### 5.1 Novel Methods That Facilitate Membrane Protein Studies and Elucidate Protein Redistribution and Phosphorylation Events

Merck KGaA, Life Science & Analytics R&D, Darmstadt, Germany

The quantitative difference in abundance of proteins is one feature to describe differences between proteomes of a cell at given time points or at changed conditions. Besides the dynamic expression level, post-translational modifications (PTMs) of proteins and the precise cellular context relating to protein subcellular localization as well as protein interaction patterns, both of which often influence activity, are additional features that are extremely informative for elucidating protein function. These important cellular events are crucial for biological processes such as signal transduction or apoptosis that often involve membrane signaling receptors, recruitment of components as for instance kinases to membranes and subsequent phosphorylation events.

The objective of this study was to develop robust sample preparation methods to investigate membrane proteins as also methods to monitor signal transduction events such as protein redistribution and identification of protein phosphorylation. To selectively isolate membrane proteins in their native, functional state, a scalable 2-step extraction procedure was set up. Membrane preparations were used in several assays including quantitative EGF-Receptor ELISA and a kinase assay. To study spatial changes of signaling molecules such as p44/42 MAP kinase, which migrates from the cytosol to the nucleus upon phosphorylation, a convenient subcellular sequential extraction scheme yielding four subproteomes enriched in (a) cytosolic, (b) membrane and membrane organelle-localized, (c) soluble and DNA-associated nuclear and (d) cytoskeletal proteins was used in parallel. Finally, a novel specific surface modified immobilized metal ion affinity chromatography (IMAC) matrix was established and used in parallel. Set up. Membrane preparations were used in several assays including quantitative EGF-Receptor ELISA and a kinase assay. To study spatial changes of signaling molecules such as p44/42 MAP kinase, which migrates from the cytosol to the nucleus upon phosphorylation, a convenient subcellular sequential extraction scheme yielding four subproteomes enriched in (a) cytosolic, (b) membrane and membrane organelle-localized, (c) soluble and DNA-associated nuclear and (d) cytoskeletal proteins was used in parallel. Finally, a novel specific surface modified immobilized metal ion affinity chromatography (IMAC) matrix was established and used in an optimized protocol for specific and efficient enrichment of phosphopeptides for phosphoproteome analysis by mass spectrometry. The approach is not biased towards a specific phosphorylated amino acid type and was shown to be superior to previous commercially available phospho-IMAC kits.

All findings demonstrate the compatibility and the broad applicability of the developed methods, which facilitate not only classical protein expression profiling but also monitoring of spatial redistribution events as well as protein activity and PTM analysis. The developed methods have recently been made available as sample preparation kits.

---

### 5.2 Deranged Expression of Protein in Lungs Between Aged and Young Mice

Guoqiang Bao¹, Subo Li¹, Yongxiang Zhang², Yangpei Zhang¹, and Shiwen Wang³
¹Beijing Institute of Transfusion, Academy of Military Medical Sciences, Beijing, China; ²Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China; and ³The Institute of Geriatric Cardiology, General Hospital of PLA, Beijing, China

In order to explore which protein play a key role in ageing processes of lung, the proteomics changes of lung were studied between young and aged mouse by means of 2D electrophoresis and MALDI-TOF-MS methods. 2D 1 to 2D 10 proteins had distinct expression were identified which might be Kit ligand precursor, CAIL, Ras-related protein Rab-8, Hyaluronan synthase 2, Numb-binding protein 2, Tudor domain containing protein 3 STANDARD VARSPLIC, Phosphatidylcholine-sterol acyltransferase precursor, Heat shock cognate 71 kDa protein, Electron transfer flavoprotein beta-subunit and Tumor necrosis factor receptor superfamily member 4 precursor respectively through searching the protein database. 2D-1 to 2D-7 expressed in the lung of young mice and 2D-8 to 2D-10 expressed in the lung of aged mice. 3 proteins which were Kit ligand precursor, Carbonic anhydrase II and Heat shock cognate 71 kDa protein had high score in the database. Pulmonary carbonic anhydrase II activity plays important roles in carbon dioxide exchange, fluid secretion, and pH regulation. It expressed insufficient might be one reason of lung failure in aged mice. The other two proteins function in lung were not clear now. All the results suggested these proteins might play important role in the lung and would benefit the research on ageing processes of lung.

