitor of PP2A (CIP2A). The knock-down of this protein in Hela cell result in decreased cell proliferation, clonogenic ability and anchorage-independent growth. The injection of the cells with depleted p90/CIP2A into nude mice will decrease the in vivo tumor formation. This protein can also substitute ST for cell transformation in the cell line which was immortalized by hTRET, LT and H-ras [62].

Later, several other groups confirmed the importance of p90/CIP2A in tumor formation in different cancer types like breast cancer, prostate cancer, gastric cancer, non small cell lung cancer, oral carcinoma, esophageal squamous cell carcinoma and chronic myeloid leukemia [64-70]. In addition, two groups also showed that p90/CIP2A can confer drug-resistance by regulating the phosphorylation of AKT in hepatocellular carcinoma and breast cancer [71, 72]. However, how this protein contributes to cancer progression besides its role in regulating the stability of c-myc is still poorly understood.

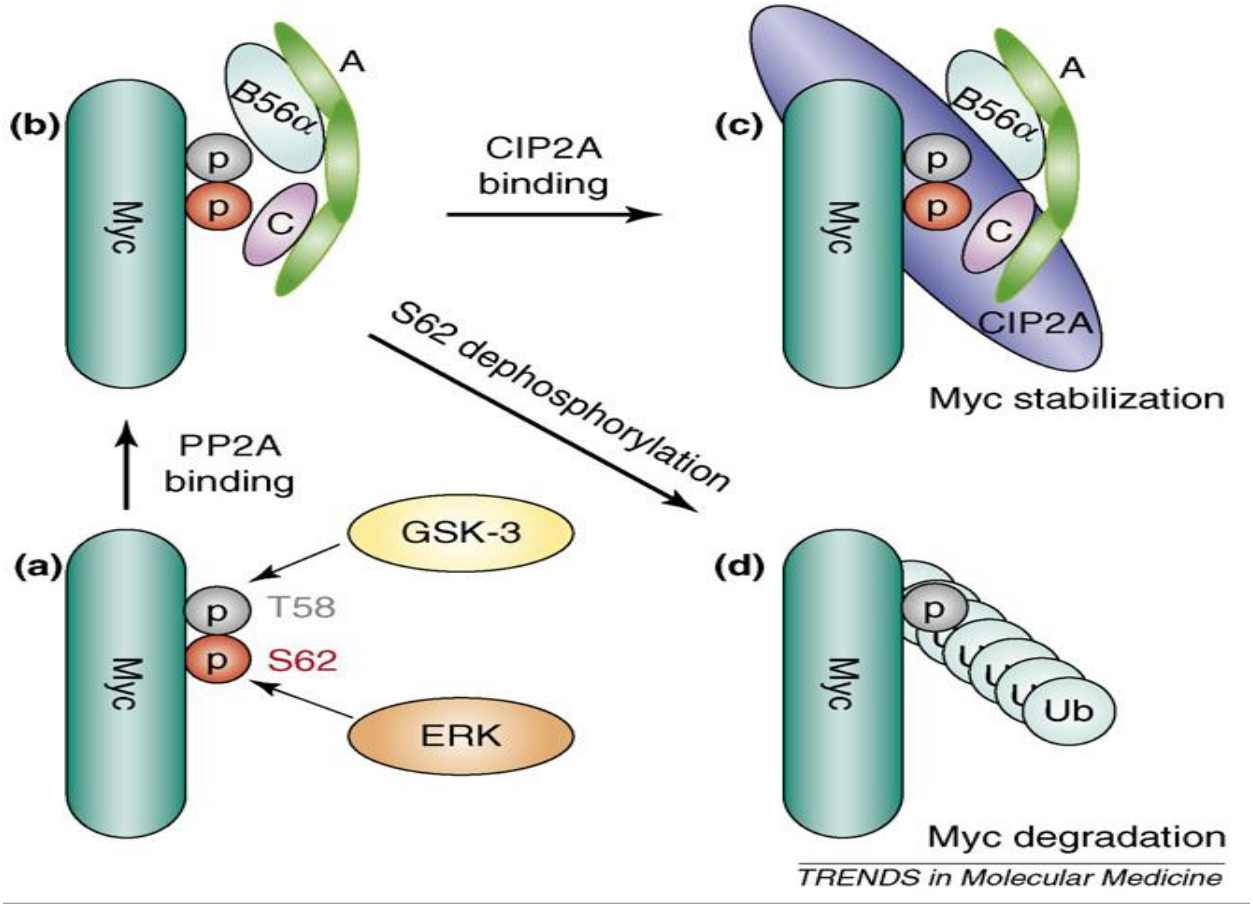


Figure 3. Proposed mechanism of p90/CIP2A-meidated c-myc stability. C-myc stability is regulated by dephosphorylation of S62 by PP2A and phosphorylation of T58 by GSK3β. The synergism of PP2A and GSK3β can lead to the proteasome degreadation of c-myc. However, p90/CIP2A is able to recognize the motif including the S62, which shield the dephosphorylation by PP2A, and extend the stability of c-myc. (Westermarck J & Hahn WC. *Trends Mol Med* ,2008)

1.6 Other endogenous PP2A inhibitor proteins

In addition to I1PP2A, I2PP2A/SET and p90/CIP2A, some other endogenous proteins inhibiting PP2A activity in cancer cells have been identified. PP2A C methylesterase protein phosphatase methylesterase-1 (PME-1) is able to demthylate PP2A catalytic domain at its C-terminal leucine 307 by binding to the inactive form of catalytic domain of PP2A [73, 74]. Another endogenous PP2A inhibitor is type 2A-interacting protein (TIP), which was shown to be expressed in human cancer cells and to interact with the PP2A holoenzyme in HEK-293 cells [75]. An *in vitro* study showed that this protein can directly inhibit PP2A phosphatase activity. TIP depletion resulted in a significant decrease in the phosphorylation of newly identified 32 kD target protein substrate of ataxia-telangiectasia mutated (ATM)/ATM- and Rad3-related (ATR) kinases [75].

1.7 Metabolism and cancer

Cancer is a type of diseases with uncontrolled cell growth, which lost the checkpoint of cell proliferation of normal cells [76]. To support the rapid cell proliferation, the cancer cells must make fundamental changes in order to meet the requirement for increased nutrient uptake and energy production. The link between metabolism and cancer was initially observed by a German physiologist, Otto Warburg. In the early 1920s, he proposed that the malignant growth of cancer cells was caused by energy production through non-oxidative breaking down of glucose to produce NADH, even in the presence of ample oxygen, which is known as glycolysis. In contrast to cancerous cells, normal cells promote energy production through mitochondrial oxidation, which breaks down pyruvate, the end product of glycosis [77]. The distinct ways used by glucose to produce energy between cancer cells and normal cells lead to the development of positron emission tomography (18-FDG PET), which monitors malignant growth by measuring glucose uptake [78].

Otto Warburg theorized that mitochondria deficiency was the primary cause of cancer. Yet, the understanding of cancer genetic lesions like oncogene activation or inactivation of tumor suppressors opposed the idea that genetic change reprogrammed the metabolic pathway to favor glycolysis rather than oxidative phosphorylation in the mitochondria, since the mitochondria remains normal in many cancer cells [79, 80]. Glucose is not only the substrates needed for energy production, but also the major carbon source for the synthesis of macromolecules; thus a critical nutrient in anabolic metabolism. Compared to mitochondrial oxidative phosphorylation, which gives rise to 36 ATP/ mol glucose, glycoslysis can only generate as low as 2 ATP/ mol glucose. This inefficient production of energy seems paradoxical to the high demands of energy in cancer cells [81]. Glycoslysis actually has at least two advantages over oxidative phosphorylation. First, glycolysis can produce ATP faster than oxidative phosphorylation when glucose is present in excess [78]. Up-regulation of glucose transporter 1 (Glu1) has been found in many different types of cancer. The increased import of glucose into cancer cells is thus providing sufficient nutrients for anabolism process. Another important feature of glycolysis are the intermediate products that are important for cell growth. For example, glucose can be metabolized in either oxidative or non-oxidative way of pentose phosphate pathway (PPP). The intermediate product, ribose-5-phosphate, is an important molecule for nucleotide synthesis. NADH can also be produced by PPP pathway and used for nucleotide and fatty acid biosynthesis [82-84].

The reprogramming of metabolic signaling networks is considered as a hallmark of cancer [85]. More and more evidence has shown that almost every oncogene has downstream effectors to be associated with cancer metabolism. Frequently mutated oncogens like c-myc, Ras, Src and AKT can stimulate the anabolism in transformed cells and thus constitute major metabolic pathways in cancer cells [79]. C-myc is a proto-oncogens regulating nearly 15% of genes in the human genome [86]. C-myc is activated by growth factors and modulates cell proliferation and cell-cycle process in normal cells. However, deregulated c-myc expression due to genetic amplification is frequently observed in cancer [87], which

resulted in the over-expression of its regulated genes. Many of the c-myc targeted genes are metabolism genes, such as LDH-A, which is an enzyme that converts pyruvate into lactate and recovers the NAD+ to maintain glycolysis to support the tumor growth [88]. Recent evidence has shown that c-myc promotes cell proliferation by enhancing glycolysis, but also participates in glutamine metabolism, known as glutaminolysis [89, 90]. Another important molecules is phosphotydilinositol 3 kinase (PI3K), which is a lipid kinase, that phosphorylates the 3 hydroxyl group of inositol and thus regulate the level of phosphorylated phosphotydilinnositol (PIP3) at the plasma membrane in response to growth factor stimulation [91-93]. Activation of PI3K will lead to the activation of downstream molecule AKT, which plays central roles in cell proliferation and cell growth by phosphorylating a wide variety of substrates. AKT activation can stimulate cell growth by increasing enzymatic activity of glycolytic enzymes like hexokinases and phosphofructose [94, 95]. AKT can also increase gene expression related to nutrient transporters like amino acid transporter and glucose transporter [96]. In addition, AKT can also regulate other proliferation promoting molecules like mammalian target of rapamycin (mTOR).

1.8 Project hypothesis and specific aims

Our hypothesis is that overexpression of p90/CIP2A in lung cancer cell could promote cell proliferation by different ways, simply classified as c-myc-dependent and c-myc-independent. To find the mechanism of the p90/CIP2A-associated cell proliferation, we have the following specific aims:

Specific aim 1: Evaluate the expression of p90/CIP2A in lung cancer specimen and lung cancer cell lines.

In this specific aim, we will use immunohistochemistry (IHC) and western-blot to analyze the expression level of p90/CIP2A in tumor tissue array and primary lung cancer cell lines. Results obtained from this specific aim could allow us to know whether p90/CIP2A is associated with lung cancer formation.

Specific aim 2: Functional analysis of p90/CIP2A-induced proteome change.

In this specific aim, we intend to use proteomic approach to identify differentially expressed protein between the wild-type cancer cell line and the p90/CIP2A-deficient cells. The loss of certain proteins may result in the change of the expression of its regulated proteins, which can be detected by proteomic approach. Further analysis of these proteins through bioinformatic tools may uncover novel functions.

Specific aim 3: Identification of novel p90/CIP2A targets by phosphoproteomics.

In this specific aim, we will adopt phosphoproteomic approach to identify novel targets of p90/CIP2A by examining the phosphorylation of key signaling molecules when p90/CIP2A is overexpressed. Identified targets will be reconfirmed with phosphorylation-specific antibody. This information will help us to establish the link between p90/CIP2A with other known signaling molecules and thus have implication for novel function.

Specific aim 4: Analysis of p90/CIP2A in cancer metabolism by metabolomic analysis.

Serving as an oncoprotein in human cancer, p90/CIP2A significantly increases cell proliferation, a phenotype linked cancer metabolism. By systematic profiling of the metabolites in the context of

depleted or overexpressed p90/CIP2A, we will be able to identify p90/CIP2A-associated metabolic pathway.

1.9 Significance

Understanding the underlying mechanisms of tumorigenesis and cancer progression is the key to develop efficient drug targets for cancer treatments. Genetic lesions have been considered as the key players during cellular transformation and cancer progression. However, directly targeting these molecules is sometimes disappointed in practice due to the complicated inter-relationship between cancer cells and their microenvironment. Thus, the choice of the optimal drug targets is of great importance. We found that p90/CIP2A can be a potential biomarker for cancer detection and is a possible drug target based on the following reasons. First, p90/CIP2A is overexpressed in lung cancer tissue while only a few cases of normal human tissue are p90/CIP2A positive. In addition, the high frequency of autoantibody against p90/CIP2A in lung cancer patients makes this protein as a potential serological biomarker for lung cancer detection. Second, p90/CIP2A is tightly regulated by development. The expression of this protein is silenced in normal adult tissues and thus targeting this protein may have less off-targets. At last, the complexity of cancer makes it difficult to cure by targeting individual molecules. The data presented in this dissertation can provide the researcher the rationale for the role of how p90/CIP2A affects the signaling in cancer cells, which may be important for combinatorial use of drugs to treat cancer. In addition, the inhibition of p90/CIP2A can restore the PP2A phosphatase activity which leads to the impediment of cancer cell growth.

Chapter 2: p90/CIP2A is a tumor-associated autoantigen in human lung cancer

2.1 Introduction

Lung cancer causes most cancer-related death both in men and women throughout the world. Based on the size of cancer cells examined under microscopy, lung cancer can be classified to small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC), which have different cell origins, genetic lesions and therefore respond differently to treatments [97]. However, NSCLC is more common than SCLC, which account for 85% cases of lung cancer diagnosed. The five year survival rate of lung cancer is as low as 15% [98]. Therefore, early detection of this disease allows the patients to receive appropriate treatment with more options and less invasive surgery, which leads to the increased five-year survival rate [98]. Unfortunately, one challenging issue for the early detection of lung cancer is the disease-associated symptoms, which can be hardly felt by the patients at the early stage. Therefore, lung cancer is usually diagnosed at late stage when the cancer cells are distantly localized. Therefore, the development of reliable biomarkers for early detection of lung cancer is a novel field since there is no currently approved screening test for lung cancer detection [98].

Blood proteins have always been attractive targets for biomarker identification due to their change of expression with the progression of diseases [99, 100] . Studies of autoimmune diseases have provided abundant evidence suggesting that autoantibodies are antigen-driven responses that can be viewed as reporters from the immune system, which reveal the identity of antigens that might be playing roles in the pathophysiology of the disease process [100, 101]. The highly specific autoantibody response in systemic autoimmune disease generally predicts the biologic phenotype of the disease, making autoantibodies clinically valuable and diagnostically useful. Of notice, most of the autoantigens targeted by host's immune system were identified as the proteins which play important roles in cell transformation and cancer progression like mutant p53, K-ras, insulin-growth factor II mRNA binding protein family (IMPs), c-myc, p90/CIP2A, p16, p21, cyclin A, B and E [101, 102]. Therefore the

identification of tumor-associated autoantigen is a way of understanding the antigens that are recognized by the host's immune system during cellular transformation and cancer progression. It is also a way to identify the proteins that are aberrantly regulated during the transformation of normal cell to cancerous cell.

p90/CIP2A is a tumor-associated autoantigen that was initially identified in gastric patients which has unknown functions due to it lacks the homology to any other proteins [63]. It was later shown that this protein is overexpressed in many different types of cancer and promotes cancer cell proliferation by inhibiting c-myc-associated protein phosphatase 2A (PP2A) phosphatase activity, which resulted in the extended half-life of c-myc oncoprotein [62]. Furthermore, the analysis of other known PP2A substrates like MEK (mitogen-activated protein kinase-kinase) and MDM2 were unaffected, which implied the target selectivity of p90/CIP2A in cancer cells [62]. In addition, the recent studies showed that p90/CIP2A is able to regulate the phosphorylation of another substrate: protein kinase B (PKB/AKT). p90/CIP2A is positively regulating AKT phosphorylation in hepatocellular carcinoma and confer cancer cell with the resistance to bortezomib, an anti-cancer drug [71, 103]. However, whether p90/CIP2A has other functions is still poorly understood.

In search of novel tumor-associated autoantigen in lung cancer patients, we established if p90/CIP2A can also elicit autoimmune response in lung cancer patients. Further analysis demonstrated that p90/CIP2A is highly expressed in lung cancer specimen but with very low expression in normal human lung tissue. In addition, p90/CIP2A is also expressed in different lung cancer cell lines. These data again validated the possibility of using p90/CIP2A as a biomarker for early cancer detection. In addition, the functional analysis of p90/CIP2A in lung cancer cell lines showed the participation of p90/CIP2A in cell proliferation through a novel mechanism, which regulated the JNK-c-Jun MAPK pathway.

2.2 Materials and methods

Cell culture. The non-small cell lung cancer cell lines, A549, NCI-H838, NCI-H1299 and NCI-H460 and two small cell lung cancer cell lines, H69 and H146 were purchased from Amercian tissue collection center (ATCC). All of the cell lines, except A549, were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) (Invitrogen) at 37^{o}C with 5% CO_2. A549 is cultured with the same conditions as for other cell lines, except that it is cultured in F12K medium (Invitrogen).

Serum sample. In this study, sera from 207 patients with lung cancer and 82 normal human sera were obtained from the serum bank in the investigator's (Zhang) laboratory at The University of Texas at El Paso (UTEP), which were originally provided by a collaborator in Sun Yat-sen University, Guangzhou, China. Normal human sera were collected from adult individuals who had no obvious evidence of hepatic diseases during annual health examinations. Histopathological staging of liver fibrosis was defined using METAVIR scoring system. All lung cancer patients were diagnosed according to the criteria described in a previous study [104]. This study was approved by the Institutional Review Board of The University of Texas at El Paso and collaborating institutions.

ELISA. Recombinant GST-p90/CIP2A fusion protein was purified using GST glutathione beads. The protein purity was >95% by SDS-PAGE. Proteins were diluted in PBS to a final concentration of 0.5 μg/mL for coating polystyrene 96-well microtiter plates (Dynatech Laboratories, Alexandria, VA). A volume of 200 μL of each human serum samples at 1:200 dilutions was added to the antigen-coated wells and incubated for 1.5 h at RT. Horseradish peroxidase-conjugated goat antihuman IgG (Caltag Laboratories, San Francisco, CA) at 1:5,000 dilution and the substrate 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (Boehringer Mannheim GmbH, Mannheim, Germany) were used as

detecting reagents. Each sample was tested in duplicate, and the average OD at 405 nm was used for data analysis. The cutoff value designating positive reaction was the mean optical density (OD) of 82 normal human sera plus 3 standard deviations (SD). The detailed protocol of ELISA was used as described.

Tumor tissue array, H&E staining and immunohistochemistry (IHC). Formalin fixed and paraffin embedded Lung cancer tissue arrays and normal human lung tissue was obtained from *Cybrdi* (Rockville, MD). The lung cancer tissue array contained 71 individual cases and 72 cores. Each of the cores represents one donor. The array contained normal human lung tissues from 23 individuals, spotted in triplicates. For both of these two tissue slides, each array spot was 1.5mm in diameter and 5 μm in thickness. All the slides were stained with H&E staining and verified by pathologists. IHC was performed according to manufacturer's protocol. Tumor tissue arrays were deparaffinized in Xylene, followed by rehydration in alcohol and then boiled in citrate buffer for antigen retrieval. Before antibody incubation, tissue array slides were incubated with hydrogen peroxide to block any endogenous peroxide activity and normal mouse antibody to reduce the nonspecific binding. The mouse monoclonal p90/CIP2A antibody was added to the slides at a concentration of 1:50 and incubated at room temperature for 1 hr, and goat anti-mouse HRP was used as secondary antibody. After washing, the peroxides reaction was developed with 3,3'-diaminobenzidine (DAB). At last, the slides were covered with cover slides mounted with mounting media.

Both of the slides were examined by two independent, blinded investigators. Five views were examined per slide, and 100 cells were observed per view at 400× magnification. IHC staining of p90/CIP2A was scored by following a semi quantitative scale (− to +++), which evaluated the intensity in tumor areas, and the percentage of cells showing significantly higher immunostaining than control cells in normal lung tissues. Nuclear and/or cytoplasmic immunostaining in tumor cells was

considered positive staining. The intensity of CIP2A staining was scored as 1 (mild), 2 (weak), and 3 (strong). Percentage scores were assigned as 0, 0%; 1, 1–50% and 2, 50–100%. The scores of each tumor sample were multiplied to give a final score of 0–6, and the tumors were finally determined as lower expression, score 0-3 and high expression, score 4-6.

Western-blot analysis. Cells were plated in 6-well tissue culture plates at 80% confluency and incubated overnight. Cell lysates were obtained from transduced cells 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 were separated in 10% SDS-PAGE gels and wet transferred to nitrocellulous membrane (GE Healthcare Life Sciences), then blocked for 1 h at room temperature in TBS-T [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] buffer containing 5% nonfat milk. Membranes were then incubated overnight at 4°C or 1 h at room temperature with the respective primary antibodies: p90/CIP2A (1:500), pJNK (1:1,000), pATF2 (1:1000), pc-Jun (1:1000) and actin (1:1,000). Anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (Santa Cruz Biotechnology).

Production of lentivirus-containing *p90/CIP2A* short hairpin RNA: Five p90/CIP2A short hairpin RNAs (knock-down), ligated in pLKO.1 vector and pLOC-KIAA1524 (overexpression) were obtained from Open Biosystems. The knock down efficiency of these shRNAs was evaluated in H1299 cells by Western-blot, where the two showing better knock-down 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, or pLOC-KIAA1524 with pMD2.G, and pCMV-VSVG into HEK293T packaging cell lines. The supernatants containing lentvirus of HEK293T were harvested at 36hrs and 72hrs post transfection. Supernatant were pooled, centrifuged to remove cells and then filtered through

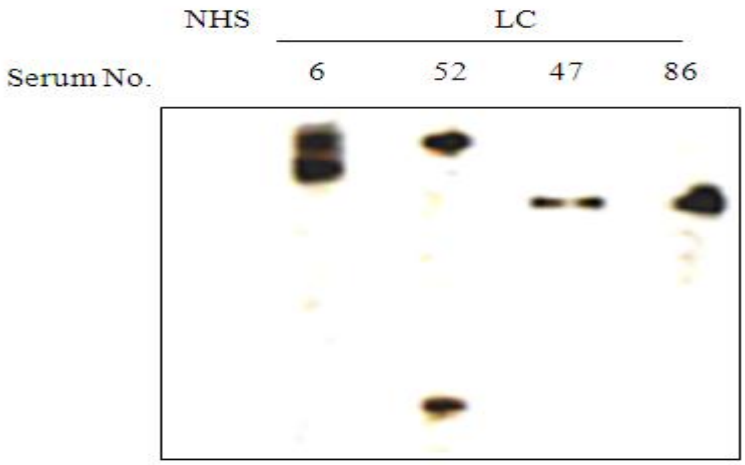
0.45μm low protein binding filter. Cells were plated in monolayer at different densities and infected with lentivirus constructs using 8 ng/mL polybrene. The stable cell lines were selected in the presence of 1μg/ml puromycin (knock-down) or 10ug/ml blastidinin (overexpression) for two weeks.

2.3 Results

p90/CIP2A elicits auto-immune response in lung cancer patients.