---

### 5.3 Redox Proteomics: New Challenge for the Characterization of Cys Containing Proteins

Serena Camerini, Maria Letizia Polci, and Angela Bachi
DIBIT, S. Raffaele Scientific Institute, Milan, Italy

Cys is the more reactive residue present in proteins and it is involved in the first post-translational modification that a protein undergoes that is the formation of disulphide bonds. This modification represents a reversible oxidation that is very important for protein function and structure. Other different reversible changes of the redox state of Cys involve the formation of sulfenic acid or nitrosothiols. These reactions occur in physiological conditions regulating protein function, therefore the redox state in a cell is strictly controlled. Nevertheless there are many cases in which the generation of reactive compounds (ROS or RNS) exceeds the detoxification capacity of the cell generating oxidative stress. Cellular redox changes are crucial both in physiological and pathological conditions: the recognition of redox-sensitive molecular targets are essential to understand many mechanisms in which Cys residues oxidations are involved.

We developed a proteomic strategy useful to analyse proteins whose Cys are sensitive to redox changes. This methodology requires the blocking of all the reduced Cys residues, the reduction of the Cys residues that are object of interest and their alkylation by a specific agent bound to a biotin tag that allows the purification of the labeled proteins by affinity chromatography. In this way the proteins labeled are enriched and identified using classical strategies coupled with MALDI-ToF MS or LC-MS-MS.

This strategy allows the identification of the modified sites and is powerful to study the dynamics of redox equilibrium of cysteine-containing proteins in different cellular compartments and in cells under several biological stimuli in order to understand the physiological role of this post-translational modification.
Identification of Phosphorylation Sites in Human Tristetraproline That Affect Its Electrophoretic Mobility

Heping Cao1, Wil S. Lai1, Leesa J. Deterding2, John Venable3, Elizabeth A. Kennington1, Timothy A. J. Haystead2, John R. Yates III3, Kenneth B. Tamer2, and Perry J. Blackshear1,5

1Office of Clinical Research and Laboratories of Signal Transduction and Neurobiology and 2Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC; 3Department of Cell Biology, Scripps Research Institute, La Jolla, CA; and 4Departments of Pharmacology and Cancer Biology and 5Departments of Biochemistry and Medicine, Duke University Medical Center, Durham, NC, USA

Tristetraproline (TTP) is a zinc finger protein that binds to AU-rich elements within certain mRNAs, and causes deadenylation and destabilization of those mRNAs. Mice deficient in TTP develop a profound inflammatory syndrome with erosive arthritis, autoimmunity and myeloid hyperplasia, apparently due to excessive production of tumor necrosis factor α (TNF) and granulocyte-macrophage colony-stimulating factor, both of whose mRNAs are direct targets of TTP. Previous studies showed that TTP is phosphorylated extensively in intact cells, and this phosphorylation resulted in multiple species differing in electrophoretic mobility on SDS gels, implying stoichiometric changes in phosphorylation. However, limited information is available about the identities of these phosphorylation sites. In this study, we investigated the phosphorylation sites of human TTP by mass spectrometry (MS) and site-directed mutagenesis. MS and protein sequencing identified a number of phosphorylation sites including S66, S182, T205, T211, S214, T228, S238, S276, and S296. In addition, Shotgun tandem MS identified other phosphopeptides with 26 additional sites. In addition, Shotgun tandem MS identified other phosphopeptides with 26 additional sites. Alanine mutations at S197, S218, and S228 of the human protein each resulted in multiple species differing in electrophoretic mobility on SDS gels, implying stoichiometric changes in phosphorylation. However, limited information is available about the identities of these phosphorylation sites. In this study, we investigated the phosphorylation sites of human TTP by mass spectrometry (MS) and site-directed mutagenesis. MS and protein sequencing identified a number of phosphorylation sites including S66, S182, T205, T211, S214, T228, S238, S276, and S296. In addition, Shotgun tandem MS identified other phosphopeptides with 26 additional sites. In addition, Shotgun tandem MS identified other phosphopeptides with 26 additional sites. Alanine mutations at S197, S218, and S228 of the human protein each significantly increased TTP’s gel mobility, but mutations at S88, S90, S93, S186, S214, T271, or S296 exhibited little effect on its gel mobility. Dephosphorylation and in vivo labeling studies showed that mutant proteins with multiple mutations were still phosphorylated; which were able to bind TNF mRNA probes in electrophoretic mobility-shift assays. All three phosphorylation sites whose mutations affected mobility were potential sites for proline-directed protein kinases and conserved in mammalian species. The mutant proteins with the three sites have exhibited normal TTP “behavior” in binding assays, but this cluster of phosphorylation sites remains an important possible domain for regulatory control.

Baculovirus-mediated Parallel Expression Systems for Spodoptera frugiperda Cells, Drosophila melanogaster Cells and Mammalian Cells

Zhai Chao and Ma Lixin

Institute of Biochemistry and Molecular Biology, Hubei University, Wuhan, China

The baculovirus expression vector system (BEVS) is one of the useful tools for the expression of recombinant proteins in insect cells such as sf9 (Spodoptera frugiperda ovarian) cells. In recent years, its host cell lines have been broadened. Several studies about the expression of foreign genes with BEVS in Drosophila cells and human liver cells have been reported. But every system has its own advantages or drawbacks. Since baculovirus genome is large and hard to handle, no comparative data about large-scale protein expressing in all these expression systems has been published. In this study, with baculovirus as vector backbone, we have constructed a series of recombinant baculovirus vectors with three different promoters (polh, CMV, Actin5) to guide the expression of foreign genes in Spodoptera frugiperda ovarian cells, Drosophila melanogaster cells and mammalian cells, respectively. More, we have designed different tags, such as His6 tag, GST-His6 tandem tag, GST -foreign gene- His6 cassette etc., which can give facility for the purification of foreign protein after fusion expression. Furthermore, all of the recombinant baculovirus vectors have the same cloning site—two tandem Bsu361 site (with different recognized sequence), the baculovirus expression vectors digested with Bsu361 will produce two distinctive stick ends compatible with the overhangs of foreign fragments, which have been amplified with special primers (with extra 3–4 defined bases on the S′-end) and treated with T4 DNA polymerase. Therefore, a single foreign fragment can be ligated into all these vectors simultaneously.

With the above-mentioned expression systems, foreign genes can be expressed in large scale and high throughput in three different eukaryotic host cells parallel and the probability of obtaining plentiful of functional protein is highly increased, which are vital to subsequent work, such as protein microarray. But more importantly, we can combine all the information attained from these different systems and get more elaborate understanding about the expressed proteins.

Now we have set to express systematically human liver cDNAs in these three systems. Along with the accumulation of related data, we can make it clear to select more suitable expression systems for special proteins.
5.6 A Genomic and Proteomic Research for the Regulatory Networks of ATRA-induced Differentiation of Acute Promyelocytic Leukemia Cells

Qunye Zhang, Qiu-Hua Huang, Shuhong Shen, Peizhen Zheng, Jing Lu, Yi Jin, Ji Zhang, KanKan Wang, Saijuan Chen, and Zhu Chen
State Key Lab for Medical Genomics and Shanghai Institute of Hematology, Rui Jin Hospital, Shanghai Second Medical University, Shanghai, P.R. China

The treatment of acute promyelocytic leukemia (APL) with all trans retinoic acid (ATRA) has set a model for the differentiation induction-based new therapy. Although a substantial body of data related to ATRA-induced differentiation mechanisms has been obtained, the molecular events downstream of RA receptor complexes remain obscure. To gain further insights into regulatory networks underlying ATRA-induced differentiation in APL, we applied 2DG, MS and cDNA microarray combined with bioinformatics analysis such as self-organizing map (SOM) to profile NB4 cells treated with ATRA over 3 time points (0h, 12h, 48h). Our results showed that cell cycle was arrested and proliferation was suppressed. For instance, the Origin recognition complex subunit 3 that is involved in initiating DNA replication that make the cell switch to S phase was downregulated significantly at the protein (67.55 times) and mRNA levels; Breast cancer replication that make the cell switch to S phase was downregulated approximately 40 kDa was identified that could produce the same effects as the crude muscle extract. We also tested the ability of semipurified (30–50 kDa) muscle extract to promote muscle repair in adult rats. Surgical intervention was used to induce muscle damage in the tibialis anterior. The semipurified muscle extract (fraction H) was injected subcutaneously over the tibialis anterior for a period of 5 days. It was found that the damaged muscle fibers were replaced by newly regenerated muscle fibers. These newly regenerated fibers originated from the fusion of differentiated satellite cells as revealed by BrdU-labeling analysis. In contrast, the injured site of muscles treated with BSA control protein contained mainly fibroblasts.