To identify novel tumor-associated autoantigens in lung cancer patients, we screened the presence of autoantibodies in 102 sera from lung cancer patients and 82 sera from normal human. Forty-nine out of 102 (48.0%) lung cancer sera contained autoantibody while 5 out of 82 normal human sera were autoantibody positive (6.1%) (Table 2). Interestingly, among these 49 autoantibody positive sera, 10 sera contained autoantibodies against a protein that migrates around 90 kD in SDS-PAGE gel, which is not detected in normal human sera (Fig. 4A). Interestingly, this migration pattern is very similar as previously identified p90/CIP2A [63]. Therefore, we directly tested whether this protein is the previously identified p90/CIP2A by using the recombinant protein. As shown in Figure 4B, five 90 kDa positive sera were randomly chosen for the validation. As expected, all of these five sera contain autoantibodies against p90/CIP2A. To further validate this, recombinant p90/CIP2A was used to immunodeplete the autoantibodies from the sera and these sera were used for Western-blot. As shown in Fig 4B, the positive band disappeared with the p90/CIP2A immune-depleted sera. Furthermore, frequency of p90/CIP2A autoantibodies was examined by ELISA with 105 lung cancer sera samples. Compared to the frequency of p90/CIP2A positive sera in normal human sera (4.9%), twenty seven of the 105 lung cancer sera (25.7%) are p90/CIP2A positive (Table 3). Taken together, p90/CIP2A elicits auto-immune response in lung cancer patients, and therefore can be a potential serum biomarker for lung cancer detection.

A.



B.

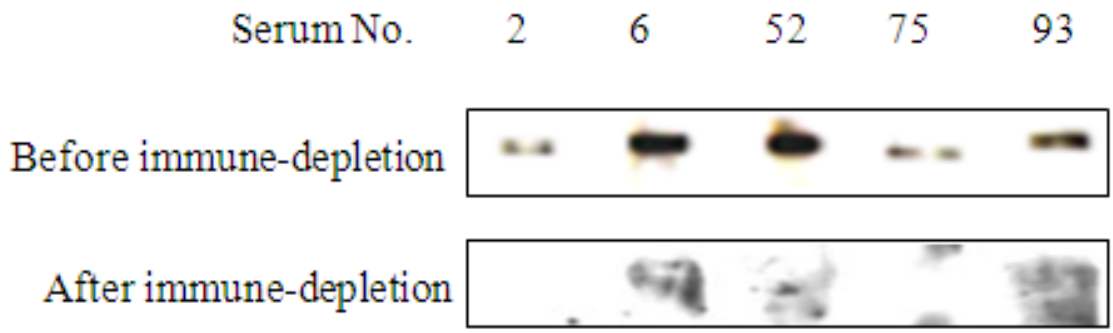


Figure 4. p90/CIP2A elicits autoimmune response in lung cancer patients. (A) Representative Western-blot results of autoantigen screening using human sera. Figure shows the some lung cancer sera containing autoantibody against a band between 75 kD and 100 kD (serum 6 and serum 52). NHS: normal human sera; LC: lung cancer. (B) Validation of p90/CIP2A autoantigen. Five randomly chosen p90 positive sera were used for this validation. Antibodies against p90 containing sera were depleted with recombinant protein and used for Western-blot.

Table 2. Frequency of autoantibody-containing sera in lung cancer patients and normal human sera.

Type of diseases	No. tested	Frequency (Patients with autoantibody/ total patients with disease)
Lung Cancer	102	48.0% (49/102)**[a]
Normal human sera (NHS)	82	6.1% (5/82)

[a]. *P* value relative to NHS: * $p < 0.05$; ** $p < 0.01$

Table 3. Frequency of autoantibody to p90/CIP2A in sera from patients with lung cancer.

Type of diseases	No. tested	Frequency Patients with anti-p90/CIP2A/ total patients with disease)[a]
Lung cancer	105	25.7%（27/105)**[b]
Normal human sera (NHS)	82	4.9% (4/82)**[b]

[a] Cutoff value: Mean + 3 SD of NHS

[b] *P* value relative to NHS: * $p < 0.05$; ** $p < 0.01$

p90/CIP2A is overexpressed in lung cancer

To gain insight into whether p90/CIP2A functions in lung cancer pathogenesis, we used commercially available tumor tissue array to examine the expression level of p90/CIP2A in lung cancer tissue specimen. A set of 72 samples of lung tumor tissues, and 63 samples of normal lung tissues were analyzed with immunohistochemistry (IHC) by using monoclonal p90/CIP2A antibody. The p90/CIP2A positive tissue sample is defined by having a score ranging from 1-6 (Fig. 5). Therefore, 7 out of 63 normal lung tissues showed positive immunoreactivity to p90/CIP2A, while 61 samples among the 72 lung tumor tissues are p90/CIP2A positive (Table 4). This data implied that p90/CIP2A is overexpressed in lung cancer. The incidence of lung cancer increases with age, especially in people who are 65 years or older. We therefore tried to define whether there is a correlation between p90/CIP2A expression and age. However, our data did not support this link. In addition, our statistical analysis also showed that p90/CIP2A overexpression is not correlated with gender, histology, grade or stages (Table 5). In consistence with the previous findings, our results also showed cytoplasmic staining of p90/CIP2A (Fig. 5) [62, 63].Taken together, p90/CIP2A is overexpressed in lung cancer compared to normal lung tissue and the resulted overexpression can be further considered as a diagnostic factor for clinical lung cancer detection.

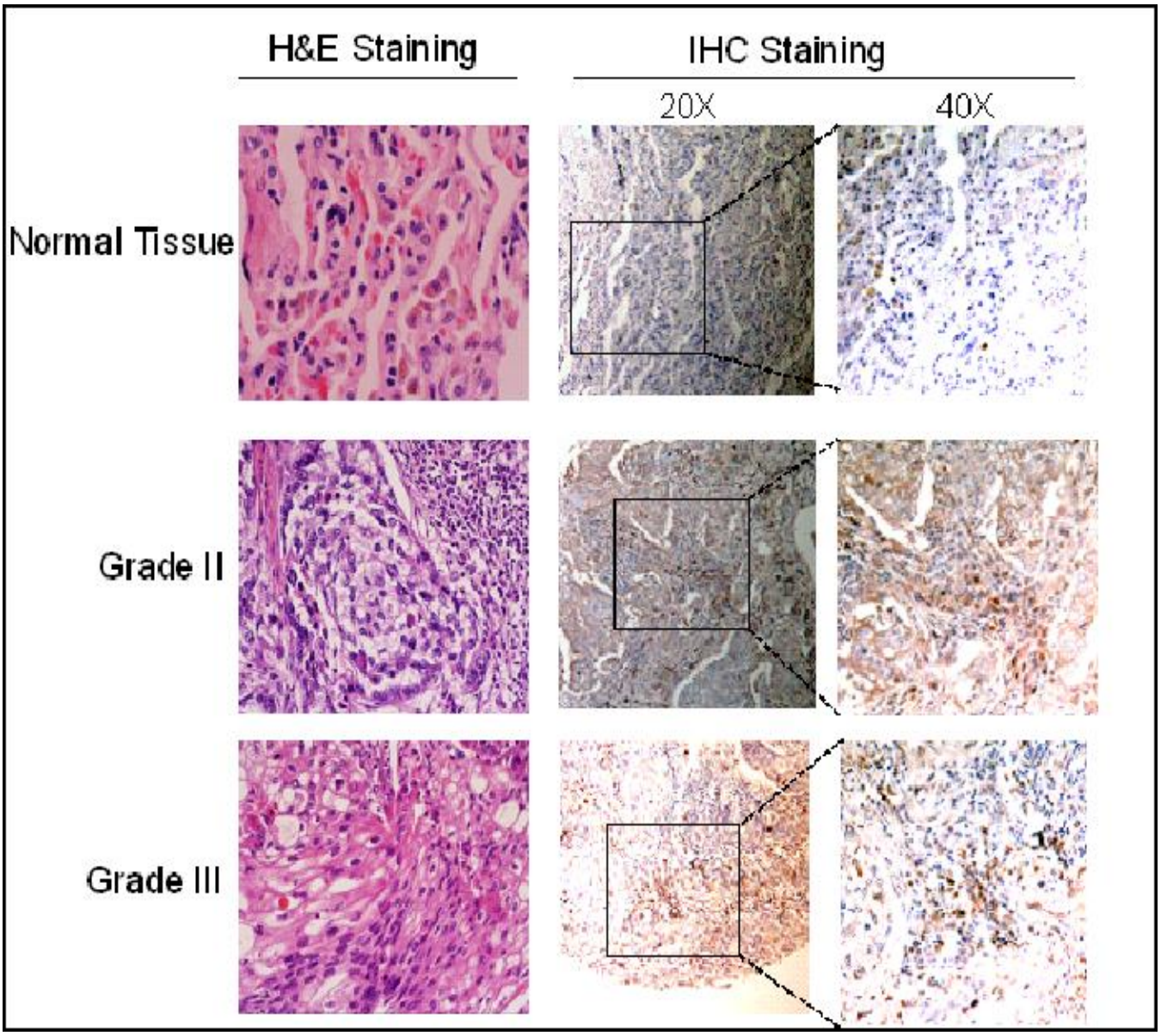


Figure 5. p90/CIP2A expression in human lung cancer tissue. Representative images of IHC results of human lung cancer tissues and normal human tissues using monoclonal mouse anti-p90/CIP2A antibody.

Table 4. IHC results showing expression level of p90/CIP2A in lung cancer tissue specimen.

Tissue status	Total No. of Samples	No. of p90/CIP2A positive	*p* value
Normal human tissues	63	7 (11.1%)	
Lung cancer tissues	72	61(84.7%)	<0.001 **[a]

a. *p value relative to normal human lung tissue. *p<0.05, **p<0.01*

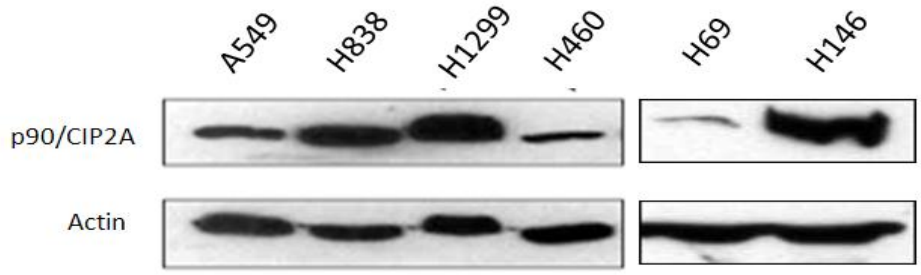
Table 5. p90/CIP2A expression does not correlate with age, gender, stage and histology.

Characteristics	p90/CIP2A low expression (score 0-3)	p90/CIP2A high expression (score 4-6)	*p* value
Age			
65<	28 (54.9%)	23 (45.1%)	1.00
≥ 65	11 (55%)	9 (45%)	
Gender			
Male	25 (47.1%)	28 (52.9%)	0.5936
Female	11 (57.9%)	8 (42.1%)	
Stage			
I-II	19 (52.8%)	17 (47.2%)	0.0935
III-IV	11 (30.6%)	25 (69.4%)	
Histology			
Adenocarcinoma	11 (37.9%)	18 (62.1%)	0.0951
Non-adenocarcinoma	27 (60%)	18 (40%)	

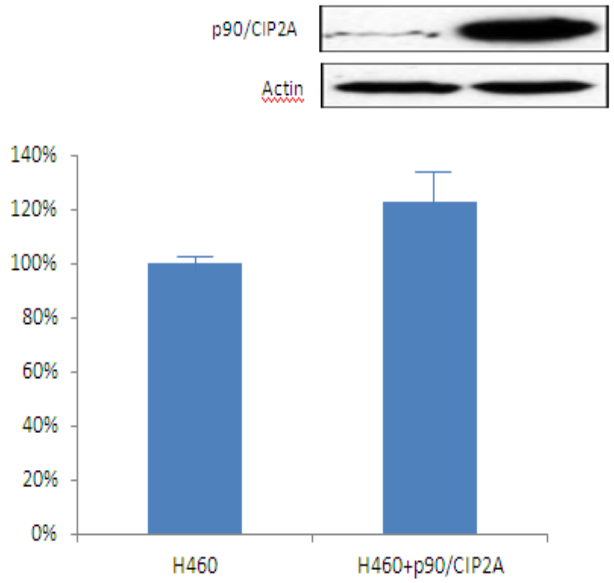
p90/CIP2A promotes cell proliferation of lung cancer cells.

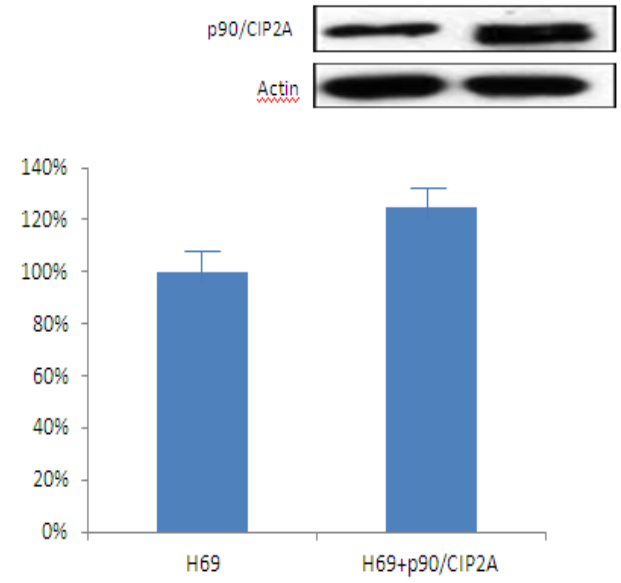
To examine the role of p90/CIP2A in lung cancer, we evaluated the expression level of p90/CIP2A in four non small cell lung cancer (A549, H460, H1299 and H838) and two small cell lung cancer cell lines (H69, H146). All of these six lung cancer cell lines are expressing endogenous p90/CIP2A (Fig. 6A). And in non-small lung cancer cell lines, H1299 is expressing relative high level of p90/CIP2A while H460 is expressing low levels. In small cell lung cancer, H146 express relatively higher level of p90/CIP2A than H69. To examine the role of p90/CIP2A in lung cancer cell growth, we either deplete p90/CIP2A by shRNA or increase the expression by ectopic expression of the recombinant p90/CIP2A. As shown in Fig. 6B, down-regulation of p90/CIP2A impaired cell proliferation in the H1299 and H146 cell lines, while the overexpression of p90/CIP2A is associated with increased cell proliferation. These studies suggested that p90/CIP2A promote cell proliferation of lung cancer cells.

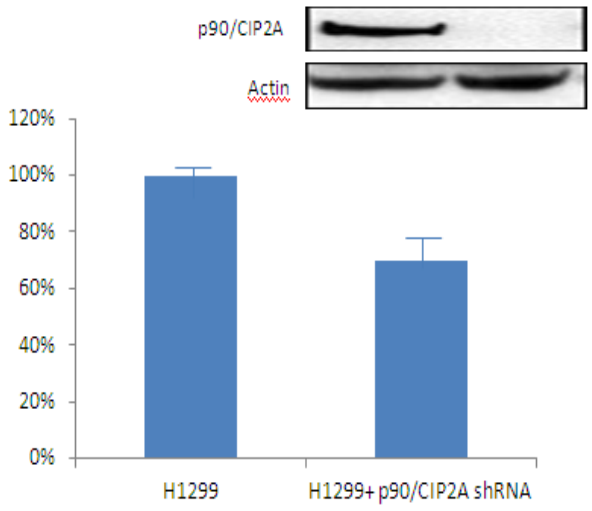
A.


A549
H838
H1299
H460
H69
H146
p90/CIP2A
Actin

B.


p90/CIP2A
Actin
140%
120%
100%
80%
60%
40%
20%
0%
H460
H460+p90/CIP2A


p90/CIP2A
Actin
140%
120%
100%
80%
60%
40%
20%
0%
H69
H69+p90/CIP2A


p90/CIP2A
Actin
120%
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H1299
H1299+ p90/CIP2A shRNA

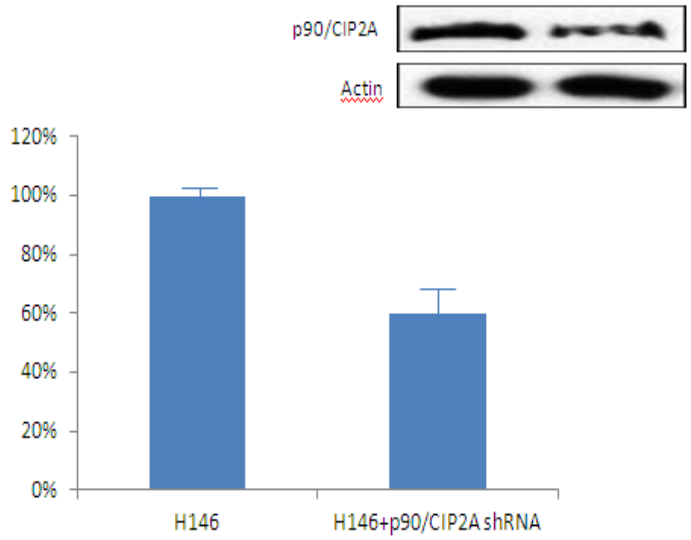

p90/CIP2A
Actin
120%
100%
80%
60%
40%
20%
0%
H146
H146+p90/CIP2A shRNA

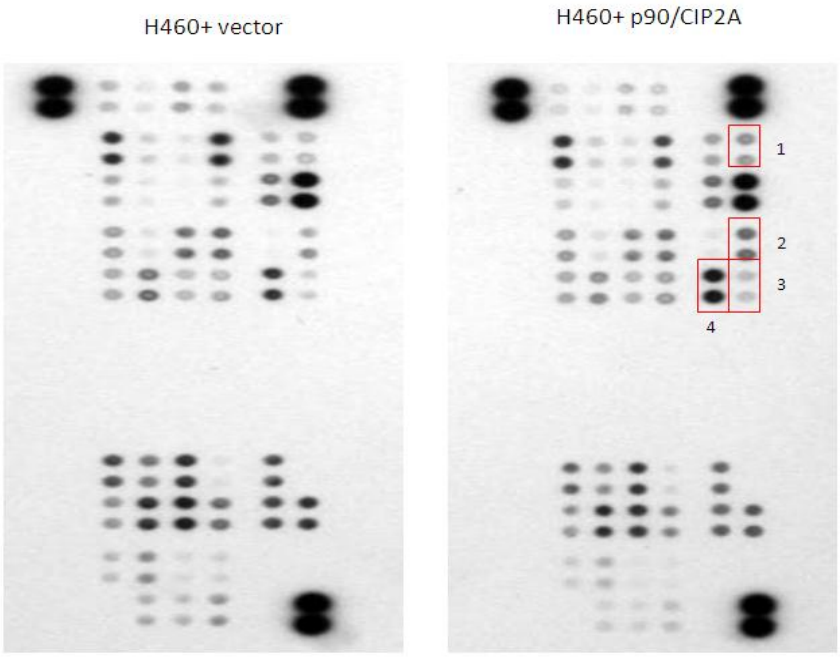
Figure 6. Expression of p90/CIP2A in different lung cancer cell lines and effect of p90/CIP2A in lung cancer cell proliferation. (A) Expression of p90/CIP2A is evaluated in four NSCLC cell lines and two SCLC cell lines using Western-blot analysis. (B) Cell proliferation were performed on four lung cancer cell lines with either knock-down (+p90/CIP2A shRNA) or overexpression (+p90/CIP2A). For adherent cell lines (H1299 and H460), three thousands cells for each cell line were plated triplicate in 96-well plates and MTT solution was added at day 1 and day 3. For suspension cells (H69 and H146), cells were resuspended as single cell and 4000 cells were plated triplicate in 96-well plate, and cell proliferation was measured the same as adherent cell. O.D absorbance was read at 570 nm. Statistical significance was done with Students't test.

p90/CIP2A positively enhances c-Jun N-terminal kinase (JNK) pathways in lung cancer cells

To identify novel p90/CIP2A targeted proteins which may promote cancer cell proliferation, we performed a global analysis of phosphorylation of key intracellular molecules from phosphoproteome level. Total proteins extracted from H460 (H460C) cells transfected with vector control or from H460 overexpressed with p90/CIP2A (H460OV) were used as antigen to react with the phosphor-antibody array, which was then detected with phosphor-antibody cocktails. Several molecules displayed differentially regulated phosphrylation between the H460C and H460OV groups (with >1.3 fold change), including: p38 alpha, pan-JNK, GSK3alpha/beta and AKT (Table 6). Of notice, the most altered phosphorylation molecus is pan-JNK, which increased as much as 1.7 fold upon the increased expression of p90/CIP2A.

JNK belongs to the family of mitogen-activated protein family (MAPK) which responds to stress stimuli like UV radiation, cytokines, heat shock and osmotic shock. Activated JNK had been found to be associated with increased cell proliferation, cell migration and cell invasion in non-small cell lung cancer. Therefore, it is possible that p90/CIP2A may be able to regulate lung cancer progression through this pathway. To validate our finding that p90/CIP2A regulates JNK phosphorylation in lung cancer cells, we treated the cells with EGF to activate the growth factor-induced JNK activation. Consistent with the finding in our phosphoproteome study, the activity of JNK is correlated with the expression level of p90/CIP2A, as demonstrated in Fig. 7B. Furthermore, the phosphorylation of downstream substrates of JNK, c-Jun and ATF2 were also correlated with the phosphorylation of JNK (Fig.7B).

A.


H460+ vector
H460+ p90/CIP2A
1
2
3
4

B.

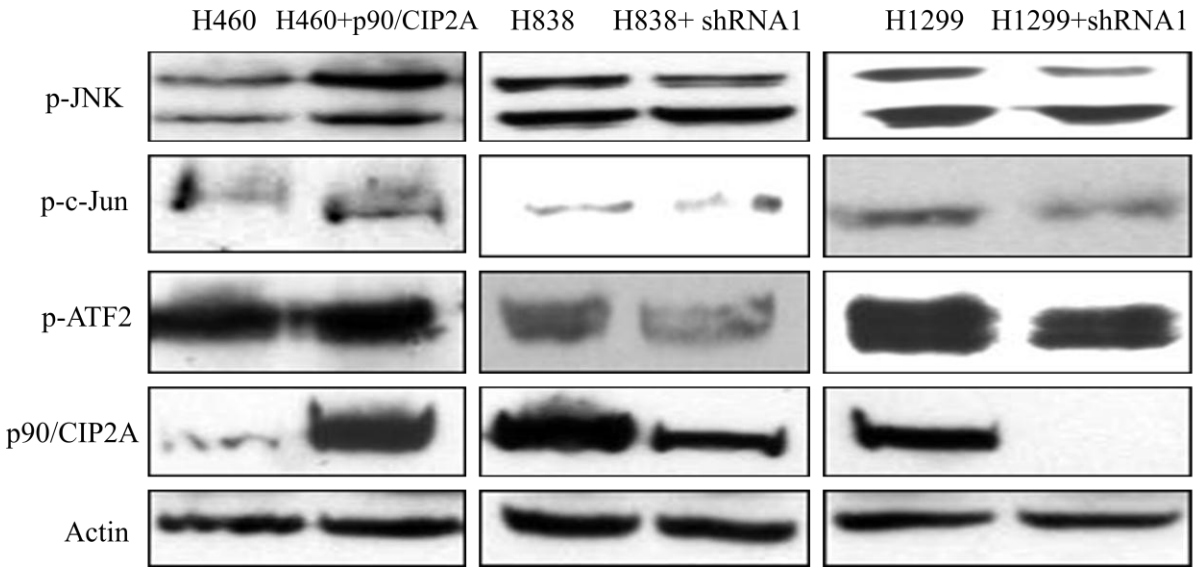

H460
H460+p90/CIP2A
H838
H838+ shRNA1
H1299
H1299+shRNA1
p-JNK
p-c-Jun
p-ATF2
p90/CIP2A
Actin

Figure 7. p90/CIP2A promote c-Jun N-terminal kinase (JNK) phosphorylation in response to EGF stimulation. (A) Phosphoproteomic analysis of the phosphorylation of key molecules in signaling pathways using Human phosphorproteome array. Highlighted spots (1-4) are top molecules showing difference between H460 cell line and H460 overexpressing p90/CIP2A cell lines. Fold of change is measured by densitometry. (B) p90/CIP2A regulates JNK phosphorylation at different lung cancer cell lines: H460, H838 and H1299.