5.7 Identification and Purification of an Intrinsic Human Muscle Myogenic Factor That Enhances Muscle Repair and Regeneration

Ming Li, Lei Cheng, C. M. Yu, Lihong Zhang, and J. E. Sanderson
The Chinese University of Hong Kong, China

The limited ability of damaged muscle to regenerate after gross injuries is a major clinical problem. To date, there is no effective therapeutic treatment for muscle injuries. In the present study, we have examined the ability of crude and fractionated human skeletal muscle extracts to promote myogenic cell proliferation and differentiation. It was found that the crude muscle extract could significantly stimulate BrdU incorporation in C2C12 myogenic cell line. In addition, the extract also promoted myogenic cell alignment and fusion. Using electrophoresis techniques, in conjunction with in vitro refolding technique, a protein with molecular weight of approximately 40 kDa was identified that could produce the same effects as the crude muscle extract. We also tested the ability of semipurified (30–50 kDa) muscle extract to promote muscle repair in adult rats. Surgical intervention was used to induce muscle damage in the tibialis anterior. The semipurified muscle extract (fraction H) was injected subcutaneously over the tibialis anterior for a period of 5 days. It was found that the damaged muscle fibers were replaced by newly regenerated muscle fibers. These newly regenerated fibers originated from the fusion of differentiated satellite cells as revealed by BrdU-labeling analysis. In contrast, the injury site of muscles treated with BSA control protein contained mainly fibroblasts.

5.8 Functional Proteomic Analysis of a Human Ovarian Epithelial Cancer Model

T. W. Young, F. Mei, G. Yang, J. A. Thompson-Lanza, J. Liu, and X. Cheng
Department of Pharmacology and Toxicology, University of Texas Medical Branch at Galveston, TX, USA

Cellular transformation is a complex process involving genetic alterations associated with multiple signaling pathways. Development of a transformation model using defined genetic elements has provided an opportunity to elucidate the role of oncogenes and tumor suppressor genes in the initiation and development of ovarian cancer. To study the cellular and molecular mechanisms of Ras-mediated oncogenic transformation of ovarian epithelial cells, we used a proteomic approach involving two-dimensional electrophoresis and mass spectrometry to profile two ovarian epithelial cell lines, one immortalized with SV40 T/t antigens and the human catalytic subunit of telomerase and the other transformed with an additional oncogenic rasV12 allele. Of approximately 2200 observed protein spots, we have identified more than thirty protein targets that showed significant changes between the immortalized and transformed cell lines using peptide mass fingerprinting. Among these identified targets, one most notable group of proteins altered significantly consists of enzymes involved in cellular redox balance. Detailed analysis of these protein targets suggests that activation of Ras-signaling pathways increases the threshold of reactive oxidative stress (ROS) tolerance by up-regulating the overall antioxidant capacity of cells, especially in mitochondria. This enhanced antioxidant capacity protects the transformed cells from high levels of ROS associated with the uncontrolled growth potential of tumor cells. It is conceivable that an enhanced antioxidation capability may constitute a common mechanism for tumor cells to evade apoptosis induced by oxidative stresses at high ROS levels.
5.9 Characterization of Multisite Phosphorylation in Translation Initiator Factor 5 and 6 (eIF5 and eIF6) by Mass Spectrometry and Site-directed Mutagenesis

Haiteng Deng, Joe Fernandez, Uttiya Basu, A. Bandopadhayay, Romit Majumdar, and Umadas Maitra

Department of Development and Molecular Biology; Albert Einstein College of Medicine, Bronx, NY, USA

Eukaryotic translation initiation factor 5 and 6 (eIF5 and eIF6) play different roles in eukaryotic translation. eIF5 is a key regulatory enzyme that stimulates hydrolysis of eIF2-bound GTP to initiate eukaryotic translation, and eIF6 is necessary for the formation of 60S ribosomal subunits. Lack of eIF6 prevents the processing of pre-rRNA to form the mature 25S and 5.8S rRNAs, the constituents of the 60S ribosomal particle. Previous studies showed that both eIF5 and eIF6 were phosphorylated in vivo and in vitro on serine residues. The purpose of this work was to characterize the phosphorylation sites of eIF5 and eIF6 and to determine how phosphorylation of eIF5 and eIF6 regulates their functions by using a combination of enzymatic digestion, mass mapping, tandem mass spectrometry, enzymatic/chemical derivation and site-directed mutagenesis.

Wild-type and mutant rat translation initiation factor 5 (eIF-5) was expressed in E. Coli as a GST fusion protein and purified using affinity chromatography. Then, eIF-5 was phosphorylated by casein kinase II purified from rabbit reticulocyte lysate in Tris buffer in the presence of 100 µM of ATP for 30 minutes. It was found that eIF-5 was phosphorylated at residues Ser174, Ser387, and Ser388 by casein kinase II using a combination of trypsin digestion and mass mapping. The phosphorylation efficiencies for Ser387 and Ser388 were four times higher than that for Ser174. This result was confirmed by site-directed mutagenesis and kinetic studies in which phosphorylation at Ser387 and Ser388 were accounted for 90% of the total in vitro eIF5 phosphorylation. Previous studies showed that formation of eIF5-eIF2 complex occurred by interaction between the conserved lysine residues at N-terminal of eIF2 and the C-terminal region of eIF-5. Phosphorylation of eIF5 at Ser174 and Ser388 may enhance this interaction.

On the other hand, Tif6p, the yeast homologue of mammalian eIF6 was phosphorylated by eIF6 kinase from rabbit reticulocyte lysate. Using mass mapping, we identified that the eIF6 kinase was casein kinase I. The phosphorylated Tif6p was Glu-C digested and analyzed. A peptide containing two serine residues Ser174 and Ser175 was found to be mono-phosphorylated. This peptide was further cleaved by trypsin and chemically derivatized with propanethiol to produce a short peptide that is amenable for MS/MS analysis. Using tandem MS, we found that Ser174 was the major phosphorylation site for Tif6p. Mutation of Tif6p at Ser174 to alanine reduced phosphorylation drastically and caused loss of cell growth and viability. While wild-type Tif6p was distributed both in nuclei and the cytoplasm of yeast cells, the mutant Tif6p (with Ser174Ala and Ser175Ala) became a constitutively nuclear protein. These results suggest that phosphorylation at Ser174 and Ser175 plays a critical role in the nuclear export of Tif6p.

5.10 Proteomic Study of Proteins Associated with Lipid Droplets in 3T3-L1 Adipocytes

Georgia Dolios¹, Dawn L. Brasaemle², Lawrence Shapiro³, and Rong Wang¹

¹Department of Human Genetics, Mount Sinai School of Medicine, New York, NY; ²Department of Nutritional Sci., Rutgers, The State University of New Jersey, New Brunswick, NJ; and ³Department of Biochem. & Mol. Biophys., Columbia University College of Physicians & Surgeons, New York, NY, USA

The adipocyte lipid droplet is a largely uncharacterized organelle. Few lipid droplet-associated proteins have been identified that are associated with lipid droplet, yet these proteins play important roles in the control of triacylglycerol storage and lipolysis. To identify additional lipid droplet-associated proteins, we have conducted a proteomic study of lipid droplets isolated from 3T3-L1 adipocytes incubated under either basal or lipolysis-stimulating conditions. To date, we have identified more than 40 proteins that associate with lipid droplets under either basal, or stimulated conditions, or both. These proteins include known structural lipid droplet proteins such as perilipins A and B, TIP47, and adipophilin, but also several enzymes involved in lipid metabolism including hormone-sensitive lipase, long chain fatty acid coenzyme A ligase 2, lanosterol synthase, NAD(P) dependent steroid dehydrogenase-like protein, 17-beta-hydroxysteroid dehydrogenase, and CGI-58. The associations of several of these proteins with lipid droplets were confirmed by immunofluorescence microscopy and immunoblotting of subcellular fractions; expression of CGI-58 as a GFP fusion protein also confirmed its localization to lipid droplets. We further employed bioinformatics analyses of newly identified proteins to help gain understanding of their molecular functions and potential involvement in adipocyte biology. This study has identified a substantial number of largely unstudied proteins that may help us to understand how lipid droplet-associated proteins contribute to the regulation of energy storage and release in adipocytes, and in turn, whole body energy metabolism.