Table 6. Top four molecules which showed significant changes in response to the overexpression of p90/CIP2A in H460 cell line.

Spot No.	Protein name	Phosphorylation site	Fold of change
1	p38 alpha	Thr180/Tyr182	1.35
2	JNK	Thr183/Tyr185	1.71
3	GSK3 (alpha/beta)	Ser9/Ser21	1.38
4	AKT	Ser473	1.52

2.4 Discussion

Relationship between the immune system and tumorigenesis is not only an important aspect to the understanding the relationship between immune response and progression of disease, but can also be fundamental for the therapeutic design of the cancer treatment. Proteins involved in tumorigenesis and cancer progression are always the target of the host's immune system. The detection of the abnormalities of these proteins can be a sign that the immune system is able to respond to such deregulated molecules. Thus, the identification of novel tumor-associated autoantigens is of great importance both in clinical development of reliable non-invasive approaches for early cancer detection, and is also a way to identify proteins whose expression were changed during the cellular transformation and disease progression. In this study, we found the previously identified tumor-associated autoantigens p90/CIP2A also initiates an immune response in lung cancer patients. This result indicated that p90/CIP2A may not be a specific antigen of gastric cancer, but it can be prognostic factor for the diagnosis of many types of cancers.

Tumor-associated autoantigens are always expressed in early stage of cellular transformation, which were thought to be a possible clue for the sense of danger by the immune system, even though the mechanism of the production of tumor-associated autoantibody remains unknown. The overexpressed tumor-associated autoantigens always confer cancer cell tumorigenic ability by interrupting normal signaling pathways. p90/CIP2A is present in embryonic development, but is silenced in most normal adults tissues. In consistency with the previous findings, we found that p90/CIP2A is overexpressed in lung cancers. Taken together with the results from other groups, our data further confirm the role of p90/CIP2A in promoting tumorigenesis.

Although abundant studies had shown that p90/CIP2A is overexpressed in many types of cancer, the role of this protein in cancer progression is still unknown. In our study, we found that p90/CIP2A overexpression is correlated with increased phosphorylation of JNK. JNK bears both of tumor-promoting and tumor-suppressive function dependent on the upstream stimuli. The activity of

JNK increased along with overexpression of p90/CIP2A, which implied that p90/CIP2A may prime the JNK to tumor-promoting function in response to mitogen stimulation. Interestingly, the two identified PP2A inhibitors, I2PP2A and small t antigen have similar roles to p90/CIP2A in promoting JNK phosphorylation and AP-1 transcriptional activity. However, the exact mechanism of how these PP2A inhibitors, including p90/CIP2A, are able to modulate the JNK phosphorylation is still unknown, since PP2A is able to target several molecules in this pathway. Further investigation should be focused to resolve this issue.

Taken together, our study found that p90/CIP2A is a cytoplasmic tumor-associated autoantigen in lung cancer, whose overexpression can be sensed by host immune system. The aberrantly overexpressed p90/CIP2A is associated with the increased cell proliferation of lung cancer cells. The underlying mechanism of p90/CIP2A for cancer progression is through the c-myc-dependent pathway and c-myc-independent pathway JNK-c-Jun/ATF2, identified in this study.

Chapter 3: p90/CIP2A regulates CREB phosphorylation by modulating AKT-associated PP2A phosphatase activity

3.1 Introduction

Lung cancer is one of the most common cancer types, and one that causes most of the cancer-related deaths throughout the world in recent years [1]. According to the latest data from American Cancer Society, there will be about 221,130 new cases of lung cancer (both small cell and non-small cell) in the United States in 2011 and about 156, 940 people would die from it at the same year [1]. Although the factors that cause lung cancer have not been well defined, like many other types of cancer, some common genetic lesions have been identified as important triggers for lung cancer formation. Genes like epidermal growth factor receptor (EGFR), V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-ras), c-myc, APC, LKB1, p53 and protein phosphatase 2A (PP2A) are the most commonly activated oncogenes or inactivated tumor suppressors in lung cancer [13, 18, 36, 105].

PP2A, a large protein family with intrinsic serine/threonine phosphatase activity, is a known tumor suppressor by targeting cancer-related molecules or pathways like AKT, MAPK and beta-catenin [48]. The holoenzyme consists of a regulatory subunit (B subunit), which confers substrate selectivity, a catalytic subunit (C subunit), and a scaffold subunit, which brings B and C subunits together [48]. Inhibition of PP2A phosphatase activity has been found to be a prerequisite for cellular transformation in epithelial cells [37]. The mechanisms involved in the inactivation of PP2A phosphatase activity include genetic mutations, epigenetic silencing or inhibition [36]. 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 [36]. The restoration of PP2A phosphatase activity by re-introduction of the wild type PP2A subunit to cancer cell lines can lead to decreased cell proliferation, cell scencese or apoptosis [36].

Besides mutations, PP2A can also be inactivated by exogenous or endogenous factors. Chemicals like okadaic acid, viral proteins like small t antigen and cellular proteins like I1PP2A and I2PP2A were all identified as PP2A inhibitor , which promote cellular transformation and cancer progression [36]. However, few endogenous PP2A inhibitors had been identified so far. p90/CIP2A was a recently identified endogenous PP2A inhibitor in human malignancies . This protein was actually identified as a tumor-associated autoantigen in gastric patients ten years ago. Although this protein was found to be tightly regulated during development, the function of this protein was largely unknown due to the lack of homologies to any other function-known proteins at the time of identification. In search of PP2A interacting proteins in Hela cells, Juttia et al. used PP2A A subunit as the bait in tandem-affinity purification, and established that the protein pulled-down by PP2A was the p90 autoantigen. The knock-down of this protein can greatly impair cell proliferation and in vivo tumor growth. One of the mechanism is involved in the ability of p90/CIP2A to inhibit c-myc-associated PP2A phosphatase activity by an unknown manner, thus named as cancerous inhibitor of PP2A (CIP2). Following studies by examining tissue specimen of different cancer types revealed that p90/CIP2A was overexpressed in cancer tissues but not in normal tissue. Although p90/CIP2A is able to inhibit PP2A phosphatase activity, previous data strongly suggest that it has substrate selectivity, since the phosphorylation of other PP2A substrates like p53 and MDM2 were unaffected. So far, only a few p90/CIP2A targets have been identified. In addition to c-myc, UNC5B and AKT were also found to be targets of p90/CIP2A. Interestingly, both of these proteins raised the involvement of p90/CIP2A in anti-apoptotic function.

The molecular mechanism of p90/CIP2A in cancer has been detected by transcriptomic studies as a whole. However, it still lacks studies at the proteome level, which do not necessarily correlate with mRNAs. Two-dimensional electrophoresis-based proteomic approach is a widely-adopted method to study the function of a gene by interpreting the global protein profile change. The integration of

differentially expressed proteins in database, including all the known pathways, and transcription factors have proved to be an effective way to uncover novel function of certain proteins.

3.2 Materials and methods

Production of lentivirus-containing *p90/CIP2A* short hairpin RNA: Five p90/CIP2A short hairpin RNAs, ligated in pLKO.1 vector, were obtained from Open Biosystems (Huntsville, AL). The knock down efficiency of these shRNAs was evaluated in H1299 cells by Western-blot, where the two showing the better knock-down 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 and pCMV-VSVG into HEK293T packaging cell lines. The supernatants containing lentivirus of HEK293T were harvested at 36hrs and 72hrs post transfection. Supernatant were pooled, centrifuged to remove cells and then filtered through a 0.45μm low protein binding filter. Cells were plated in monolayer at different densities and infected with lentivirus constructs using 8 ng/mL polybrene. The stable cell lines were selected in the presence of 1μg/ml puromycin for two weeks.

Cell proliferation, clonogenic assay, and anchorage-independent cell growth assay. The shRNA for *p90/CIP2A* lentivirus-transduced cells and control-transduced cells were plated quadruplicate in 96 wells plates at a density of 3×10^3/100μl medium for each well. After a 3-day culture, 15μl of the dye solution was added to each well and incubated at 37°C for another 4 hrs. At the end of the incubation, 100μl stop solution was added to each well and colometric absorbance was read in the SpectraMax Plus (Molecular Devices) at 570nm. Cell free medium was used as mock control.

To measure the effects of knockdown on tumor cell colonogenic expansion, *p90/CIP2A* shRNA–transduced cells and control shRNA transduced cells were trypsinized and resuspended in complete growth medium to form single cells. For each group of cells, 5×10^3 cells were plated in 100-mm petri

dish (BD bioscience) and incubated for 15 days. Medium was changed every three days. At the end of incubation, colonies were fixed with 10% formalin and stained with crystal violet. Number of colonies were counted using Image J software (NIH.)

Colony formation ability on soft agar was evaluated. Briefly, 2.5 mL base layer consisting of 0.4% Nobel agar medium were prepared in 100mm petri dish (BD biosciences, Bedford, MA). p90/CIP2A shRNA–transduced and control lentivirus–transduced cells were trypsinized, centrifuged, resuspended in 0.4% agarose medium (equal volumes of 0.8% agarose and 2X RPMI1640 containing 20% FBS culture medium), and plated onto the top agar at 12,500 cells per well. The cells were kept wet by adding a small amount of 2.5ml complete RPMI 1640 growth medium (Life Technologies), which contained 10% FBS (Carlsbad, CA), and incubated for 3 wk at 37°C. Colonies were stained with crystal violet and counted under the microscope.

PP2A phosphatase activity assay. Measurement of PP2A phosphatase activity was performed by using the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit (Invitrogen, CA) . 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 incubate with 1XPP2A phosphatase reaction buffer for 30mins at 37^{o}C. Fluorescence was measure using excitation at 355nm and emission at 485nm. The fluorescence intensity was normalized to the expression level of PP2A catalytic domain.

Western-blot analysis. Cells were plated in 6-well tissue culture plates at 80% confluence and incubated overnight. Cell lysates were obtained from transduced cells 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, were wet-transferred to nitrocellulose membrane (GE Healthcare Life

Sciences) and then blocked for 1 h at room temperature in TBS-T [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20] buffer containing 5% nonfat milk. Membranes were then incubated overnight at 4°C or 1 h at room temperature with the respective primary antibodies: anti-p90/CIP2A (1:500), anti-c-myc (1:1,000), anti-PP2A catalytic domain (1:500) and anti-actin (1:1,000). Anti-mouse or anti-rabbit secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA) was used to visualize the stained bands with an enhanced chemiluminescence visualization kit (Santa Cruz Biotechnology, Santa Cruz, CA).

Sample preparation and 2-Dimensional electrophoresis: Cells transduced with either control lentivirus or p90shRNA1 were directly lysed in 2-DE rehydration sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 80 mM DTT, and 1% carrier ampholyte pH 3−10). Protein concentration was measured with Brandford assay (Bio-rad). One hundred and twenty five micrograms of protein were loaded to 11cm pH 3-10 Isoelectric focusing (IEF) gel strip, where IEF was carried out for 8000 Vh. IEF gels are equilibrated in equilibration buffer containing 2% DTT for 15 mins, followed by equilibration in equilibration buffer containing 2.5% iodiactomine for another 15 mins. The gels were transferred to the second-dimension electrophoresis using 10% acrylamide gel. Protein spots were visualized by staining with Coomassie blue (CBB) R250.

Protein digestion. The CBB-stained protein spots were cut from the gel and destained three times with 100 μL of 50 mM ammonium bicarbonate/50% acetonitrile (ACN) for 30 min at ambient temperature. The destained gel pieces were then completely dried in a SpeedVac vacuum concentrator (Savant Instruments, Farmingdale, NY). Gel pieces were reswollen with 8 μL of 25 mM ammonium bicarbonate containing 10 ng of trypsin at 4 °C for 1 h. After 13.5 h of incubation at 37 °C, the gel pieces were vacuum-dried, 8 μL of 50 mM ammonium bicarbonate was added, and the samples were incubated at 37 °C for 1 h. The supernatant was transferred into another microtube, and 8 μL of 2.5%

trifluoroacetyl (TFA)/50% ACN was used for the second step of extraction at 30 °C for 1 h. Then both supernatants were combined with the first one. Finally, in order to extract hydrophobic peptides, 8 μL of 100% ACN was used at room temperature for 1 h, and the supernatant was also combined with the previous supernatants. The combined supernatants were then dried in a SpeedVac and redissolved with 2 μL of 0.5% TFA.

MALDI-TOF/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.

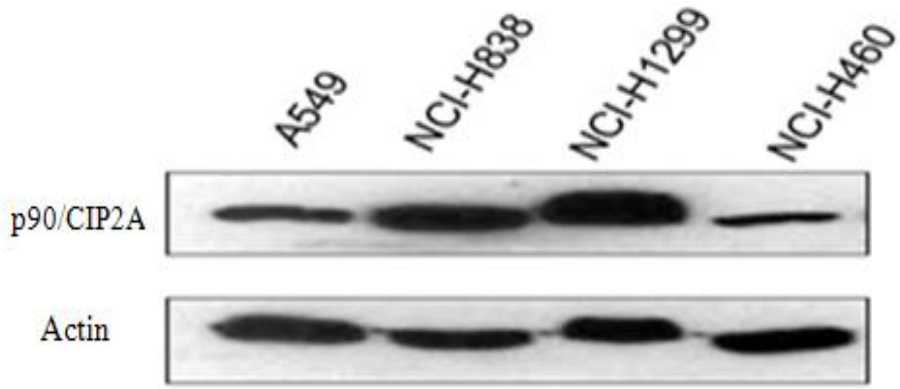
Bioinformatics analysis: The gene interaction network was built with Pathway Studio software. All the differentially expressed proteins are entered into the database and set filter as “Promoter Binding" to create the shortest pathways, which give predictions on transcription factor candidates.

3.3 Results

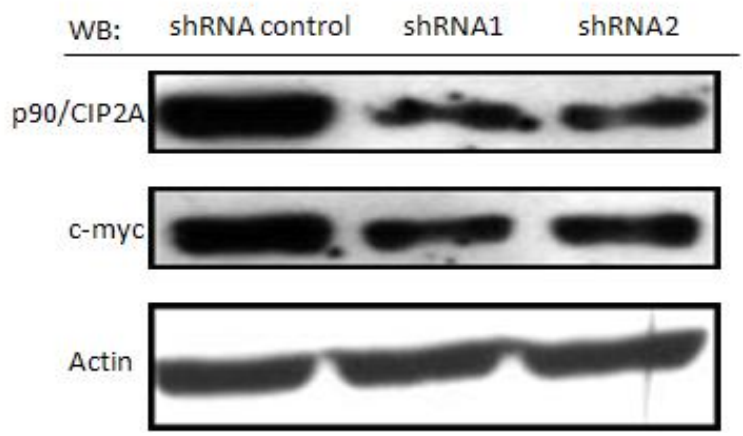
Establishment of stable cell line expressing p90/CIP2A shRNA.

First, we examined the expression of p90/CIP2A in four different lung cancer cell lines: A549, NCI-H838, NCI-H1299 and NCI-H460. As shown in Figure 8, all of the examined lung cancer cell lines expressed endogenous p90/CIP2A. While NCI-H1299 contained the most abundant protein, NCI-H460 expressed the least amount of p90/CIP2A in these four cells lines, as compared to the expression level of actin. Therefore, the H1299 cell line was used as our research model to generate stably expressing p90/CIP2A shRNA cell lines by lentiviral-mediated transduction which achieved >90% knocking-down effect as determined by western-blot. According to previous data showing that the loss of p90/CIP2A will induce the degradation of c-myc by restoring c-myc associated PP2A phosphatase activity in Hela cells, we also measured the expression level of c-myc and PP2A phosphatase activity. In consistence with previous results, the knock-down of p90/CIP2A in H1299 also lead to decreased levels of c-myc (Fig. 8B) and increased PP2A phosphatase activity in the total cell lysates (Fig. 8 C). Therefore, this stable cell line reinforced what the previous results showed.

A.


A549
NCI-H838
NCI-H1299
NCI-H460
p90/CIP2A
Actin

B.


WB:
shRNA control
shRNA1
shRNA2
p90/CIP2A
c-myc
Actin

C.

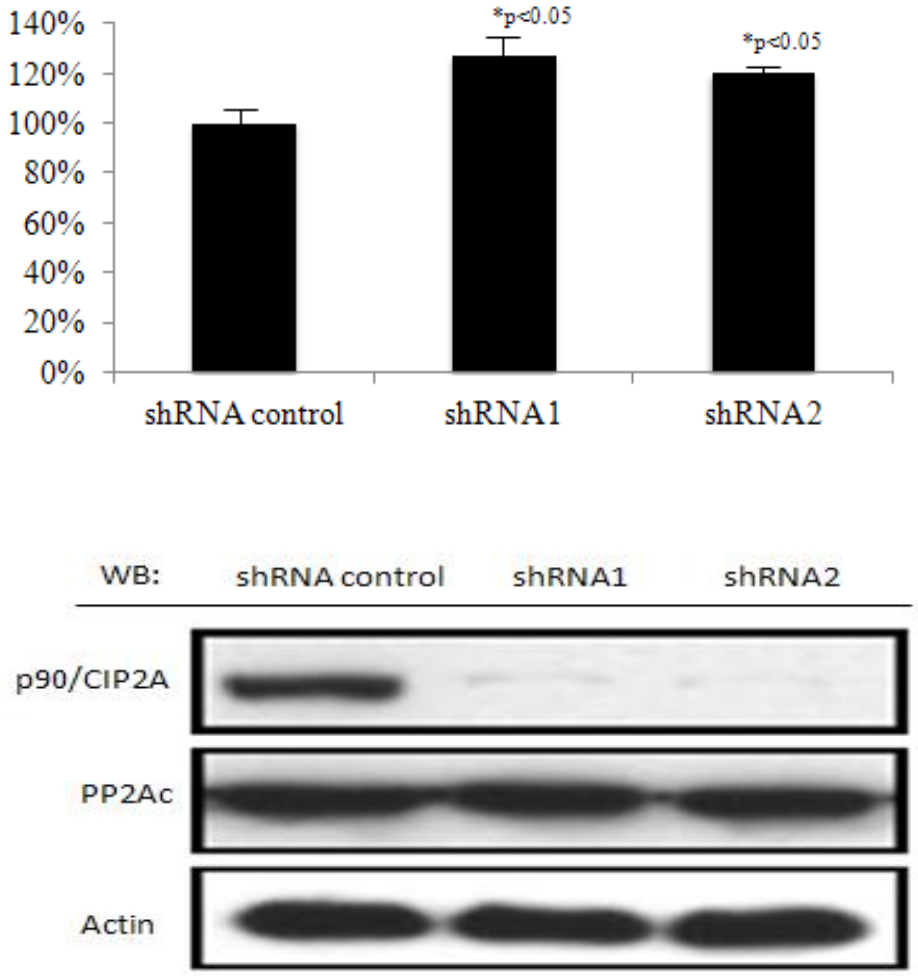


Figure 8. p90/CIP2A expression in lung cancer cell lines and p90/CIP2A knock-down in H1299. A) p90/CIP2A expression was examined in four different lung cancer cell lines: A549, NCI-H838, NCI-H1299 and NCI-H460. B) Western-blot results of p90/CIP2A, c-myc and actin after knock-down of p90/CIP2A in H1299 lung cancer cells (C) PP2A phosphatase activity assay. Total cell lysates extracted from either control group or shRNA p90 treated groups are loaded to measure the PP2A phosphatase activity by using DiMUF as the substrates (upper panel). Fluorescence readings were normalized to the expression level of PP2A catalytic subunit (lower panel).

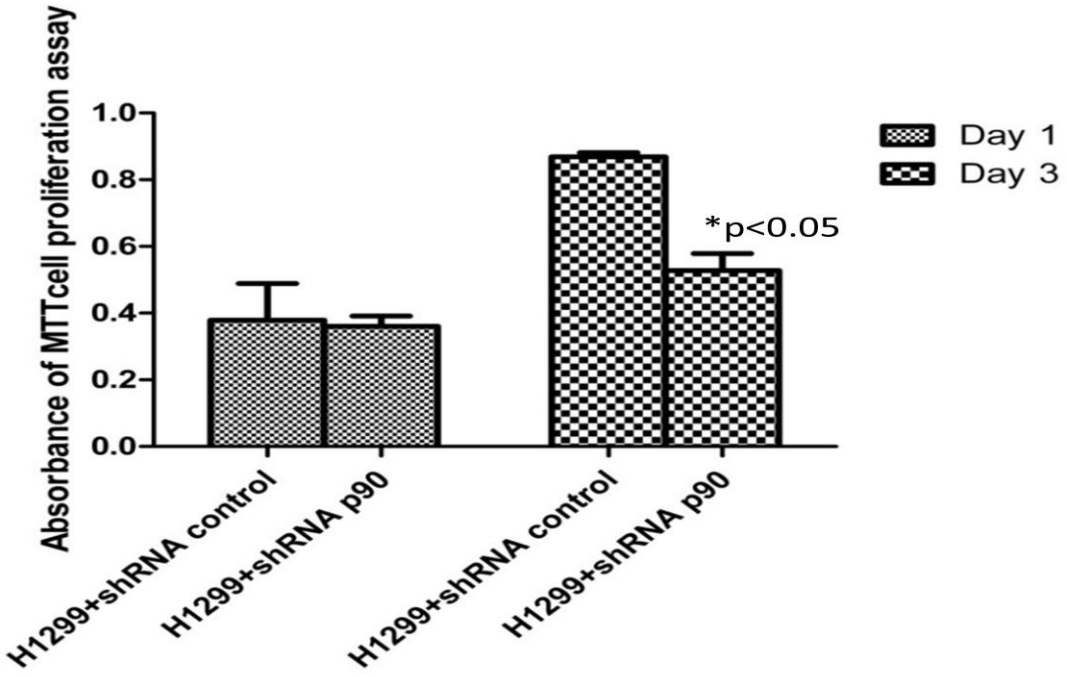
Depletion of p90/CIP2A impairs cell proliferation, clonogenic ability and anchorage-independent growth of H1299 cell line.

As previously reported, p90/CIP2A plays crucial roles in cell proliferation by regulating the expression of oncoprotein c-myc. We intended to determine whether this protein could have a similar function in lung cancer cells. By knocking-down p90/CIP2A in H1299 lung cancer cell line via shRNA, we found that the proliferation rate of p90/CIP2A shRNA transduced cell line was greatly impaired. Proliferation decreased to only 59% (Fig 9A, upper panel) when compared to the cells infected with shRNA control. These data suggested that p90/CIP2A increase cell proliferation of lung cancer cells.