Acknowledgment: This study is partially supported by NIH-NCI-CA88325 (R.W.), NIH R01 DK54797 (D.L.B.), AHA EI award (D.L.B.), and a Johnson & Johnson Discovery Award (D.L.B.), an ADA career development award (L.S.), and a Jules and Doris Stein award from the RPB foundation (L.S.).
5.11 Differential Proteomic Analysis of an Induced and Non-induced Cell Line for the Expression of the TRPM-2/Clusterin Gene

N. Smargiasso1, H. Ammar2, A. Dubus3, J. Closset2, E. De Pauw1, and G. Mazzucchelli1

1Laboratory of Mass Spectrometry, University of Liège, Liège, Belgium; 2Laboratory of Endocrinology and Biochemistry, University of Liège; and 3GIGA Proteomics, University of Liège, Belgium

Clusterin (TRPM2) is, in its major form, a secreted heterodimeric glycoprotein of 75–80 kDa with an ubiquitous distribution in all mammalian biological fluids. Its exact role remains ambiguous although recent reports indicate that clusterin can function as a secreted chaperone. However its up-regulation is associated with many diseases such as Alzheimer’s disease or renal diseases. In addition, the role that clusterin plays in apoptosis is controversial. It is known that the most common form is a secreted survival protein protecting the cell from apoptosis but on the other hand, it has been suggested that the nuclear form (nCLU) could induce apoptosis. We have compared the proteomes of lung metastatic cells issued from the rat prostatic cell line MAT-LyLu (MILL) with that of the same cells induced for the expression of clusterin. We expect that the identification of differentially expressed proteins between these two conditions will reveal new indications about the functional role of clusterin.

Proteins from the nuclear and cytoplasmic subfractions and the medium of each cell culture conditions were separated by two dimensional gel electrophoresis (2D-PAGE) and the differential expression was analysed using the DIGE system (Amersham Biosciences). Potentially interesting protein spots were excised trypanised and identified by MS/MS ion search (Mascot algorithm, Matrix Science) from data collected by nanoLC-MS/MS on an ion trap spectrometer. (Esquire HCT, Bruker Daltonics). The results of these experiments are presented and discussed on our poster.

5.12 Over-expression of Functional Characteristics Proteins in Low Different Nasopharyngeal Carcinoma Tissue and CNE2 Cell Line

Xue-ping Feng1, Zhi-qiang Xiao1, Yi-xuan Yang1, Guo Zhu1, Ming Li1, Cui Li2, Feng Li2, Zhu-chu Chen1,2, Peng-fei Zhang1, and Jian-yun Xiao3

1Key Laboratory of Cancer Proteomics of Chinese Ministry of Health, Xiangya Hospital, Central South University, Changsha, P.R. China; 2Cancer Research Institute, Xiangya School of Medicine, Central South University, Changsha, P.R. China; and 3Otolaryngology Department, Xiangya Hospital, Changsha, P.R. China

This most promising prospect for clinical proteomics is the study of patterns of proteins. It is possible for over expression of the same unique functional characteristics proteins between low different nasopharyngeal carcinoma tissues and low different CNE2 cell line by patterns of proteins. The proteome were separated from low differentiated cell line CNE2 and high differentiated cell line CNE1, 4 cases low differentiated nasopharyngeal carcinoma and chronic nasopharyngitis by using immobilized pH gradient two-dimensional polyacrylamide gel electrophoresis, silver staining. 136 spots of these different protein spots (56 spots in tissues and 78 spots in cell line) were analyzed and identified using Image Master 2D software, peptide mass fingerprint based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and database searching. Over expression of 3 functional characteristics proteins was found in advanced low differentiated nasopharyngeal carcinoma tissues and low differentiated cell line CNE2. One of 3 proteins is involved in tumor metastasis and invasion.