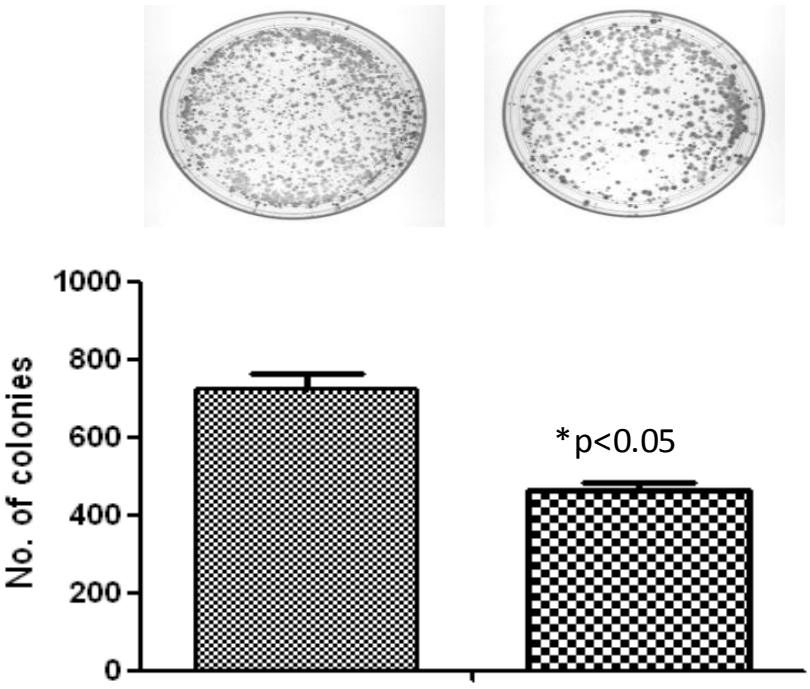
Clonogenic expansion is an ability of tumor cells to grow from a single cell. Previous results showed that knock-down of p90/CIP2A in Hela cells can impair its ability to form foci in culture dish. Therefore, we tested whether it had the same effects in lung cancer cell lines. By plating on the petri-dish for two weeks, the down-regulation of p90/CIP2A in H1299 decreased the foci locus to 62% as compared to vector control cell line (Fig 9 B). In addition, the number of foci of the knock-down cell line is smaller than the control group (Data not shown).

Similarly, anchorage-independent growth showed that the p90/CIP2A down-regulation has only 50% of colonies formed in soft agar of the cells transduced with control shRNA (Fig 9C). This data suggest that p90/CIP2A is essential for tumor cell growth *in vitro*.

A.


Absorbance of MTTcell proliferation assay
1.0
0.8
0.6
0.4
0.2
0.0
Day 1
Day 3
*p<0.05
H1299+shRNA control
H1299+shRNA p90
H1299+shRNA control
H1299+shRNA p90

B.


No. of colonies
1000
800
600
400
200
0
*p<0.05

C.

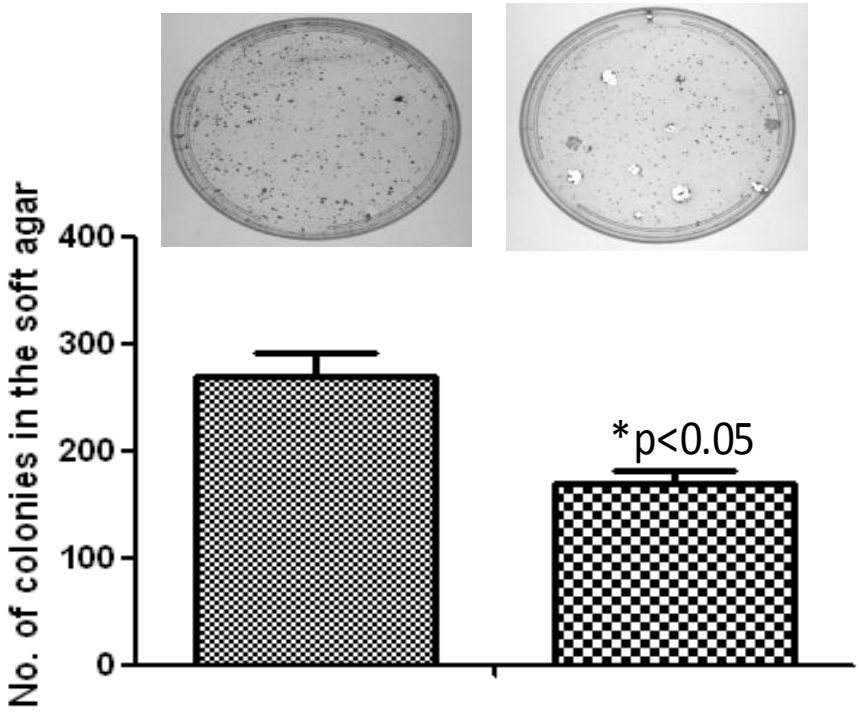


Figure 9. Depletion of p90/CIP2A induce decreased cell proliferation, clonognic expansion, soft agar growth and PP2A phosphatase activity. A) Cell proliferation assay of the effect of knock-down p90/CIP2A in H1299 using MTTs assay. B) Clonogenic expansion assay in H1299 cancer cells. $5X10^3$ control cells or cells transduced with shRNA are seeded in 100-mm petri dish for 15 days. Foci are fixed in 10% formalin, stained with crystal violet, scanned with Quantity One software and analyzed with Image J. C) Anchorage-independent cell growth. $1.25X10^4$ cells are resuspended in 0.4% agarose, poured to base agar containing 0.8% Nobel agar and incubated to three weeks. Colonies are stained with crystal violet and counted under microscope. D) PP2A phosphatase activity assay. Total cell lysates extracted from either control group or shRNA p90 treated groups are loaded to measure the PP2A phosphatase activity by using DiMUF as the substrates. All statistic significance is calculated by using Students' t test.

Proteomic analysis of the output upon the loss of p90/CIP2A in H1299 lung cancer cell line.

The current understanding of the function of p90/CIP2A is still limited to its ability to regulate the expression of c-myc. To gain insight into how p90/CIP2A regulates tumor growth, we utilized 2-D electrophoresis and mass spectrometry-based proteomic approach to systematically investigate the differential expression profile of H1299 cancer cell line on the context of wild type cell line or cell line stably expressing p90/CIP2A shRNA. By comparing the 2-D profile to these two groups, we quantitatively detected 49 differentially expressed protein spots, which showed at least 1.5 fold change (Fig. 10).

Figure 10. Representative 2-DE gel. Equal amount of proteins were loaded to IEF strip (11cm, pH3-10) and isoelectric focusing for 8000 vhrs. Gels are equilibrated before loading for the second-dimension. The SDS-PAGEs are stained with Coomassie R-250, scanned and analysed with PDQUEST software. Protein spots with more than 1.5 fold changes are considered as differentially expressed proteins, which are indicated as arrows in the gel.

Protein classification and annotation

According to the reported function of these proteins, we found that a majority of these altered proteins belong to functional classes of metabolism, signal transduction and transcriptional and translational control (Fig 11). Proteins like phosphoglycerate mutase 1 (PGM), α-enolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are involved in different steps of glycolysis by catalyzing chemical reactions (Table 7) [106-108]. Tumor cells always exhibited increased glycolysis to generate ATP to support their growth under aerobic conditions, which is known as Warburg effect [77]. The loss of p90/CIP2A impairs the expression of these proteins and indicates that p90/CIP2A may indirectly affect their expression through c-myc, since c-myc is known to promote the aerobic glycolysis of cancer cells. Other metabolism-related proteins like nucleoside diphosphate kinase (NDK) play roles in catalyzing the exchange of phosphate groups between different nucleoside diphosphates [109]. Nucleophosmin, which is encoded by nm23-H1 gene, has been reported to be overexpressed in many different type of malignancies [110]. The clinical study showed that its overexpression is strongly associated with tumor cell motility, invasion and metastasis [110].

Twenty four percent of the differentially expressed proteins are associated with transcriptional and translational control. Among these proteins, SAP-domain containing ribonucleoprotein (CIP29) and heterogeneous nuclear ribonucleoprotein H1 (hnRNP H1) are known to be able to bind nucleotide and regulate RNA stability and localization (Table 1) [111, 112]. CIP29, like p90/CIP2A, is a fetal protein that is only expressed during embryonic stage and becomes silenced in adult tissue [111]. This protein is overexpressed in breast and pancreatic tumors. hnRNP H1 is a mRNA binding protein and its interaction with target mRNA can determine their subcellular localization [112]. The function of this protein in cancer is not well understood, but it has been reported to be involved in anti-cancer drug resistance [112]. Protein DJ-1 and far upstream element-binding protein 2 (FUBP-2) can facilitate gene transcription [113, 114]. For example, protein DJ-1 can positively regulate the transcription of androgen receptor (AR) in

the nucleus [113]. FUBP-2 and its family protein FUBP-1 are known to be involved in the transcriptional control of c-myc. The knock-down of this protein will lead to decreased cell proliferation and xenographt tumor growth [114]. Other proteins like elongation factor 2 (eEF2), acidic leucine-rich nuclear phosphoprotein 32 (ANAP32), ruvB-like 1 et al are participating in diverse cellular function such as translation elongation, nuclearsome assembly and transcription [115-117].

Another major functional class is related to signal transduction and protein degradation (Table 6). This functional class protein consists of several cancer-related genes (Table 6). Annexin A5 and 14-3-3 ε are proteins of great importance in regulating cancer cell proliferation [118, 119]. Proteins like Annexin A1 and EBP1 have anti-proliferative effects in cancer. 14-3-3 ε exerts its effect through binding to other signal transduction molecules by recognizing the phosphoserine-containing residue. It can promote cell proliferation by interacting with the key components in the RAS and MAPK pathways [119]. EBP1 is an ErbB-3 binding protein, which inhibit cell proliferation and induce cell differentiation in breast cancer cells. Yet, it is unknown how this protein inhibits cell proliferation, since it act as transcription factor or transcription co-regulator, which is normally sequestered in the cytoplasm as inactive form until it has been stimulated with proper ligands [120].

Some proteins related to the cytoskeleton have also been identified: septin, F-actin-capping protein subunit-beta, stathamin-1 and microtubule-associated protein RP/EB family member (MAPRE1). These proteins participate in chromosome stability, cell polarity and cytokenesis [121, 122]. Except for F-actin-capping protein subunit-beta, all of the other three proteins have been confirmed to be related to cancer (Table 8, 9).

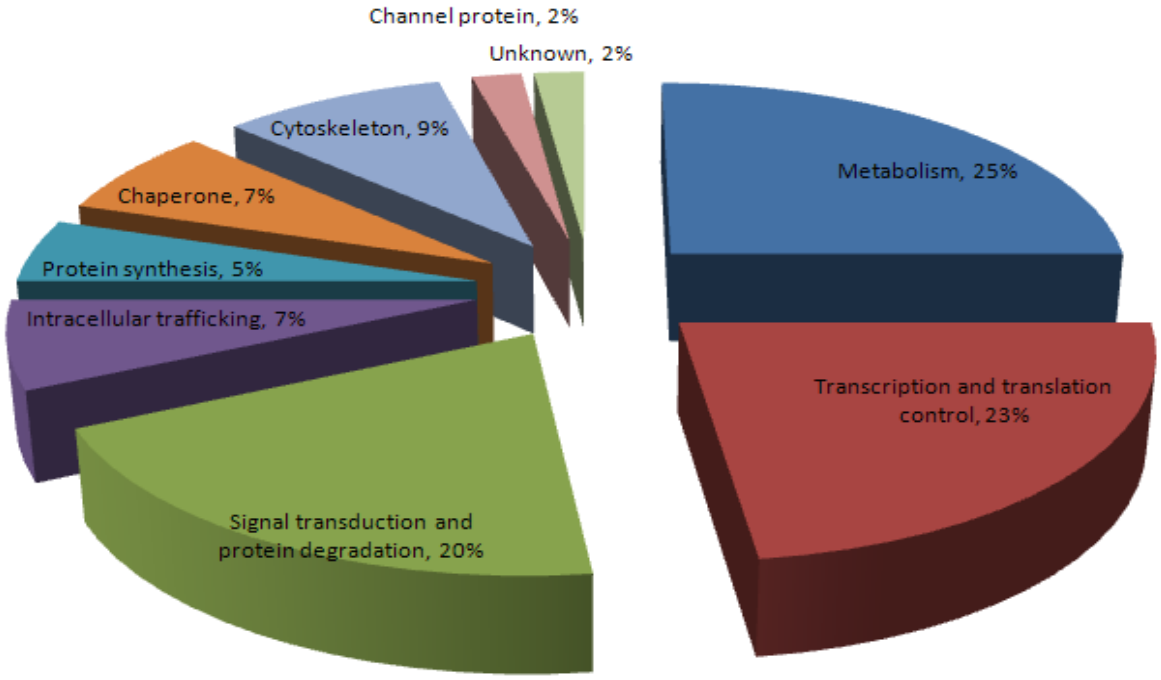


Figure 11. Protein classification by function. The differentially expressed proteins are classified by their reported function. The gene function was obtained from Gene Ontology (GO) database.

Table 7. Summary of differentially expressed proteins.

Spot No.	Protein Name	Accession No.	Score	MW/pI	Function
Metabolism					
5	Glucosidase II	gi|2274968	242	107,289 Da/5.74	breaking down complex carbohydrates
9	UDP-N-acteylglucosamine pyrophosphorylase 1	gi|3273316	313	57,421 Da/5.92	catalyzes the final step of UDP-N acetylglucosamine (UDP GlcNAc) biosynthesis from fructose 6 phosphate.
15, 16	alpha-enolase	gi|693933	420	47,421 Da/7.01	catalyzes glycolysis
20	glyceraldehyde-3-phosphate dehydrogenase	gi|7669492	269	36,201 Da/8.57	catalyzes glycolysis
24	L-lactate dehydrogenase	gi|4557032	313	36,900 Da/5.71	It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply
26	dimethylarginine dimethylaminohydrolase 2	gi|7524354	267	29,911 Da/5.53	degrading methylarginines
32	glutathione S-transferase	gi|2204207	318	23,595 Da/8.91	catalyse the conjugation of reduced glutathione to electrophilic centers on a wide variety of substrates
34	Phosphoglycerate mutase 1	gi|4505753	344	28,900 Da/6.67	Catalyzes glycolysis
37	nucleoside diphosphate kinase A	gi|35068	305	20,740 Da/5.83	
43	inositol monophosphatase 1	gi|443382	283	30,437 Da/5.16	
Transcriptional and translational regulation					
6	elongation factor 2	gi|4503483	395	96,246 Da/6.41	Translation elongation
7	far upstream element-binding protein 2	gi|119589502	449	77,060 Da/6.84	Facilitate c-myc transcription
12	ruvB-like 1	gi|4506753	569	50,538 Da/6.02	involve in transcriptional activation of select genes principally by acetylation of nucleosomal histone H4 and H2A.
13,17	heterogeneous nuclear ribonucleoprotein H1	gi|5031753	576	49,484 Da/5.89	influence pre-mRNA processing and other aspects of mRNA metabolism and transport
21	nascent polypeptide-associated complex subunit alpha	gi|5031931	253	23,370 Da/9.6	prevents short recently synthesized ribosome-associated polypeptides from inappropriate interactions with cytosolic proteins

22	acidic leucine-rich nuclear phosphoprotein 32	gi|5453880	141	28,682 Da/3.99	E4F1-mediated transcriptional repression.
29	prohibitin	gi|4505773	588	29,843 Da/5.57	modulate transcriptional activity by interacting with various transcription factors, including nuclear receptors
33	SAP domain-containing ribonucleoprotein	gi|32129199	271	23,713 Da/6.1	participate in important transcriptional or translational control of cell growth, metabolism and carcinogenesis
35	protein DJ-1	gi|31543380	279	20,050 Da/6.33	positive regulator of androgen receptor-dependent transcription
40	eukaryotic translation initiation factor 3	gi|10801345	156	25,329 Da/4.72	Translation iniation
48	chromobox protein homolog 1	gi|5803076	237	21,519 Da/4.85	Recognizes and binds histone H3 tails methylated at 'Lys-9', leading to epigenetic repression
Signal transduction and protein degradation					
14	erbB3 binding protein EBP1	gi|4099506	292	38,320 Da/6.13	Inhibit androgen receptor-mediated transcription
39	translationally-controlled tumor protein	gi|15214610	177	19,759 Da/4.84	regulation of apoptosis, and microtubules stabilization
30	ubiquitin carboxy-terminal hydrolase L1	gi|4185720	447	23,354 Da/5.33	Protein degradation
27	14-3-3 protein epsilon	gi|5803225	297	29,326 Da/4.63	mediate signal transduction by binding to phosphoserine-containing proteins
41	annexin A5	gi|4502107	581	35,971 Da/4.94	anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex
28	proteasome subunit alpha type-1 isoform 2	gi|4506179	246	29,822 Da/4.24	Protein degrdation
49	ubiquitin-conjugating enzyme E2 K	gi|4885417	235	22,507 Da/5.33	ubiquitin conjugating enzyme, protein degradation
31	proteasome subunit beta type-4	gi|22538467	442	29,242 Da/4.21	Protein degradation
42	proteasome subunit alpha type-3	gi|4506183	202	28,643 Da/4.26	Protein degradation
Protein trafficking					
1	nuclear autoantigenic sperm protein isoform 2	gi|27262628	493	85,471 Da/4.26	transporting histones into the nucleus of dividing cel
19	annexin A1	gi|119582950	607	40,475 Da/6.57	promotes membrane fusion and is involved in exocytosis
23	Clathrin chain A	gi|4502899	243	23,704 Da/4.43	endocytosis
44	Ran-specific GTPase-	gi|542991	144	23,610	GTPase activator for the nuclear Ras-related

	activating protein			Da/5.19	regulatory protein Ran, converting it to the putativel inactive GDP-bound state
47	endoplasmin precursor	gi|4507677	379	92,696 Da/4.76	processing and transport of secreted proteins
Cytoskeletal proteins					
2	Drebrin	gi|18426913	336	72,078 Da/5.02	actin-binding protein thought to play a role in the process of neuronal growth
18	septin-2	gi|4758158	342	41,689 Da/6.15	causes actin stress fibers to disintegrate and cells to lose polarity.
25	F-actin-capping protein subunit beta	gi|4826659	407	30,952 Da/5.36	regulates growth of the actin filament
38	Stathmin 1/oncoprotein 18	gi|5031851	255	17,292 Da/5.76	regulation of the microtubule filament system by destabilizing microtubules. It prevents assembly and promotes disassembly of microtubules.
46	microtubule-associated protein RP/EB family member	gi|6912494	314	30,151 Da/5.02	regulate microtubule dynamics and their interactions with intracellular structures
Protein synthesis					
10	transfer RNA-Trp synthetase	gi|340368	263	53,396 Da/5.32	have aminoacylation activity
Channel protein					
45	nuclear chloride channel	gi|4588526	480	27,249 Da/5.09	stabilization of cell membrane potential
Chaperon					
3	Hsp90AA1	gi|83699649	449	98,652 Da/4.94	Help protein folding in general and prevent protein aggregation
4	heat shock cognate 71 kDa	gi|62897129	527	71, 083 Da/5.37	binds to nascent polypeptides to facilitate correct protein folding
11	T-complex protein 1 subunit beta	gi|5453603	589	57,794 Da/6.01	molecular chaperone help the folding of actin and tubulin
36	prostaglandin E synthase 3	gi|23308579	80	18,971 Da/4.35	for proper functioning of the Glucocorticoid and other steroid receptors.
8	calreticulin precursor	gi|4757900	243	48,283 Da/4.29	Molecular calcium-binding chaperone promoting folding, oligomeric assembly and quality control in the ER

Table 8. Up-regulated proteins after the loss of p90/CIP2A

Spot No.	Protein Name	Fold of change	Cancer-related?
5	Glucosidase II	1.56	No
6	elongation factor 2	1.53	Yes
11	T-complex protein 1 subunit beta	1.72	Yes
9	UDP-N-acteylglucosamine pyrophosphorylase 1	1.73	No
10	transfer RNA-Trp synthetase	1.65	No
13, 17	heterogeneous nuclear ribonucleoprotein H1	1.57	No
12	ruvB-like 1e	1.82	No
14	erbB3 binding protein EBP1	2.13	Yes
19	annexin A1	2.34	Yes
18	septin-2	2.1	Yes
45	nuclear chloride channel	1.78	No
47	endoplasmin precursor	2.03	Yes
43	inositol monophosphatase 1	1.96	Yes
33	SAP domain-containing ribonucleoprotein	1.54	Yes
41	annexin A5	2.03	Yes
46	microtubule-associated protein RP/EB family member	1.79	Yes
49	ubiquitin-conjugating enzyme E2 K	1.63	Yes
48	chromobox protein homolog 1	1.97	Yes

Table 9. Down-regulated proteins after the loss of p90/CIP2A.

Spot No.	Protein Name	Fold of change	Cancer-related?
1	nuclear autoantigenic sperm protein isoform 2	2.31	No
2	Drebrin	2.27	No
3	Hsp90AA1	1.77	Yes
4	heat shock cognate 71 kDa	1.68	Yes
8	calreticulin precursor	1.54	No
7	far upstream element-binding protein 2	2.13	Yes
36	prostaglandin E synthase 3	1.79	Yes
39	translationally-controlled tumor protein	1.43	Yes
30	ubiquitin carboxy-terminal hydrolase L1	1.25	Yes
29	prohibitin	1.98	Yes
25	F-actin-capping protein subunit beta	2.1	No
21	nascent polypeptide-associated complex subunit alpha	1.98	Yes
27	14-3-3 protein epsilon	2.15	Yes
32	glutathione S-transferase	1.73	Yes
26	dimethylarginine dimethylaminohydrolase 2	1.73	No
23	clathrin light chain A	1.84	Yes
22	acidic leucine-rich nuclear phosphoprotein 32 family member A	2.15	Yes
38	Stathmin 1/oncoprotein 18	2.31	Yes
28	proteasome subunit alpha type-3	1.56	No
15, 16	alpha-enolase	1.99	No
20	glyceraldehyde-3-phosphate dehydrogenase	2.12	Yes
24	L-lactate dehydrogenase	2.32	Yes
31	proteasome subunit beta type-4	1.57	No
35	protein DJ-1	1.64	Yes
34	Phosphoglycerate mutase 1	1.74	No

40	eukaryotic translation initiation factor 3	1.57	No
42	proteasome subunit alpha type-3	1.91	No
44	Ran-specific GTPase-activating protein	1.23	Yes
37	nucleoside diphosphate kinase A	2.15	Yes

Validation of differentially expressed proteins.

To confirm the differentially expressed protein identified in the 2-D gels, three cancer-related proteins, Annexin A1, 14-3-3 epsilon and EBP1, were chosen to confirm the altered expressions with Western-blot. As shown in figure 7, the protein changes are in consistent with our 2-DE results (Fig. 12).

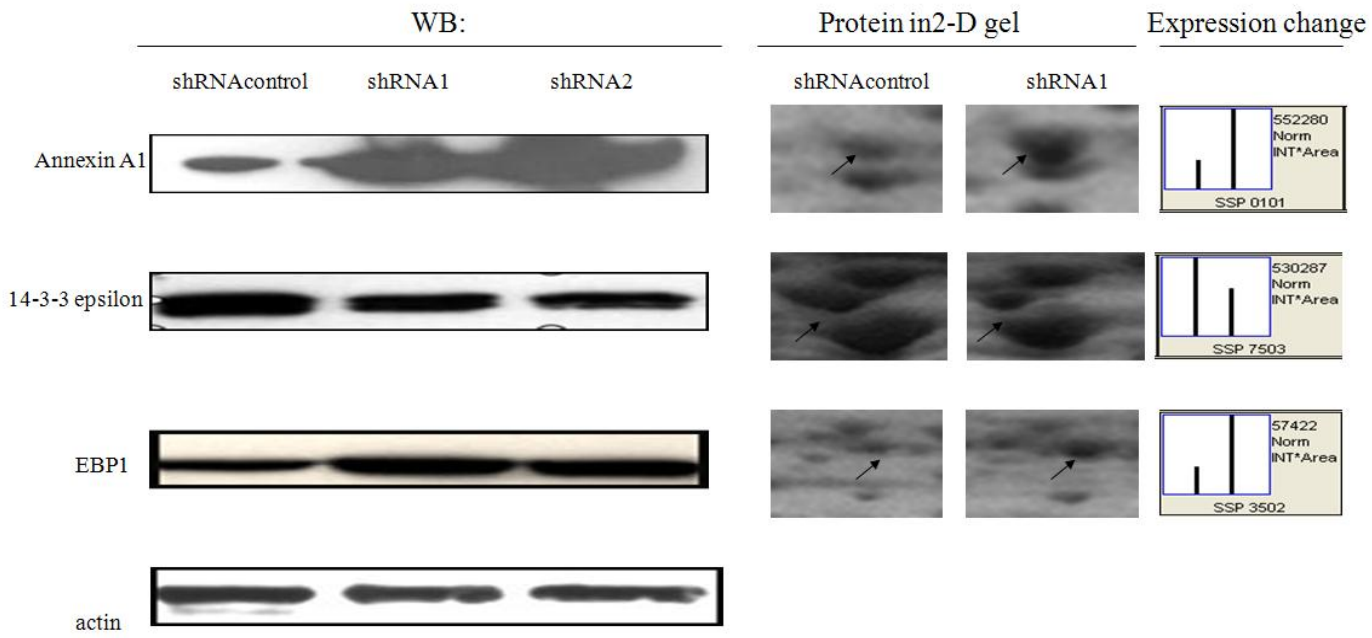


Figure 12. Validation of the differentially expressed proteins by Western-blot. H1299 cancer cell lines are transiently transfected with shRNAcontrol or shRNA1 or shRNA2. Protein expression level of EBP1, Annexin A1 and 14-3-3 epsilon were evaluated with Western-blot with specific antibodies (Left panel). The corresponding protein spots were enlarged (middle panel) and the expression changed was quantified with PDQUEST (Right panel).

Gene interaction network predict p90/CIP2A may involve in the regulation of c-myc, CREB, ETS-1 and ESR-1 transcriptional factors.

To examine whether there is an interaction between the differentially expressed proteins, these proteins were entered into the software Pathway Studio. By searching the promoter binding filter, we found that several of the differentially expressed proteins were either independently regulated by four transcription factors, including c-myc, CREB, ETS-1 and ESR-1 or co-regulated among them (Fig. 13).

ESR-1 (Estrogen receptor 1) is a ligand-activated transcription factor. It normally resides in the plasma membrane in an inactive form. Upon hormone binding, ESR-1 can translocate to the nucleus and form homodimers or heterodimers with estrogen receptor 2 (ESR-2) to drive downstream gene transcription. Overexpression of estrogen receptors have been observed in many different types of cancer, especially in hormone-dependent cancers like breast cancer and prostate cancer [123]. ETS-1 (protein c-ETS-1), encoded by ETS-1 gene, belongs to ETS gene family, which shares the ETS domain. ETS-1 is expressed both in normal cell and cancer cells. This proto-oncogene regulates the expression of several angiogenic and extracellular matrix remodeling factor, which promote an invasive phenotype [124]. c-myc is a transcription factor playing roles in development and cell transformation. It is involved in a wide range of biological processes including metabolism, DNA replication, transcription, cell growth, differentiation and apoptosis by controlling ~ 15% genes from flies to mammalian cells [57, 125]. c-myc is an essential proto-oncogene for cell transformation, since the c-myc knock-out mouse embryonic fibroblasts cannot be transformed by other oncogenes. The mutation of gene by gene amplification is very common in different types of cancers, which promotes cell proliferation and metabolic rates [57, 126]. CREB (cyclic AMP responsive element binding protein), is a transcription factor involved in diverse range of biological processes like cell proliferation, cell survival and glucose metabolism [126]. The most significant role of this protein is in neural plasticity and long-term formation [126]. The relationship between CREB and cancer is inferred from the fact that CREB can

be activated by growth factors, which normally promote cell proliferation in cancer cells [126]. Of notice, two of the transcription factors, c-myc and ETS-1, have been reported to be associated with p90/CIP2A. p90/CIP2A can regulate the c-myc stability by inhibiting its associated PP2A phosphatase activity while ETS-1 promote the overexpression of p90/CIP2A through MEK-ERK pathway [127].

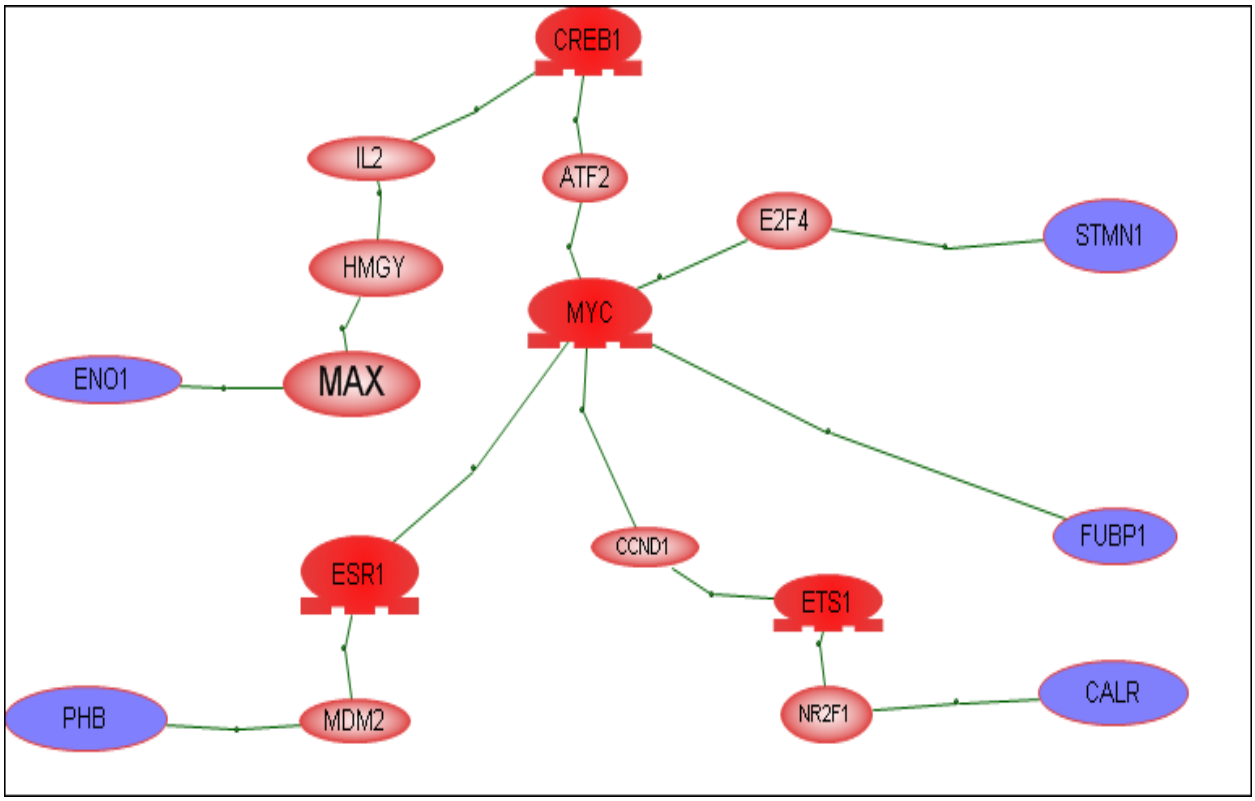


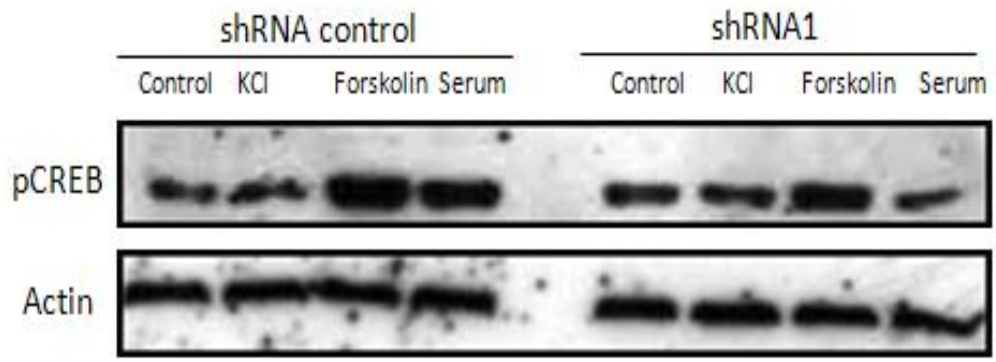
Figure 13. **Construction of gene interaction network by using Pathway Studio.** The differentially expressed proteins are searched against potential transcription factors in Pathway studio database. The four highlighted proteins, MYC, ESR1, CREB and ETS are the nexus proteins, which share common protein targets.

p90/CIP2A may regulate CREB transcription through growth factor mediated AKT-CREB pathway.

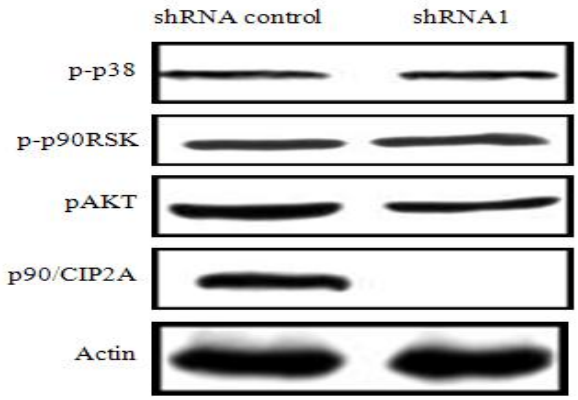
To confirm our bioinformatics prediction, we directly tested whether p90/CIP2A can regulate the activity of CREB. The activation of CREB is dependent on the phosphorylation at its serine 133 residue [128]. There are multiple pathways which can regulate the phosphorylation event. Second messengers like calcium and cAMP activate extracellular-regulated kinases (ERK) or protein kinase A (PKA) to induce CREB phosphorylation. Growth factors like EGF can also trigger CREB phosphorylation by activating the PI3K-AKT and p38 signaling pathways [128]. Therefore, we tested whether the loss of p90/CIP2A would lead to impaired CREB phosphorylation at S133 by using phosphor specific antibody. After being starved for 24 hrs, H1299 and H1299-p90/CIP2A shRNA cells were treated with either KCl, which depolarized cell membrane and induced calcium influx, forskolin or serum. Interestingly, the treatment with KCl and forskolin did not significantly alter the phosphorylation of CREB. In contrast, the serum treated H1299-p90shRNA showed large reduction of phosphorylated CREB (Fig. 14A). This result implied that p90/CIP2A may affect the CREB phosphorylation through growth factor stimulated pathway.

One of the most important growth factors associated with lung cancer is the EGF. Upon ligand binding, the downstream MAPKs will be activated, which lead to cell proliferation. Therefore, we examined the phosphorylation of the proteins, which are known to regulate the phosphorylation of CREB. Interestingly, only the phosphorylation of AKT is downregulated, while the phosphorylation of p90RSK and p38 is not affected (Fig. 14B). Furthermore, the overexpression of p90/CIP2A also promote the phosphorylation of CREB at S133 through AKT, whose phosphorylation was also elevated (Fig. 14C&D). Taken together with the previous results that p90/CIP2A can decrease AKT associated PP2A phosphatase activity, we conclude that one of the downstream targets of AKT is CREB (Fig. 14E).

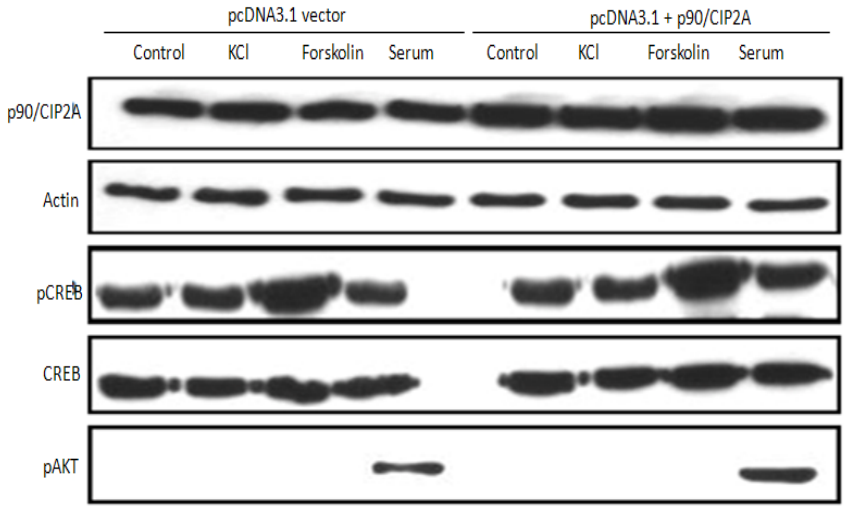
A.


shRNA control
shRNA1
Control
KCl
Forskolin
Serum
Control
KCl
Forskolin
Serum
pCREB
Actin

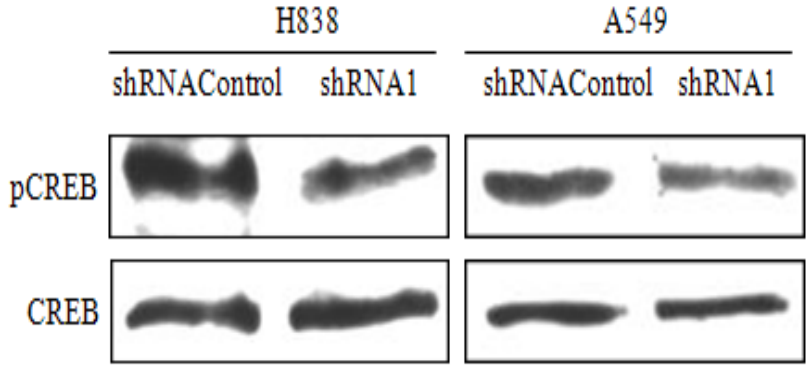
B.


shRNA control
shRNA1
p-p38
p-p90RSK
pAKT
p90/CIP2A
Actin

C.


pcDNA3.1 vector
pcDNA3.1 + p90/CIP2A
Control
KCl
Forskolin
Serum
Control
KCl
Forskolin
Serum
p90/CIP2A
Actin
pCREB
CREB
pAKT

D.



E.

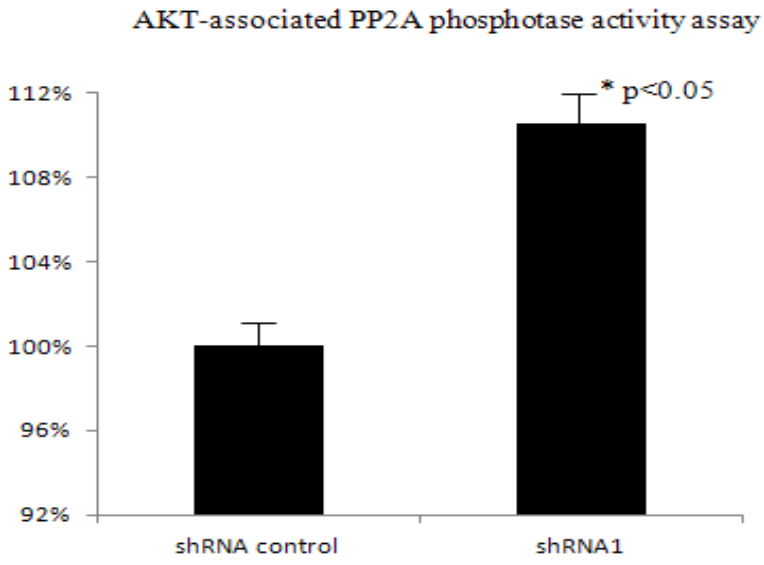


Figure 14. p90/CIP2A regulate CREB phosphorylatio through AKT. A) H1299 transduced with either control shRNA or shRNA1 were serum starved for 16hrs and then treated with either KCl (60mM), forskolin (10μM) or complete growth medium containing 10% FBS. B) Western-blot results shows that treatment with EGF resulted in reduced phosphorylation of AKT but not p90RSK and p38, all of which are upstream kinases of CREB. C) & D) p90/CIP2A is overexpressed in H1299 or knock-down in H838 and A549 cell lines and the phosphorylation of CREB and AKT was evaluated. E) Evaluate of AKT-associated PP2A phosphatase activity.

3.4 Discussion

Although the protein p90/CIP2A was identified more than ten years ago, the function of this protein was recently discovered. Serving as an endogenous PP2A phosphatase activity inhibitor, this protein was found to specifically recognize the PP2A-c-myc complex, and extended the half-life of c-myc, leading to cellular transformation and increased cell proliferation. Previous studies demonstrated that this protein was overexpressed in a wide variety of cancer types including prostate cancer, breast cancer, non-small cell lung cancer, cervical cancer and gastric cancer [67, 68, 129, 130]. Decreased c-myc expression level, cell proliferation and in vivo tumor growth were consistently observed in almost all of the cancer cell lines upon the knock-down of p90/CIP2A via siRNA. The fact that p90/CIP2A can be expressed in premalignant stage and malignant stage indicated that p90/CIP2A may play essential roles in cellular transformation and in cancer progression. In our study, the phenotypic analysis of p90/CIP2A in lung cancer cell confirmed the results by showing the decreased cell proliferation, clonogenic ability and anchorage-dependent cell growth along with the knock-down of p90/CIP2A.

Unfortunately, no systematic study has been performed to analyze the proteome change upon the expression change of p90/CIP2A at the post-translational level. In this study, we implemented two-dimensional electrophoresis-based proteomic approach to explore the global protein expression change upon the loss of p90/CIP2A. With this method, we found that p90/CIP2A may participate in cell metabolism, transcriptional and translational control and protein signaling pathways. In search of the literature, it revealed that 30 out of the 47 proteins have been found to be associated with cancer. Twenty-five percent of the altered proteins were associated with metabolism. The relationship between metabolism and cancer was described long time ago after the discovery of cells undergoes glycolysis instead of oxidative phosphorylation in proliferating and cancer cells. Recently, it was found that metabolic-associated genes play active roles in cell transformation and tumorigenesis rather than by-stander. Oncogenic factors like Ras, c-myc and HIF1 have been reported to be able to reprogram cellular

metabolism pathways to favor the cancer cell growth [131]. C-myc is a oncogene that controls diverse cellular pathways, including metabolism. It is therefore not surprising that p90/CIP2A could affect the expression of metabolic enzymes like L-lactate dehydrodrenase via c-myc-dependent pathways [131]. The presence of the other c-myc-independent genes implied p90/CIP2A may regulate metabolic pathway through other unknown mechanisms.

Aberrant transcription and translation have been reported in cancer [132]. Besides the transcription factors, other factors in the transcription and translation machinery have also been found to be altered in cancer, which can be triggered by abnormal activation of upstream biochemical pathways [132]. Although the mechanism of how p90/CIP2A contributed to such alterations is still unknown, it is speculated that p90/CIP2A may exert such effects by regulating the upstream biochemical pathways like AKT. AKT is a central regulator which integrates different stimuli and then transmits the signal to appropriate substrates through phosphorylation. Upon the stimulation of growth factors, AKT is able to promote protein synthesis by regulating the phosphorylation of ribosomal protein p70 S6 kinase, and eukaryotic initiation factor 4E binding protein. We also found that p90/CIP2A is able to regulate the phosphorylation status of AKT, which may partially explain the resulted changes of transcription and translation machinery from p90/CIP2A knock-down [133].

Deregulated pathways are common events in cancer progressions [134]. Key signaling molecules will either be increased to promote tumor growth or down-regulated to reduce the constraint of cell proliferation. In our study, we found several signaling molecules that were altered with the loss of p90/CIP2A. We chose three of them for Western-blotting validation. 14-3-3 epsilon belongs to the 14-3-3 family which has the feature to bind to the phosphor-serine residue and regulate composition of different protein complexes. Signaling molecules like TAK1 and protein phosphatase 2C beta (PPM1B) in MAPK pathway can be regulated 14-3-3 epsilon [135]. Interestingly, molecules in the MAPK pathway are also targets of PP2A. Therefore, p90/CIP2A may be able to modulate the activity of the

MAPK pathway by directly targeting MAPK substrates or indirectly regulate through 14-3-3 epsilon. Another interesting molecule altered by p90/CIP2A is the EBP1. EBP1 negatively regulates the signaling pathway transduced by ErbB3 [136]. The observation that the expression of EBP1 was increased upon the loss of p90/CIP2A raise the possibility that p90/CIP2A might be able to regulate the down-stream signaling pathways through the regulation of EBP1.

Taking advantage of bioinformatic tool, we predicted four transcription factors that may partially contribute to the expression change of the differentially expressed proteins. Although the direct interaction network of p90/CIP2A with other known pathways was not detected in the prediction, we were able to find several differentially expressed proteins that share common transcription factors such as CREB, ESR-1, c-myc and ER-1. The presence of c-myc in the bioinformatic analysis ensures the reliability of the prediction by this method. We chose to examine the phosphorylation of CREB based on three reasons. First, CREB is a transcription factor that has been implicated in the development of lung cancer. Second, CREB is a substrate of AKT, whose phosphorylation has been shown to be regulated by p90/CIP2A in hepatocellular carcinoma. The third reason is that p90/CIP2A is mainly localized in the cytoplasm while CREB is localized in nucleus. In this case, we hypothezed that p90/CIP2A may be able to influence CREB phosphorylation by regulating the activity of upstream kinases. In fact, CREB can be activated by multiple signaling pathways, including increased concentration of cAMP, membrane depolarization and growth factors. Our study found that only the growth factor can induce the phosphorylation change of CREB, but not KCl and forskolin. This indicated that p90/CIP2A is able to enhance cell proliferation by post-translational regulation of AKT-CREB axis, which is c-myc independent.

Although p90/CIP2A regulates the phosphorylation of CREB through AKT, the mechanism is still unknown since CREB is mainly localized in the nucleus, the p90/CIP2A, localized in the cytoplasma, seems to not be able to target CREB in a direct way. Therefore, this event may occur

through the regulation of upstream kinases like AKT. Although previous studies showed p90/CIP2A can target AKT, we report novel data that p90/CIP2A can regulate the phoshporylation of certain AKT substrate.

In conclusion, our proteomic study not only extended our understanding of the role of p90/CIP2A in lung cancer progression by modulating the expression of cell-related behavior, we also found that p90/CIP2A can regulate certain AKT substrates. Follow-up studies may be performed to identify the AKT substrates that can be recognized by p90/CIP2A, which will help us elucidating the role of p90/CIP2A in AKT signaling network.

Chapter 4: p90/CIP2A promote cancer cell proliferation by regulating glycolytic pathway

4.1 Introduction

Cancer results from uncontrolled cell proliferation, which requires high demands of energy to fuel the cell growth and division. Glucose is the major source of energy production in normal and cancer cells. In normal cells, glucose is processed by glycolysis to produce pyruvate in the cytosol, which is subsequently converted to carbon dioxide in the mitochondria under aerobic condition. However, glycolysis is favored under anaerobic condition, where the little pyruvate will be dispatched to mitochondria for further processing. Otto Warburg was the first person to observe that cancer cells preferred the use of glycolysis to generate energy in the presence of ample oxygen, which is known as "Warburg effect" [77, 137]. Based on recent advances in the discovery of mutated metabolic genes in cancer, the conception that metabolic pathways in cancer cell had been reprogrammed by activated oncogenes and inactivated tumor suppressors to promote cell proliferation was proposed, and it is now accepted as a hallmark of cancer [82, 138] . Although the glycolytic pathway had been well elucidated, the oncogene regulating this process is still poorly understood.

p90/CIP2A (Cancerous inhibitor of PP2A), a recently characterized oncoprotein, promotes cell proliferation and tumor growth by stabilizing the oncogenic transcription factor, c-myc, at post-translational level [62]. c-myc regulates the transcription of almost 15% of the human genome, and promotes diverse cellular functions including cell proliferation, cell differentiation, cell cycle, metabolism and apoptosis [57, 139]. The deregulated c-myc expression has been found in many different types of cancer and is considered an essential gene for tumor progression. Under normal circumstance, the transcriptional activity of c-myc is positively regulated through phosphorylation at Serine 62 and negatively regulated by desphosphorylation of S62 by PP2A and phosphorylation of T58 by GSK3β [47]. The synergism of PP2A and GSK3β lead to the ubiquitin-dependent degradation of c-

myc[47]. Therefore, the role of p90/CIP2A in promoting c-myc stability can lead to increased cell proliferation of c-myc. In contrast, the down-regulation of p90/CIP2A can greatly inhibit the cell proliferation in many different cancer cell lines including, breast cancer cell line, prostate cancer cell line and cervical cancer cell lines. However, whether this protein has any other function in regulating cell proliferation is still unknown. Our previous studies using proteomic approach established that several metabolic-associated enzymes, and glycolytic enzymes were altered when endogenous p90/CIP2A was depleted. Given the function of p90/CIP2A in cell proliferation, we hypothesize that p90/CIP2A may play important roles in the integrity of metabolic network in cancer cells.

Recently developed metabolomics is expected to facilitate the identification of the regulation. Metabolomics can provide global qualitative and quantitative analysis of small-molecule metabolites by investigating chemical processes involving the metabolites, and shows great promise as a means to identify biomarkers of drug efficacy and understanding of metabolic mechanisms [140, 141]. The adoption of high-throughput metabolite separation method, coupled with pattern recognition analysis, could possibly explain whether the knock-down and over-expression of p90/CIP2A could affect cellular metabolites. In addition, it may help identify what crucial biomarkers play an important role in the evens. In the present study, metabolomics carrying cellular metabolites of H1299 cells were developed to analyze changes of metabolites in the absence and over-expression of p90/CIP2A. First, we were interested in identifying metabolites that, being specifically regulated in the absence and over-expression of the protein, might be involved in cell proliferation. Second, we wanted to exploit the metabolomics data to identify, among the protein-regulated metabolites, metabolites capable of functionally regulating cell proliferation, as p90/CIP2A does. Thus, providing reasonable mechanisms that described how proteins promote the cell proliferation through metabolites. We hypothesize that the achievement of the two objectives will provide new data that correlates cell proliferation with the interaction between p90/CIP2A and metabolites.

Here we present a comparative analysis of metabolic regulation in H1299 cells with over-expression of p90/CIP2A and without p90/CIP2A. We further identified the key altered levels of metabolites as biomarkers and described their differential changes. At last, we report the use of crucial metabolites that were down-regulated and up-regulated in H1299 cells with over-expression and loss of p90/CIP2A, respectively, to regulate the proliferation. These results may have significant implications, not only in representing an example of the use of differential metabolomics approach to investigate the interrelation of oncogene with metabolites, but as an indication of how this technology can be exploited in the mechanisms of oncogenesis.

4.2 Materials and methods

Metabolite extraction. Total metabolites were extracted as described previously with modifications [142]. Briefly, cells were grown in 10-cm petri dish until they reach 80-90% confluence. After the removal of growth media, cells were immediately washed three times with ice-cold phosphate buffered saline (PBS) and quenched with 1ml 100% cold methanol (-20^{o}C). Cells were detached from petri dish by cell scraper and collected in 2ml Eppendorf tubes. To lyse the cells, samples were sonicated three times with 3W for 20s in ice using Sonicator 3000. Cell lysates were centrifuged at 16,000 x g for 15 mins at 4^{o}C. Supernatants were collected into a new tube, and the cell pellets were extracted again with another 500 ul cold methanol and processed as described above. After the centrifuge, the pellets were re-extracted one more time with 500ul HPLC-grade water. The supernatants collected were combined, dried in a room-temperature vacuum-speed centrifuge, and stored at -80^{o}C for further experiment.

GC-MS analysis. GC-MS analysis is carried out with a variation on the two-stage technique as described previously [142]. In brief, samples were derivatized and then used to protect carbonyl moieties through methoximation through a 90 min 37℃ reaction with 40 μL of 20 mg/mL methoxyamine hydrochloride (Sigma-Aldrich) in pyridine. This was followed by derivatization of acidic protons through a 30 min 37℃ reaction with an addition 80 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, Sigma-Aldrich). The derivatized sample of 1 μL was injected into a 30m × 250 μm i.d. × 0.25 μm DBS-MS column using splitless injection, and analysis was carried out by Trace DSQ II (Thermo Scientific, IL). The initial temperature of the GC oven was held at 85℃ for 5 min followed by an increase to 330℃ at a rate of 15℃ min^{-1} , then held for 5 min. Helium was used as carrier gas and flow was kept constant at 1 mL min^{-1}. The MS was operated in a range of 50-600 *m/z*.

Data processing. Spectral deconvolution and calibration was performed using AMDIS and internal standards. A retention time (RT) correction was performed for all the samples. Then the RT was used as

reference. The remaining spectra were acquired and a file containing the abundant information of each metabolite from all the samples was assembled. Metabolites from the GC-MS spectra were identified by searching the National Institute of Standards and Technology (NIST) library, the NIST MS search 2.0. The resulting data matrix was normalized using the concentrations of added internal standards. The sum peak area was not used since it showed a positively significant correlation with the double increasing BLFX concentrations. The resulting normalized peak intensities formed a single matrix, with Rt-m/z pairs for each file in the dataset. This file was then used for further statistical analysis.

Independent component analysis (ICA), is a popular data-driven blind source separation technique that has been applied to a number of biological and medical applications [143]. In the analysis, the generative linear mixing model, X = EC, is used, where X is the mixture that is factorized into a mixing matrix E and independent source component C. It is able to add a further value for biological interpretation, since it is an extension of covariance analysis by looking for kurtosis thresholds or high entropy. To test for differences in the median concentrations of metabolites between experimental and control groups, one-way analysis (ANOVA) was used. Differences were considered statistically significant at $p < 0.05$.

Statistical analysis. After the normalization, the standards were removed so that the data used for modeling consisted of extracted compounds. Components that were identified as the same compound were averaged. Metabolic data were presented as means ± SDs. Transformations and manipulations were done using Excel. Significance Analysis of Microarrays (SAM), and hierarchical clustering (HCL) were performed with MeV 4.7.4. SAM was used to examine expression differences between the double increasing BFLX concentrations by using a multi-class comparison, which consisted of seven groups and 1.91 were used as δ cutoffs. Orthogonal function partial least squares (O-PLS-DA) were applied to the different variable scaling methods for comparison purposes (unit variance, Pareto and centered scaling) using SIMCA 12.0. Potential markers of interest were extracted from the S-plots

constructed after the analysis with OPLS-DA, since they showed statistically significant ($P < 0.05$) difference in abundance, and were significantly elevated (p(corr)[1] > 0.85). Ingenuity network analysis was performed with MetPA (http://metpa.metabolomics.ca./MetPA/faces/Home.jsp) based on database source including the KEGG (http://www.genome.jp/kegg/), Metabolic Pathways Database (http://metacyc.org/), *Escherichia coli* K-12 (http://ecocyc.org/) to identify the affected metabolic pathways analysis & visualization. The possible biological roles were evaluated by the enrichment analysis using the MetaboAnalyst. T-test and correlation analysis were performed using Excel.

Hexokinase assay. Hexokinase assay was performed using the Hexokinase Assay Kit (Biomedical Research Service Center, University of Buffalo, NY) according to manufacturer's instructions. Briefly, 10^5-10^6 cells were washed twice with ice-cold PBS and lysed with 100µl 1X cell lysis solution. Cell lysates were centrifuged in a refrigenerated microcentrifuge for 3 mins at maximal speed, collected and stored on ice for kinase assay.

Kinase assay was initiated by adding 50µl HK working solution (20 µl HK substrate dissolved in 1 ml HK assay solution) to 50 µl cell lysates in 96-well microplate (Corning, CA) and incubate at 37^{o}C for 10 min or 20 mins. Reaction was stopped by adding 50 µl 3% acetic acid. Colometric absorbance was measured with microplate reader at O.D. 492 nm (Thermo Scientific, IL). Kinase activity was measured by a kinetic format and calculated according to the following equation:

Enzyme activity in Units/ml= (O.D. 20min – O.D. 10 min) X 110.

4.3 Results

Effect of p90/CIP2A on characteristics of cellular metabolic response

To investigate the role of p90/CIP2A in cellular metabolism, GC/MS based metabolomics approach was used to investigate differential metabolic responses to the absence and over-expression of p90/CIP2A. Quantitative data was obtained for 97 metabolites from 176 peaks, after the deletion of seven peaks that were below the limit of quantitation or too unstable to yield reliable information (Fig. 15) . The 97 compounds were categorized into six metabolic pathways: amino acid metabolism (24), carbon metabolism (30), fatty acid metabolism (18), oxidative phosphorylation (3), nuclear acid metabolism (4) and unknown (18) (Fig. 16A) (Table. 10). The supervised OPLS model was used to enhance biomarker discovery efforts. The OPLS-DA approach distributed the five sample groups into three faces in a 3-D scatter plot. The control, plk0.1, 90.5, 90.2, and 90+ were located in the bottom face, left and right faces, respectively (Fig. 16B). Thus, the OPLS-DA approach was applied to obtain better discrimination between the control and experimental groups, based on the differences in their metabolic profiles, and the experimental groups between the loss and overexpression of p90/CIP2A. The 97 compounds were shown in a clustered heat-map format. The format showed that 90.2 and 90.5 were separately clustered together, and then p90+ was added (Fig. 16C). The three groups were closely related to each other, compared with two controls, wide type cell (WT) and plk0.1, which were clustered together. These results showed that the metabolic profiles had significantly adjusted as a result of p90/CIP2A absence or over-expression.

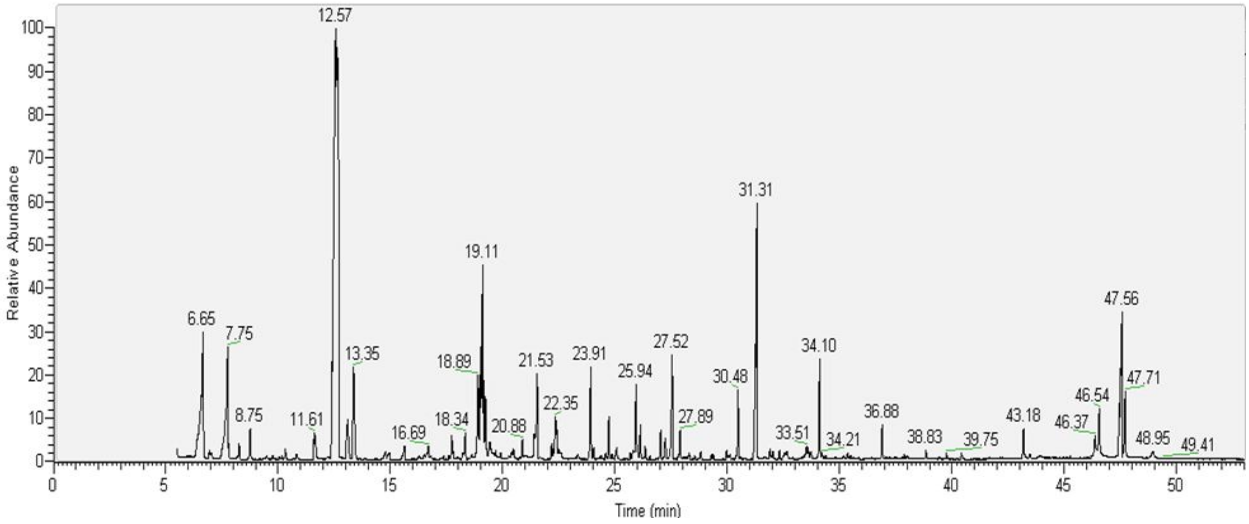
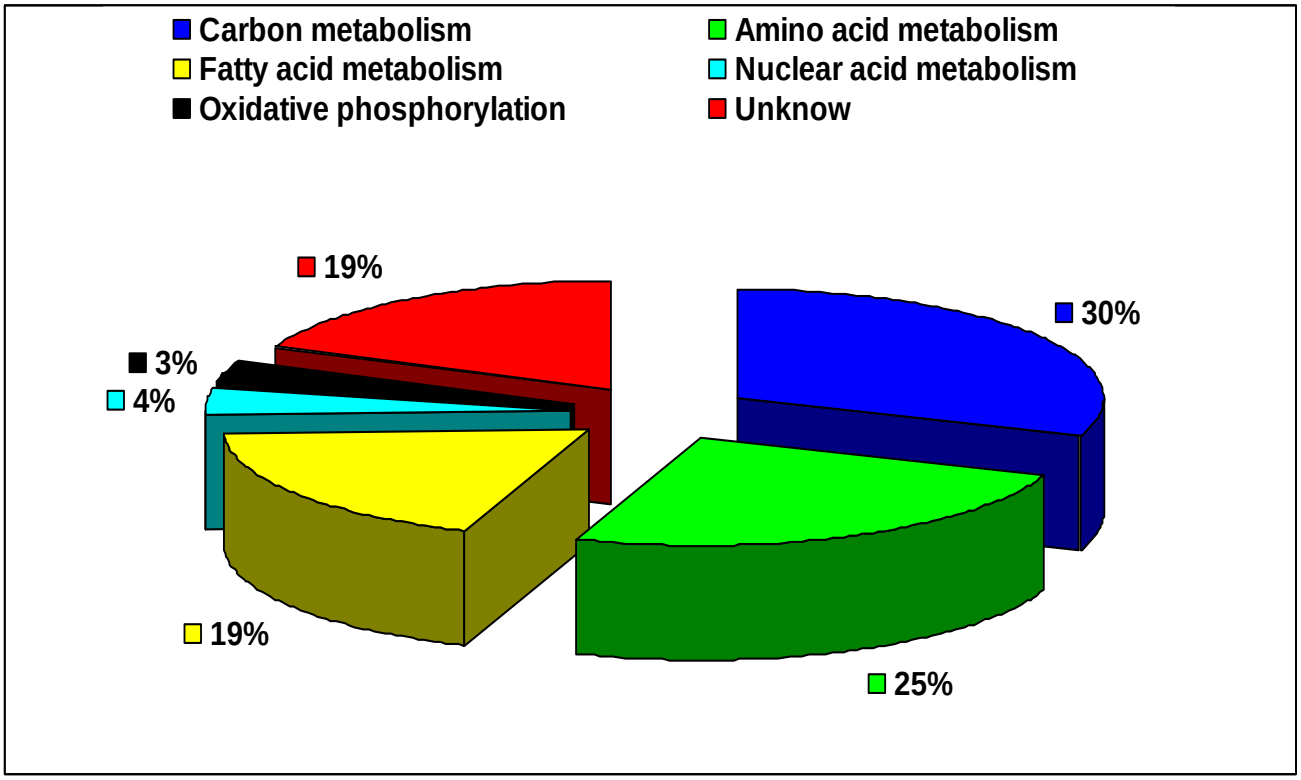
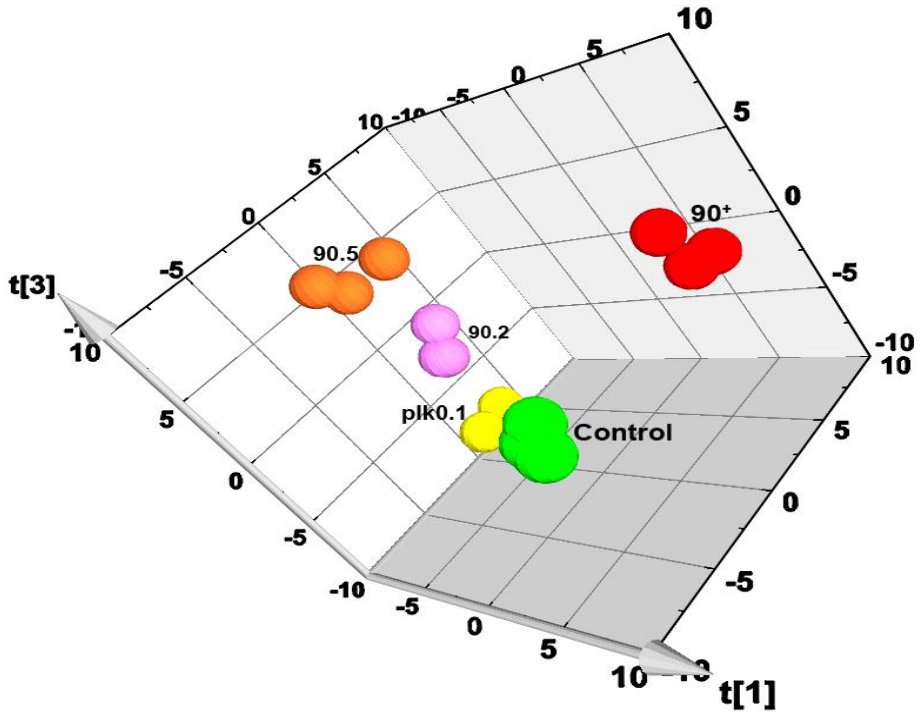


Figure 15. Metabolic profiling by GC/MS. It shows the capacity to resolve 79 compounds within 50 mins run. GC/MS chromatogram of the total metabolites extracted from H1299 lung cancer cell lines.

A.


Carbon metabolism
Amino acid metabolism
Fatty acid metabolism
Nuclear acid metabolism
Oxidative phosphorylation
Unknow
19%
30%
3%
4%
19%
25%

B.


t[3]
t[1]
90+
90.5
90.2
plk0.1
Control

C.

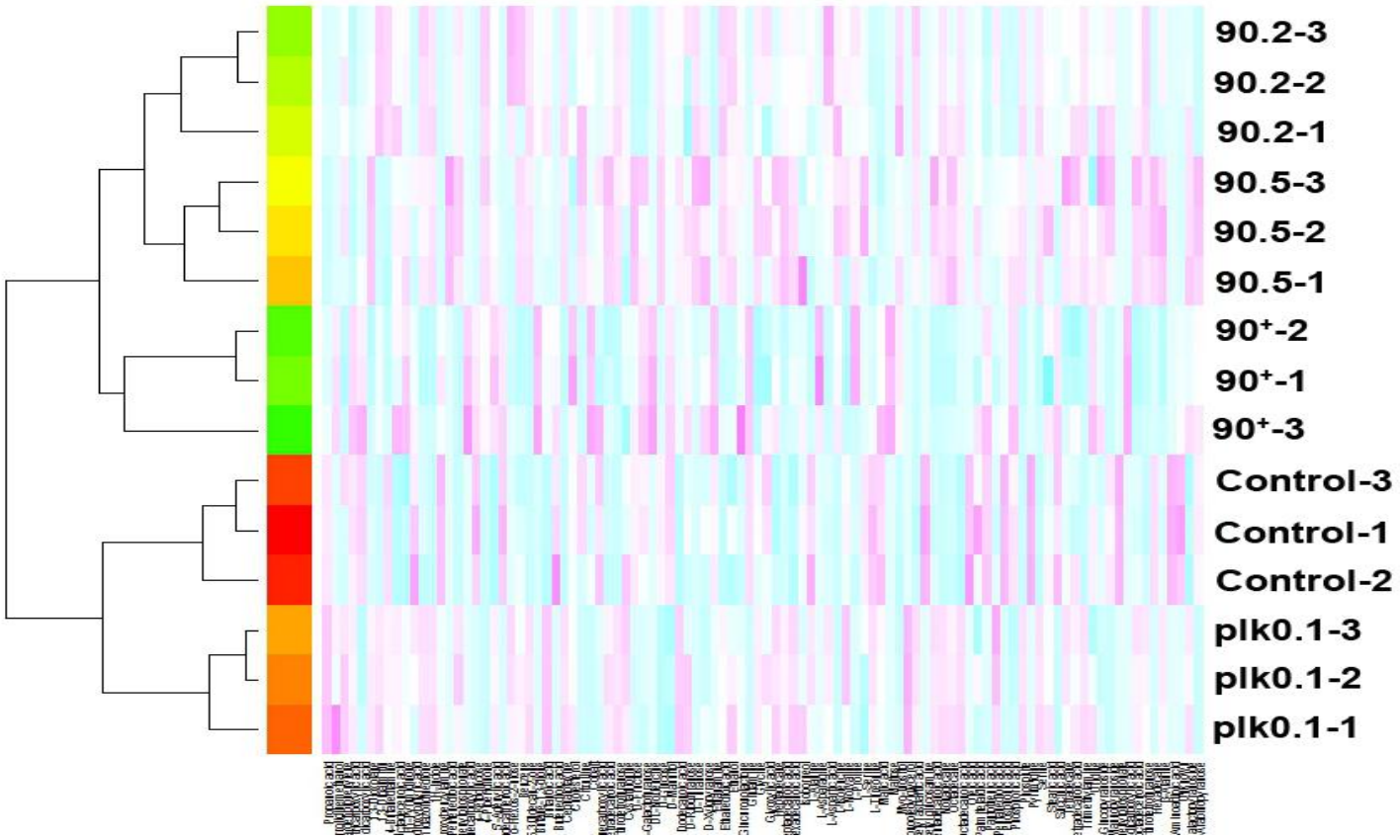


Figure 16. Profile of metabolic regulation to cells with the loss and over-expression of p90/CIP2A. A, The whole cellular spectrum profile based on metabolic pathways. B, OPLS-DA results for control and experimental groups. Significant metabolomic difference was shown between them in 3-D plot. One data point stands for one subject. C, Hierarchical clustering analysis of metabolite concentrations in cells with the loss and over-expression of p90/CIP2A. Identified metabolites were analyzed for any apparent patterns. Clustering was performed using Pearson correlation as the distance metric. Cluster tree shows how the samples and metabolites divide. Across the bottom are the metabolites, and along the side are the samples.

Table 10. Complete list of identified metabolites by GC/MS by comparing to the commercial NIST library.

Retention time	Metabolite
6.74	Propanoic acid
6.96	L-Alanine
7.67	l-Alanine
8.27	Glycine
8.77	Ethanedioic acid
9.48	Trisiloxane
9.68	(R)-(-)-2-Pyrrolidinemethanol
9.76	Phosphoric acid
10.05	Isoborneol
10.17	Glycine
10.27	Glyoxylic acid, di-TMS
10.67	2-Dimethyl(trimethylsilyl)silyloxytridecane
10.82	L-Norvaline
11.59	Phosphonic acid
12.37	Silanol, trimethyl-, phosphate
13.03	L-Isoleucine
13.08	L-Proline
13.29	Glycine
13.45	4-Piperidinone
13.85	Bis(trimethylsilyl)3,4-dimethylphenylphosphonate
14.38	L-Proline
14.79	Silanamine
14.88	L-Serine
15.51	Cobalt
15.62	N,O,O-Tris(trimethylsilyl)-L-threonine
15.94	Glycine
16.17	Glucopyranoside
16.27	Arabino-Hexos-2-ulose
16.43	Glycine

16.48	Glycine
16.68	Alanine
16.8	Benzene
17.36	Glycine
17.48	Hexadecane
17.72	Ethanamine
17.78	L-Aspartic acid
17.96	Trimethylsiloxy(trimethylsilyl)proline
18.19	Malic acid
18.61	Silane
18.89	L-Proline
19.1	L-Proline
19.42	Cyclohexanecarboxylic acid
19.68	2,3,4-Trihydroxybutyric acid tetrakis(trimethylsilyl) deriv.
19.9	Cystathionine
20.29	L-Proline
20.46	Pentanedioic acid
20.77	Citrulline
20.87	Silanamine
21.38	Glutamine
21.65	(Z,Z)-3-Methyl-3H-cyclonona(def)biphenylene
21.74	Octadecane
22.04	2-Hydroxybenzylamine
22.15	Propanoic acid
22.34	Succinic acid
23.23	Tetradecane
23.34	Heptadecane
24.04	1,4-Butanediamine
24.17	DL-Arabinopyranose
24.4	Butanoic acid
24.43	Phosphoric acid
24.72	Phosphoric acid
24.84	Uridine

25.01	N Acetyl aspartic TMS
25.07	Phosphoric acid
25.48	Ethanol
25.66	Propanoic acid
25.76	1,2,3-Propanetricarboxylic acid
26.12	d-Erythrotetrofuranose
26.4	2-Pentenedioic acid
26.54	Tetradecanoic acid
27.02	D-Fructose
27.52	d-Glucose
27.87	d-Glucose
28.04	Dodecanoic acid
28.29	D-Mannitol
28.54	n-Pentadecanoic
28.8	Phosphonic acid
29.36	Pantothenic acid
29.8	D-Glucopyranoside
29.95	2(1H)-Pyrimidinone
30.03	Palmitelaidic acid
30.1	Inositol
30.22	D-Mannopyranoside
30.48	Hexadecanoic acid
31.3	Myo-Inositol
31.63	Myo-Inositol
31.88	1,3-Dioxolane
31.99	Cyclopropanetetradecanoic acid
32.3	Heptadecanoic acid
32.54	D-Glucopyranoside
32.64	Butane-1,3-dione
32.9	Butanoic acid
33.5	D-, 2,3,4,6-tetrakis-O-(trimethylsilyl)-
33.57	11-cis-Octadecenoic acid
33.71	11-cis-Octadecenoic acid

34.1	Octadecanoic acid
34.2	16-Trimethylsilyloxy-9-octadecenoic acid
35.16	D-Galactofuranose
35.34	d-Glucose
35.58	Octadecane, 3-ethyl-5-(2-ethylbutyl)-
36.61	D-Xylopyranose
36.87	Myo-Inositol
38.82	1,2-Benzenedicarboxylic acid
38.98	Octadecane
39.64	Glucuronolactone
39.74	Hexadecanoic acid
40.4	Uridine
40.47	D-Glucopyranoside
40.49	1,3-Dipalmitin trimethylsilyl ether
40.95	D-Xylopyranose
41.93	D-Turanose
42.09	D-Glucopyranoside
42.25	1-Monooleoylglycerol trimethylsilyl ether
42.5	D-Glucopyranoside
43.15	2(1H)-Pyrimidinone
43.45	2,4-Imidazolidinedione
43.82	2(1H)-Pyrimidinone
45.23	D-Glucopyranoside
46.2	D-Ribofuranose
46.34	2(1H)-Pyrimidinone
46.54	5'-Adenylic acid
47.54	Cholesterol trimethylsilyl ether
47.7	D-Galactopyranoside
48.92	Stearic acid

Identification of sample recognition using independent component analysis (ICA)

To determining the characteristic metabolites in cells with absence and over-expression of p90/CIP2A, the multi-class unpaired method of SAM was used to identify the metabolites whose concentration differed significantly among all samples collected. SAM analyses were able to differentiate the difference. SAM (for a delta value of 1.91% and 0% false discovery rate (FDR)—median) identified 38, 50, 61, 70, 71, 73 peaks representing 30, 42, 48, 53, 54, 56 metabolites in BLFX-con64, BLFX-con32, BLFX-con16, BLFX-con8, BLFX-con4 and BLFX-2, respectively (Fig. 16). The 56 positively significant metabolites from 73 peaks detected in BLFX-2 contained all changes occurred in the other samples. These were listed in order by decreasing significance: tetradecanoic acid, palmitelaidic acid, phosphoric acid, glycine, 9H-Pyrido[3,4-b] indole and Morphinan as the first six metabolites (Fig.17A-C,). According to the parametric t-test, we found that the majority of the altered metabolites were increased with the double increasing BLFX concentrations.

Then, ICA was separately used to identify significant differences in the data set. ICA is one of the unsupervised algorithms that can extract higher-order statistical structures, from which extracted transformation vectors are able to indicate the occurrence of specific metabolites. It provides similar relative metabolite level responses for different processes, due to a time-lag effect or analogous biochemical regulation. In the present study, ICA of the metabolites gave complete separation of sample groups (Fig. 18A). The extracted transformation vectors IC1–IC2 revealed information for biomarker identification. First, the three experimental groups (90.5, 90.2 and 90+) and the two controls (control and plk) are separated on IC1. The second component (IC2) depicts p90/CIP2A and vector roles in the five groups (Fig.18A). Thus, the role of p90/CIP2A was completely resolved by combining the distinct features of metabolite data.

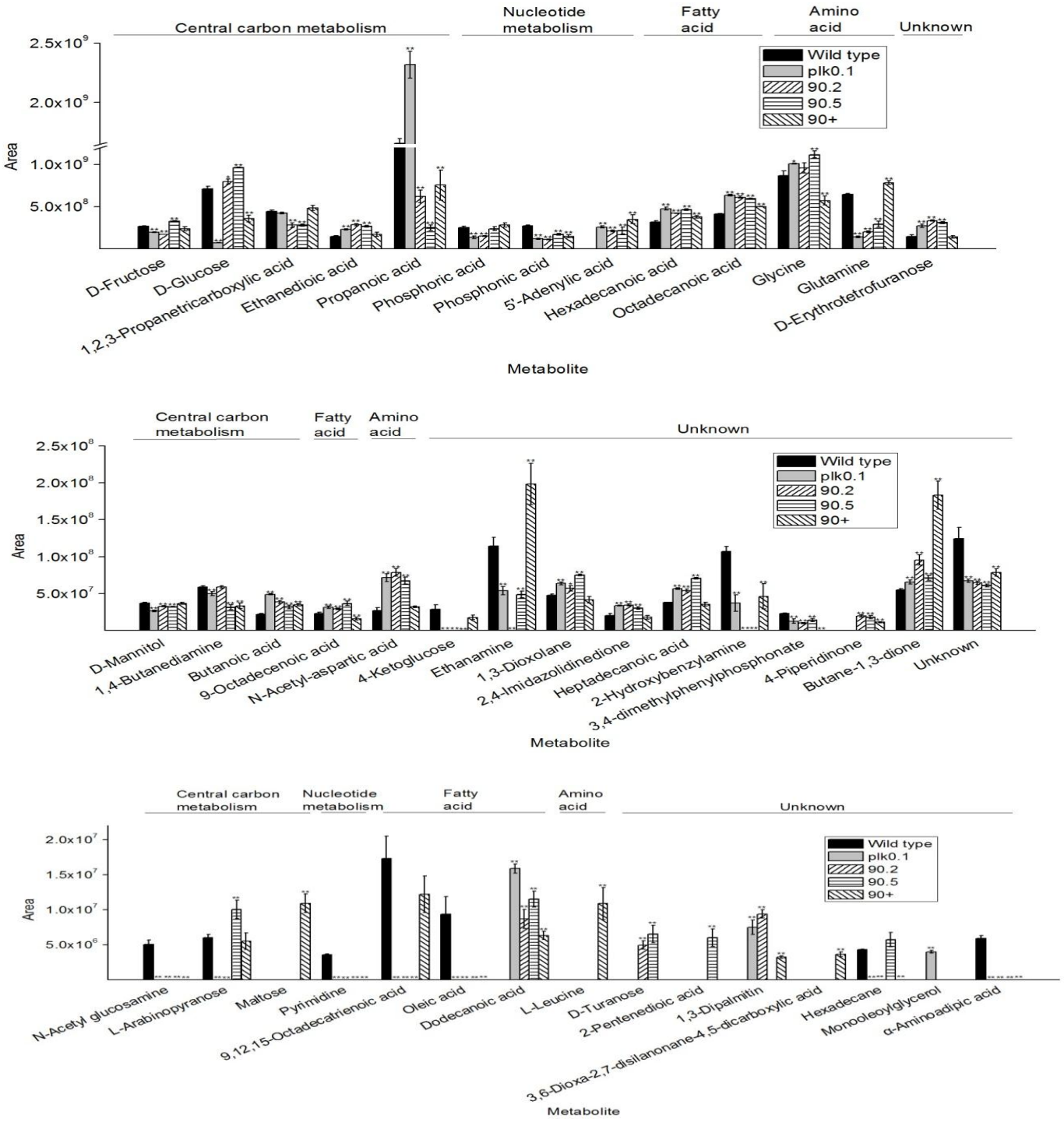
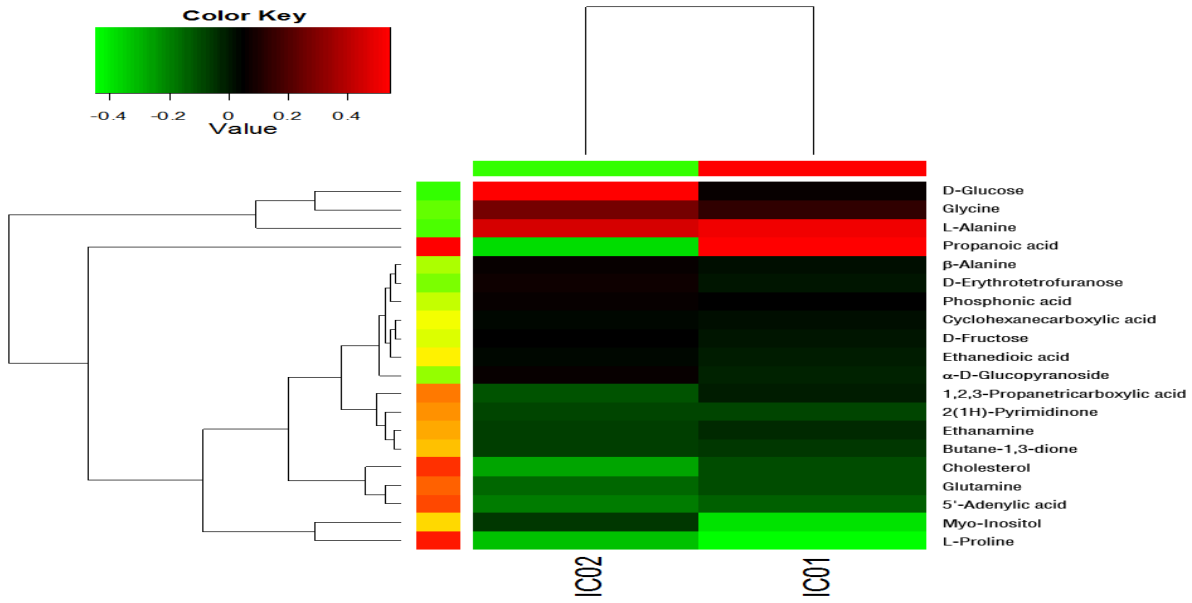


Figure 17. Histogram of significant different metabolites by SAM analysis. SAM analysis was used to differentiate the metabolites which are significantly altered in decreased or increased of p90/CIP2A expression.

A.


Color Key
-0.4 -0.2 0 0.2 0.4
Value
D-Glucose
Glycine
L-Alanine
Propanoic acid
β-Alanine
D-Erythrotetrofuranose
Phosphonic acid
Cyclohexanecarboxylic acid
D-Fructose
Ethanedioic acid
α-D-Glucopyranoside
1,2,3-Propanetricarboxylic acid
2(1H)-Pyrimidinone
Ethanamine
Butane-1,3-dione
Cholesterol
Glutamine
5'-Adenylic acid
Myo-Inositol
L-Proline
IC02
IC01

B.

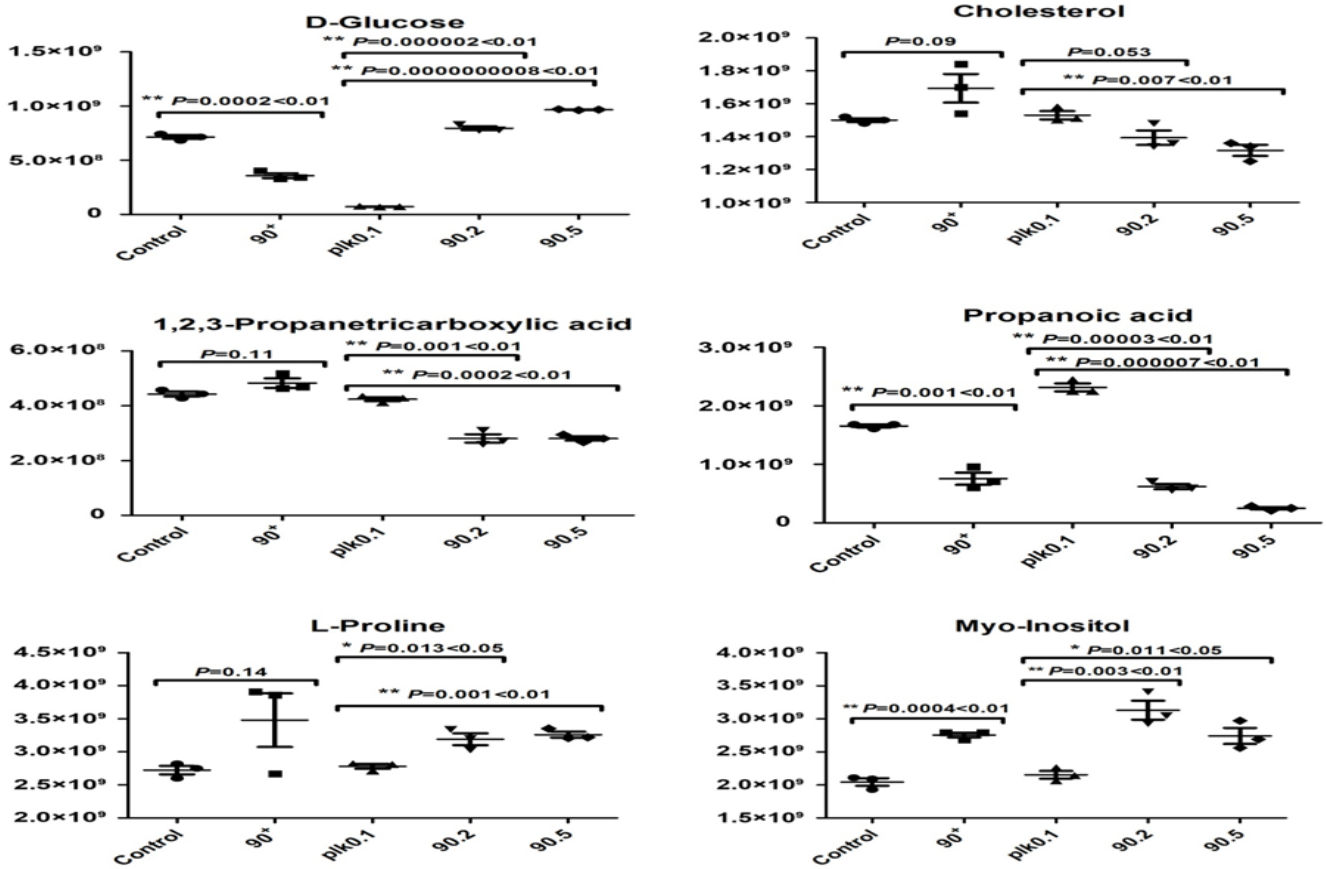

D-Glucose
** P=0.000002<0.01
** P=0.0000000008<0.01
** P=0.0002<0.01
Cholesterol
P=0.09
P=0.053
** P=0.007<0.01
1,2,3-Propanetricarboxylic acid
P=0.11
** P=0.001<0.01
** P=0.0002<0.01
Propanoic acid
** P=0.00003<0.01
** P=0.000007<0.01
** P=0.001<0.01
L-Proline
P=0.14
* P=0.013<0.05
** P=0.001<0.01
Myo-Inositol
* P=0.011<0.05
** P=0.003<0.01
** P=0.0004<0.01
Control
90+
plk0.1
90.2
90.5

Figure 18. ICA for cells with the loss and over-expression of p90/CIP2A. A, Sample pattern recognition in a metabolite covariance matrix, showing visualization of independent components 1 and 2 (IC1 and IC2). For each experiment three independent biological replicates were measured. The control and plk0.1 were used as controls of 90+, and 90.2 and 90.5, respectively. The following biological information was extracted from the plots: (a) IC1 separates two controls and three experimental groups; (b) IC2 separates the respective two comparison: control vs 90+ and 90+ vs 90.2 and 90.5. B, clustering of the ICA loadings (weights) on IC1–IC2. The loadings are proportional to the importance or significance of a metabolite for a corresponding independent component. The biclustering diagram was constructed from the first 20 ranked loadings for the corresponding IC1 and IC2. C-E, Plots of identified biomarkers for combination of IC2 and IC1. Biomarkers were identified from metabolites with high absolute weights of IC2 and low absolute weights of IC1. Glucose (C), glycine (D) and proline (E) were identified.

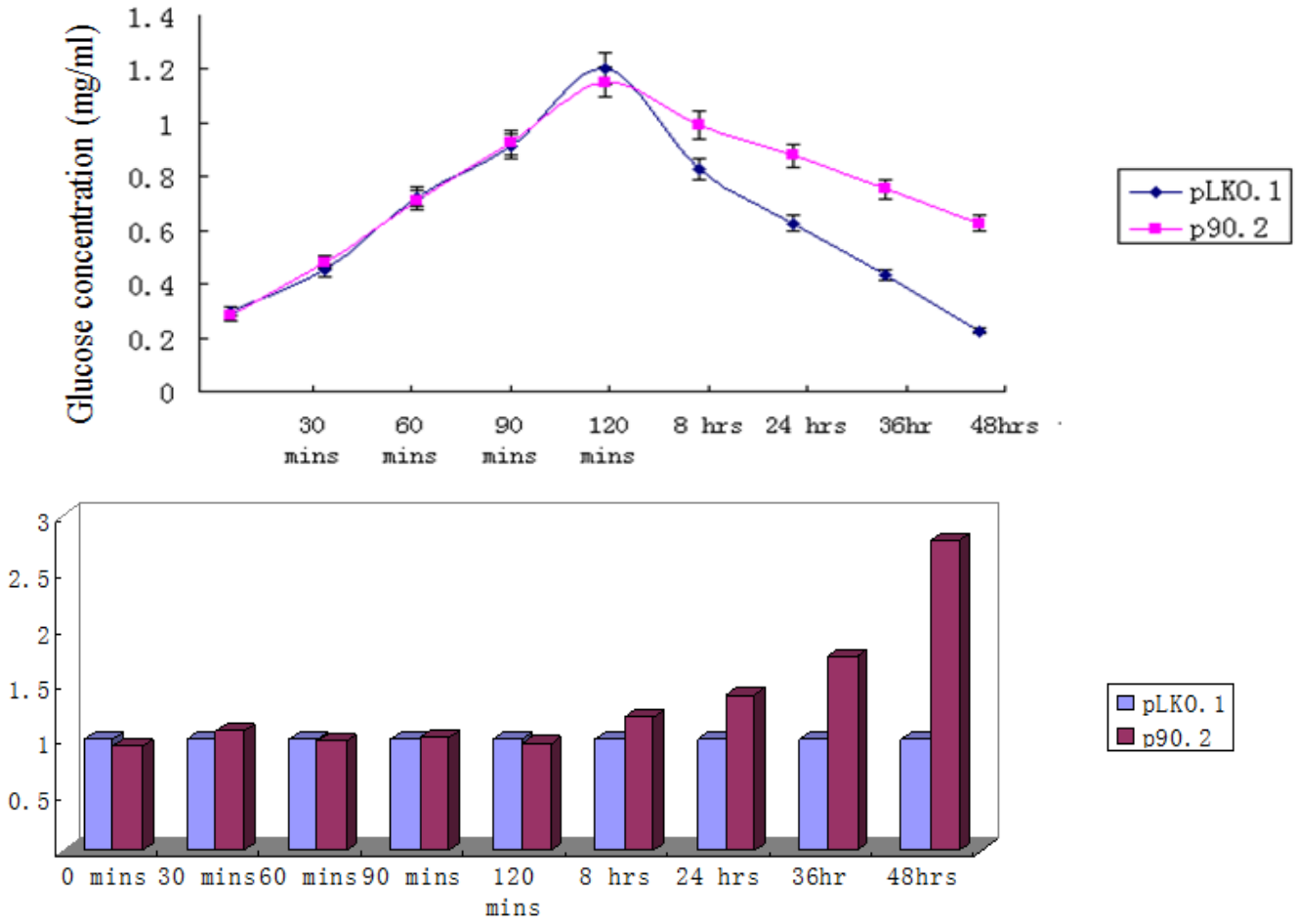
Identification of biomarkers based on metabolites of ICA

A ranked assignment of metabolite sets involved in the observed phenomena is able to be characterized based on the biological interpretation of the transformation vectors IC1-IC2, and the new independent components IC1 and IC2. The assignment is visualized in a biclustering diagram, which displays the loading of different independent components IC1 and IC2. The loading coefficients represent the relative degree of each component on the observed sample discrimination along the transformation vectors, and are directly related to their importance on this biological phenomenon. It is reasonable that crucial biomarkers are identified based on their different response effects to IC1 and IC2. Two control groups (WT and plk0.1) and three test groups (90+, 90.2 and 90.5) are clearly divided by IC1, while the samples with over-expressed p90/CIP2A (90+) and without p90/CIP2A (90.2 and 90.5) are highly related to IC2. Compared with IC1, IC2 include the following key differential metabolites: glucose, propanoic acid, L-proline, 1, 2, 3-propanetricarboxylic acid, myo-inositol, cholesterol. They have distinct difference on loading with IC1. There were two types of changes for the six metabolites: (i), The opposite regulations are detected between the loss and over-expression of p90/CIP2A compared with their respective controls, including glucose, cholesterol and 1, 2, 3-propanetricarboxylic acid. (ii) The same change trend was determined in the three tested groups, and was compared their corresponding controls, including propanoic acid, L-proline and myo-inositol. Thus, biological significance of glucose, cholesterol and 1, 2, 3-propanetricarboxylic acid was related to p90/CIP2A, and changes of propanoic acid, L-proline and myo-inositol vector was associated with the vector. The biological significance was different between the three metabolites, indicating that p90/CIP2A promotes the use of glucose and limits the use of cholesterol and 1, 2, 3-propanetricarboxylic acid. In summary, glucose, cholesterol and 1, 2, 3-propanetricarboxylic acid are three crucial biomarkers that differentiate between over-expression of p90 from the loss of p90 (Fig. 18B).

p90/CIP2A regulates glucose metabolism through hexokinase

Since the depletion of p90/CIP2A can increase the intracellular concentration of glucose, the utilization of glucose may be impaired in p90/CIP2A deficient cells. To confirm that, we quantified the glucose concentration at different time points in H1299 cell line (Fig. 19A). Interestingly, there was no significant difference of glucose concentration observed between wild type (WT) cell line and p90/CIP2A deficient cell line, which implied that p90/CIP2A does not influence the uptake of glucose (Fig. 19A). However, glucose concentration was increased to 1.2 fold in comparision to the wild type cell line (Fig. 19A) indicating the role of p90/CIP2A in the metabolic process of glucose. Hexokinase is the first enzyme to break down glucose and is subjected to the regulation of AKT. Our previous results showed that p90/CIP2A can regulate the phosphorylation of AKT in response to growth factors. Therefore, hexokinase may be affected by the loss of p90/CIP2A. As expected, the knock-down of p90/CIP2A in both of H1299 and H838 cells decreased the hexokinase activity (Fig. 19B). In contrast, overexpression of p90/CIP2A in H460 cells increases hexokinase activity to 123% compared to control cells.

A.


Glucose concentration (mg/ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
30 mins
60 mins
90 mins
120 mins
8 hrs
24 hrs
36hr
48hrs
pLKO.1
p90.2
3
2.5
2
1.5
1
0.5
0 mins
30 mins
60 mins
90 mins
120 mins
8 hrs
24 hrs
36hr
48hrs
pLKO.1
p90.2

B.

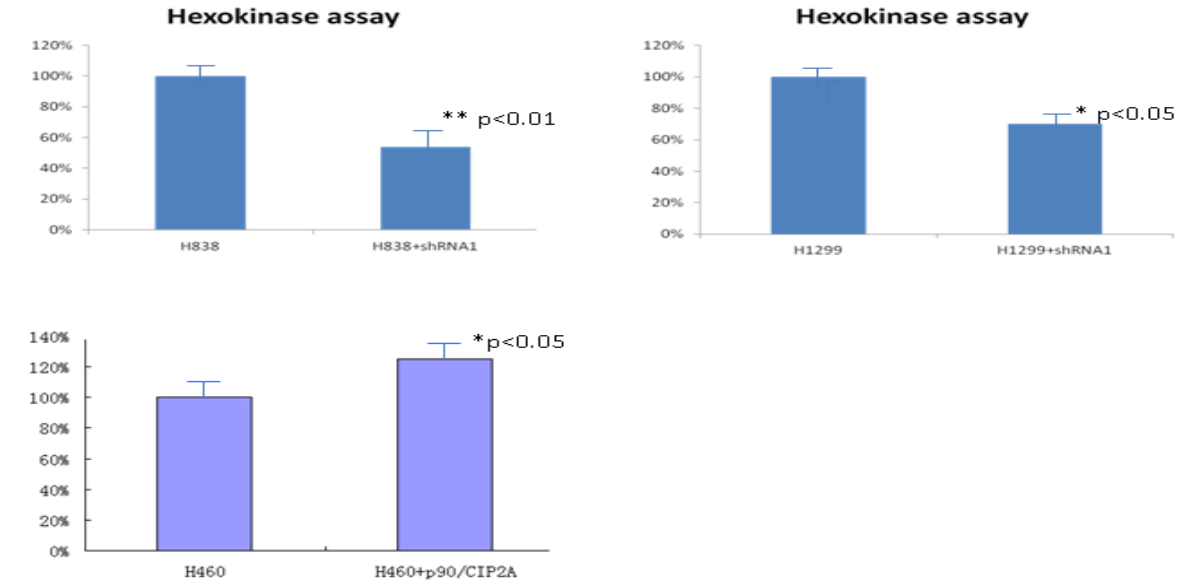

Hexokinase assay
120%
100%
80%
60%
40%
20%
0%
** p<0.01
H838
H838+shRNA1
Hexokinase assay
120%
100%
80%
60%
40%
20%
0%
* p<0.05
H1299
H1299+shRNA1
140%
120%
100%
80%
60%
40%
20%
0%
*p<0.05
H460
H460+p90/CIP2A

Figure 19. p90/CIP2A regulates glucose metabolism through hexokinase. (A) Quantification of intracellular concentration of glucose in H1299 cells. $7X10^5$ cells (H1299 shRNA control or p90/CIP2A) were seeded in 6-well plates with triplicate for 24 hrs. After incubation, cells were washed five times with glucose-free RPMI medium and incubated in glucose-free medium for 30mins to deplete the intracellular glucose. Complete growth medium was then added and cells were collected at different time points as indicated by direct lysis in RIPA buffer. Cells were centrifuged to remove the cell debris and supernants were collected for glucose quantification or for Western-blot of normalization using actin (Upper panel). Fold of glucose concentration change was normalized to control. (Lower panel) (C) Hexokinase activity was measured based on the reduction of the tetrazolium salt INT in a NADH-coupled reaction to INT-formazan, which is read at OD_{492} nm. Kinase activity was normalized to actin level.

4.4 Discussion

The reprogramming of metabolic pathways to fuel cell growth by cancer cell has been considered one of the hallmarks of cancer [85]. Both of the activated oncogenes and inactivated tumor suppressors are contributing to these alterations during cellular transformation [79]. Oncoproteins like c-myc, hypoxia-induced factor 1 (HIF1), and K-ras have been proved to be important molecules to regulate metabolism [79]. However, how these proteins contribute to cancer-associated metabolism is still poorly understood. In this study, we use global metabolic profiling to study the function of a novel oncoprotein p90/CIP2A in cancer metabolism.

We identified three metabolites that were significantly altered correspondingly with the change of p90/CIP2A level (both in knock-down and overexpression): glucose, cholesterol and 1,2,3-propanetricarboxylic acid. Of interest, glucose and 1,2,3-propanetricarboxylic acid are associated with glycolysis and Kreb cycle pathways. The knock-down of p90/CIP2A increased the intracellular glucose concentration, while the overexpression of p90/CIP2A enhanced the glucose consumption. These results implied that p90/CIP2A may positively regulate the glucose metabolism. Hexokinase is the first enzyme in the glycolytic pathway to phosphorylate glucose to produce glucose-6-phosphate. The activity of this enzyme is subjected to the regulation of AKT [92, 95, 144]. Interestingly, studies performed by our group and others had showed that p90/CIP2A is able to regulate phosphorylation status of AKT in response to growth factor stimulation and cellular stress. Therefore, the role of p90/CIP2A in promoting cell proliferation may be partially through the regulation of EGF-PI3K-AKT pathway.

Kreb cycle (tricarboxylic acid cycle, TCA cycle) is another pathway which is usually used by normal cells to generate energy. Although the defects of TCA cycle has been reported to be associated with cancer, the molecular mechanism linking TCA cycle defects to cancer remained to be defined [145, 146]. Mutations of the enzymes of TCA cycle like isocitrate dehydrogenase, succinate dehydrogenase and fumarate hydrogenase had been found in different solid tumors [147]. These mutated enzymes will

have recessive or dominant functions and produce metabolites that repress cell differentiation. However, whether there is any other factor besides mutations that interrupt normal TCA cycle in cancer, is still poorly defined. Aconitase is an enzyme in the TCA cycle that converts citrate into iso-citrate [148, 149]. Abnormal activity of aconitase has been reported in prostate cancer, which leads to high levels of citrate. The ratio of citrate to isocitrate has been considered as a marker for tumor grade differentiation [150]. However, how the activity of the aconitase is regulated in cancer is poorly understood. In our results, we find that metabolite 1,2,3-propanetricarboxylic acid, an inhibitor of aconitase, is decreased along with the overexpression of p90/CIP2A. This implied that p90/CIP2A may employ an indirect way to promote the TCA cycle in cancer cells, which leads to the cell proliferation.

Taken together, our studies showed that p90/CIP2A can promote cell proliferation by regulating metabolic pathway in cancer cells. It achieves this not only through c-myc dependent pathway, but also through a c-myc-independent pathway with the regulation of the molecules critical for glycolysis and TCA cycle. Our results expanded the understanding of the p90/CIP2A in cancer cell growth. Also, it provided an important example for the use of metabolomics in the study oncogene of function in cancer field.

Chapter 5: Final conclusions and future directions

5.1. Overview and final conclusions

Tremendous work had been done in the past decades to elucidate the molecular mechanism of oncogenesis. The inhibition of PP2A has been considered an important event for the onset of tumor formation. Therefore, the identification of factors that contribute to this inhibitory effect are of great importance. p90/CIP2A is such a protein whose function was unknown until recently. The importance of this protein in cancer has been considered valuable and an increasing number of researchers have started studying its study the biological function. Yet, little process had been made, and the physiological function of p90/CIP2A is still unknown.

In this present study, we mainly applied "-omic" approach to explore the biological function of p90/CIP2A in cancer cell behavior, especially cell proliferation. On the second chapter, we found that p90/CIP2A can regulate the phosphorylation of JNK-c-Jun pathway by using phosphoproteomic array. JNK-c-Jun is an important component of the MAPK kinase pathway, which has opposite functions in cancer by either promoting cell proliferation or initiating apoptosis. This is dependent on the extracellular stimuli. In response to the EGF treatment, p90/CIP2A can increase the phosphorylation of JNK and its substrate c-Jun and ATF2, which implied a possible target of p90/CIP2A. This regulation was not observed before.

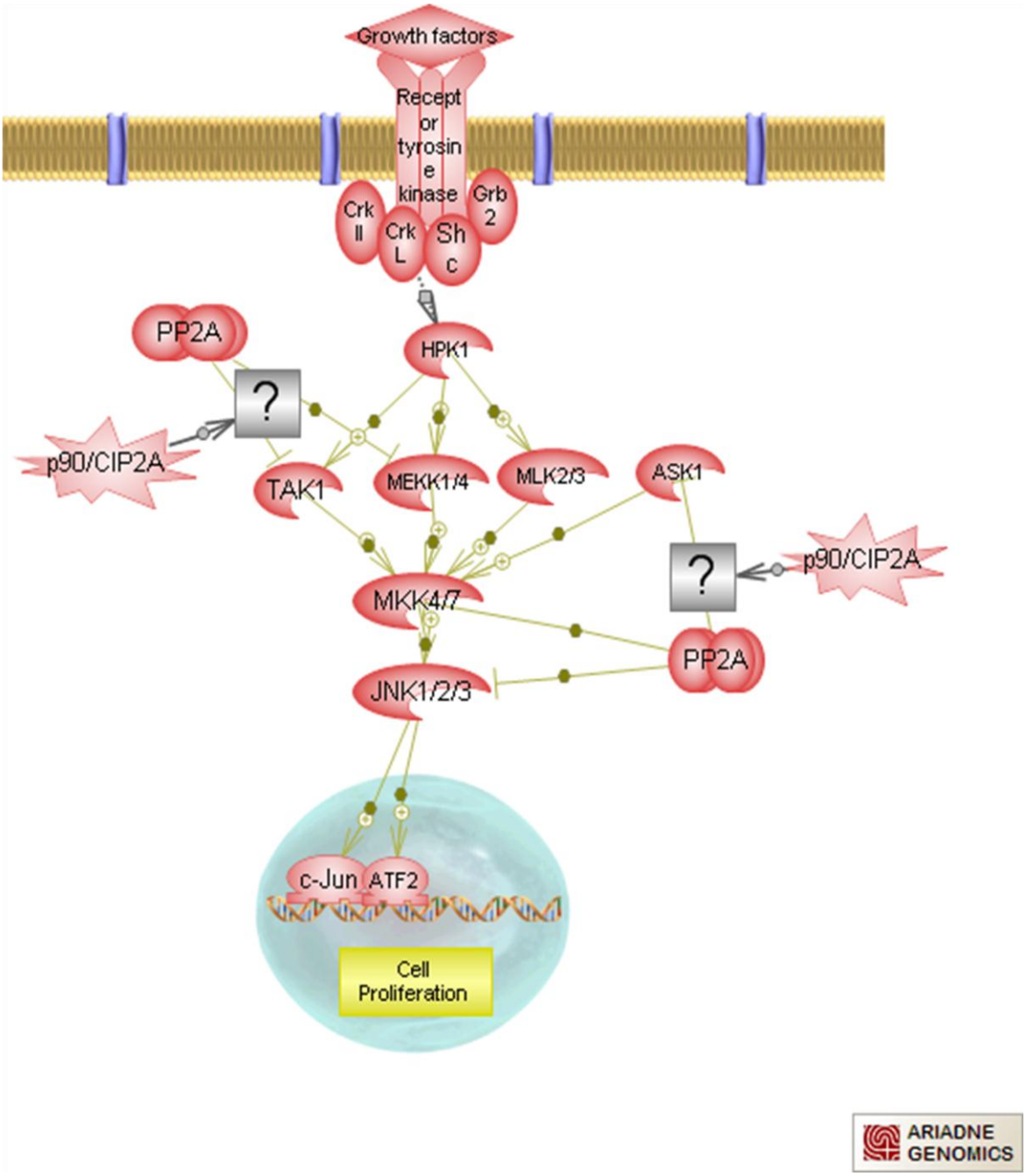
On the third chapter, we used proteomic approach to investigate the proteome change upon the loss of p90/CIP2A. By utilizing advanced bioinformatic tools, we established that p90/CIP2A can regulate the phosphorylation of CREB indirectly, which is done through AKT pathway. AKT is a central regulator of cell proliferation, cell growth and cell survival by integrating distinct upstream pathways, which in turn phosphorylate downstream targets. Although AKT has been reported to be a target of p90/CIP2A, the downstream effects had not been reported. In our study, we found that p90/CIP2A can prime the AKT for the phosphorylation of CREB, which may be a way for p90/CIP2A to promote cell proliferation.

On the fourth chapter, we described the metabolomic analysis of the loss or overexpression of p90/CIP2A. The recent proposed idea that cancer is also a metabolic diseases extends our understanding

of the underlying mechanism of cell proliferation and cancer cell growth. Our proteomic data suggested that p90/CIP2A can alter the expression levels of several metabolic enzymes, and may be a potential oncogene to regulate cancer metabolism. Interestingly, we found that D-glucose is one of the most significant metabolites during the loss or overexpression of p90/CIP2A. Further experiments also showed the correlation between the hexokinase activity and p90/CIP2A expression level. This study contributed to the cancer research field by understanding that p90/CIP2A can regulate cell proliferation through the glycolytic pathway, and by demonstrating that metabolomic can be a useful tool to investigate oncogene functions.

Taken together, we found that p90/CIP2A can regulate the JNK-c-Jun pathway, CREB phosphorylation and glycolytic pathway (Fig. 20) .

A.


Growth factors
Recept
or
tyrosin
e
kinase
Grb
2
Crk
II
Crk
L
Sh
c
PP2A
?
HPK1
p90/CIP2A
TAK1
MEKK1/4
MLK2/3
ASK1
?
p90/CIP2A
MKK4/7
PP2A
JNK1/2/3
c-Jun
ATF2
Cell
Proliferation
ARIADNE
GENOMICS

B.

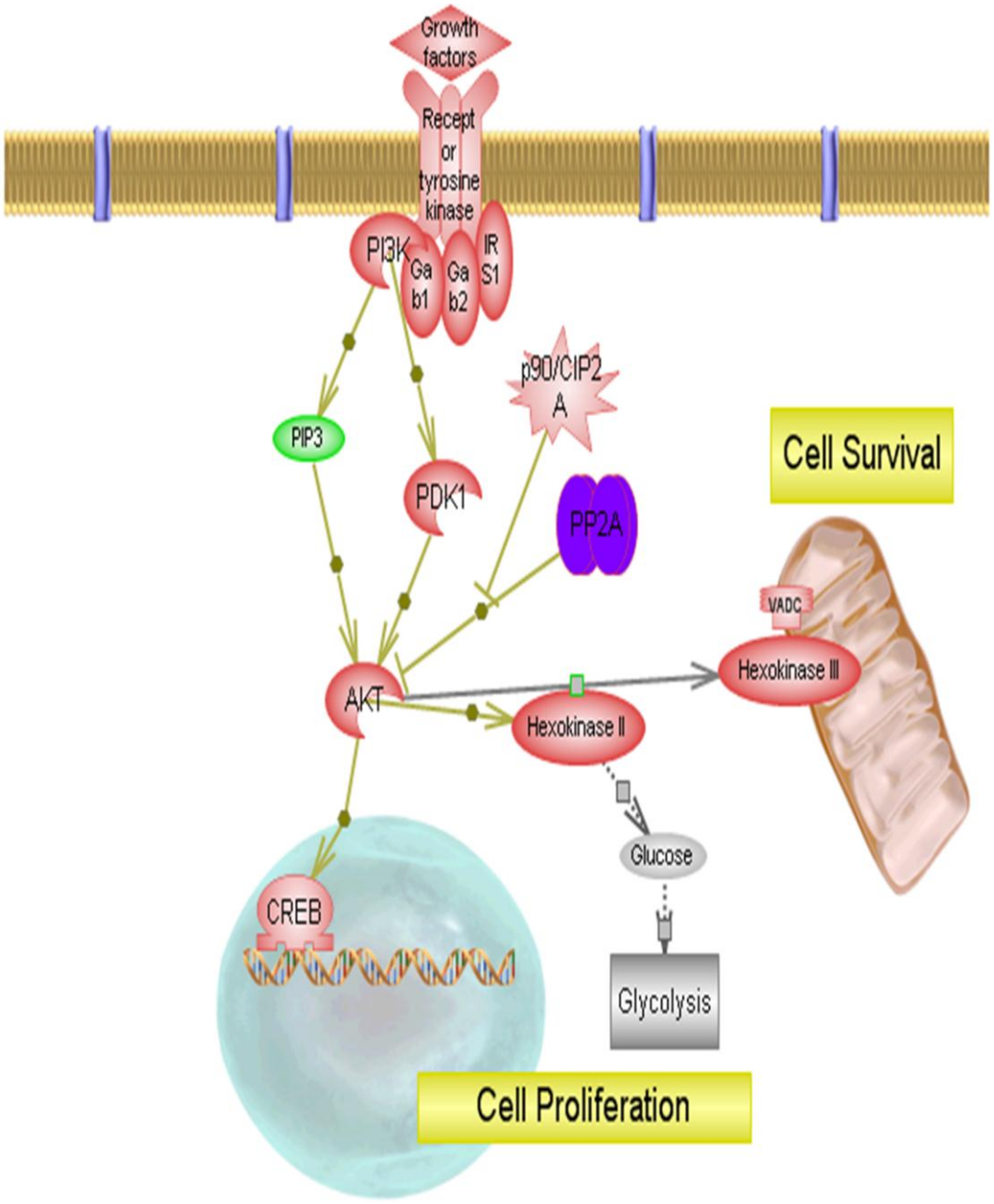

Growth factors
Recept or tyrosine kinase
PI3K
Ga b1
Ga b2
IR S1
PIP3
p90/CIP2 A
PDK1
PP2A
Cell Survival
VADC
Hexokinase III
AKT
Hexokinase II
Glucose
CREB
Glycolysis
Cell Proliferation

Fig. 20. Proposed model and research implications. (A) Upon the stimulation of growth factors, receptor tyrosine kinase (RTK) localized in the receptor recruits the adaptor proteins CrkII, CrkL, Shc and Grb2, which activate HPK1 kinase. The activation of HPK1 kinase can lead to the phosphorylation of several MAPKKK kinase like (MEKK1/4 and MLK2/3), which results in the activation of JNK kinase via the activation of MKK4/7. Activated JNK can phosphorylate the transcription factor c-Jun and ATF2, which initiate the transcription of cell proliferation genes. There are several molecules in this process that can be negatively regulated by PP2A including TAK1, MEKK1/4, ASK1, MKK4/7 and JNKs. Therefore, p90/CIP2A may have one or more targets for the regulation of this pathway. Future studies can be focused to elucidate the mechanism by identifying p90/CIP2A target(s). (B) As described previously, the activation of RTK, adaptor proteins (Gab1, Gab2 and IRS1) will be recruited by RTK which results in the membrane localization of PI3K which phosphorylate PIP3 or activate PDK-1. Both of these molecules will be able to activate AKT phosphorylation. AKT can phosphorylate the CREB, which is localized in the nucleus and can promote gene transcription. In addition, AKT can phosphorylate hexokinase II (HKII) and thus increase hexokinase activity to initiate glycolytic pathway. In addition, AKT can also promote the localization of HKII to the outer membrane of mitochondria where it forms complex with voltage-dependent channel (VADC). The resulted formation of this complex will shied the cell from oxidative stress-induced apoptosis. Although previous results had shown p90/CIP2A decrease the AKT-associated PP2A phosphatase activity, we have established that p90/CIP2A can regulate the AKT substrate to favor cell proliferation in cancer cells.

5.2 Future directions

Although we have determined that p90/CIP2A can alter the phosphorylation of JNK-c-Jun pathway, the targets in this pathway are still unknown. Since several upstream molecules of JNK are the target of PP2A, further work may be done to examine the phosphorylation of ASK1 and MEK1. This will allow us to know how p90/CIP2A can regulate this stress response pathway.

In addition, we found two AKT substrates: CREB and hexokinase are subjected to the regulation of p90/CIP2A. This data implied that p90/CIP2A may influence the AKT substrate selectivity. To further understand the interrelation between p90/CIP2A and AKT, further work can be done to identify those substrates. This will have two benefits: first, we will know which AKT-regulated pathway can be targeted by p90/CIP2A. Second, by aligning the targets AKT substrate, we may find consensus sequence recognized by p90/CIP2A, which may provide rationale for the drug design.

Although hexokinase may be a target of p90/CIP2A, we still don't know whether p90/CIP2A can confer cancer cell resistance to oxidative stress. Therefore, in future work, we can compare the sensitivity of cells to oxidative stress by depletion of p90/CIP2A or overexpression of p90/CIP2A.

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Appendix

List of abbreviations

NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
PP2A	Protein phosphatase 2A
CREB	cAMP-responsive element
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
PI3K	Phosphoinositide 3-kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
2D gel	Two-dimensional electrophoresis
mTOR	Mammalian target of Rapamycin
GC/MS	Gas-chromatography mass spectrometry
shRNA	Short hairpin RNA
WT	Wild type
TCA cycle	Tricarboxylic acid cycle
DMEM	Dulbecco’s modified Eagle’s medium
RPMI1640	Roswell Park institute medium 1640
HRP	Horseradish-peroxidase
IHC	immunohistochemistry
DAB	3,3'-diaminobenzidine
ICA	Independent component analysis
SAM	Significant analysis of microarray
OD	Optical density

List of publications and manuscripts

1. Liu W, **Peng B**, Lu Y, Xu W, Qian W, Zhang JY. (2011*)* Autoantibodies to tumor-associated antigens as biomarkers in cancer immunodiagnosis. *Autoimmune Rev*. 10(6):331-335.

2. Shao Q, Ren PF, Li Y, **Peng B**, Dai LP, Lei NJ, Yao W, Zhao G, Li LG, Zhang JY. (2012) Autoantibodies against glucose-regulated protein 78 as serological biomarkers in Hepatocellular carcinoma. Int. J. Oncology. 41(3): 1061-1067.

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5. **Peng B**, Huang XY, Nakayasu ES, Petersen JR, Qiu SM, Almeida IC, Zhang JY. Identification and characterization of alpha-enolase as an autoantigen in liver fibrosis. (Submitted)

6. **Peng B**, Lei NJ, Peng XX, Zhang JY. p90/CIP2A play a role in regulating the phosphorylation of CREB1 through AKT in lung cancer. (submitted)

Curriculum vita

Bo Peng was born on October 15th, 1982 in Anfu, Jiangxi Province, China. The only child of Xuanxian Peng and Sanying Wang, he received his Bachelor of Science from School of Life Science, Xiamen University, China in 2004. Upon his graduation, he worked as a technician at National University of Singapore for one year and pursued his Master of Science at the same lab, where he conducted the research regarding the host-pathogen interaction. After completing his M.S. in microbiology, he joined to the doctoral program of Department of Biological Science at University of Texas at El Paso in Fall of 2008, where he has been working at Dr. Jianying Zhang's lab. His research focused on the elucidation of the role of p90/CIP2A in human lung cancer progression.

He has presented his results three times in international meetings organized by American Association for Cancer Research (AACR): 100th Annual AACR meeting (Denver, 2009), 101st Annual AACR meeting (Washington D.C., 2010) and 103rd Annual AACR meeting (Chicago, 2012). In addition, he also received several travel awards from Graduate school, Department of Biological Sciences and College of Science.

He has already published four co-authored peer-reviewed papers and had submitted another two first-author manuscripts. After graduation, he will go to National Lawrence Berkeley Lab to study the infection process of virus using live imaging. His long-term goal is to become an independent researcher to study the relationship between cancer and metabolism.