5.13 Spindlin1, a Novel Human Gene Encoding a Protein Ahat Localized in the Cell Nucleus and Inducing NIH3T3 Cell’s Transformation

Yanhong Gao1, Lipeng Qin2, Peng Zhang2, Lin Chen3, Hongfeng Yuan4, Cixian Bai4, Fang Yan4, Wen Yue4, and Xuetao Pei5

Laboratory of Stem Cell Biology, Beijing Institute of Transfusion Medicine, Beijing, P.R. China

Spindlin1, a novel human gene recently cloned by our laboratory, is highly expressed in the cancer tissue. To study its biological function, a vector expressing green fluorescent-spindlin1 fusion protein was constructed and transfected into COS-7 and NIH3T3 cells by lipofectamine methods. The results showed that the fusion protein spindlin1-EGFP was localized in the nucleus of COS-7 and NIH3T3 cells. NIH3T3 cells which could stably express spindlin1 as a result of RT-PCR analysis compared with the parental NIH3T3 cells displayed a complete morphological change; improved cell growth and increased the percentage of cells in G2/M phase (12.6% vs control cells at 3.4%). Furthermore, overexpressed spindlin1 cells formed colonies in soft agar, more motile in migration assay in vitro and formed tumors in nude mice. Our findings provide direct evidence that spindlin1 gene may be a prooncogene which is associated with tumorigenesis.

5.14 The Effects of Radiation on the Expression of Mouse Bone Marrow Proteins

P. Guo1, J. F. Wang2, and S. Q. Wang3

Beijing Institute of Radiation Medicine, Beijing, P.R. China; and Beijing University of Traditional Chinese Medicine, Beijing, P.R. China

The objective of the research is to observe the effects of radiation on the expression of mouse bone marrow proteins. 34 C57BL/6J mice were divided randomly into normal control group and radiation group with 17 mice of each group. The mice of radiation group were induced by using a single dose of 3.5 Gy radiation from a 60Co γ source. The extracted proteins of the bone marrow were analyzed by proteomic technology and the differential protein spots were identified. The results showed that after radiation 18 proteins were up-regulated and several of them are hematopoietic cell protein-tyrosine phosphatase (HCP), histone deacetylase-3 (HDAC3), growth factor receptor binding protein 14 (Grb14 protein) and Lgals12. HCP plays a key role in hematopoietic regulation and could down-regulate hematopoietic cell proliferation. A transcriptional corepressor, HDAC3 inhibits gene transcription and binds with several other proteins to form complex to directly act on promoters of inhibited gene. Grb14 protein inhibits ras signal pathway. Lgals12 can make the cell cycle stay in G1 phase and then inhibit cell growth. The result also showed radiation made the expression of six proteins down-regulated. Four of them are lymphocyte-specific protein 1 (LSP1), proteasome 26S ATPase subunit 4, H-ras and glycosylaldehyde-3-phosphate dehydrogenase. LSP1 expression is up-regulated when the granulocytes and monocytes of bone marrow differentiate. Proteasome 26S ATPase subunit 4 has ATP-dependent RNA/DNA helicase function and can promote transcription. Glycosylaldehyde-3-phosphate dehydrogenase is an important enzyme in glycosyloxyenolysis metabolism. The research suggests that radiation act on multi-targets of bone marrow and thus inhibit the growth and differentiation of hematopoietic cell.
5.15 The Functional Proteomics Research of the Effects of Si-Wu-Tang on Bone Marrow of Radiation Injured Mice

P. Guo, J. F. Wang, and S. Q. Wang
Beijing Institute of Radiation Medicine, Beijing, P.R. China; and Beijing University of Traditional Chinese Medicine, Beijing, P.R. China

The objective of the research is to observe the effects of Si-Wu-Tang (SWT), a kind of traditional Chinese medicine, on protein expression of bone marrow injury mice and to elucidating the molecular mechanism of blood enriching function of the medicine. 51 C57BL/6J female mice were randomly divided into normal control, model control and SWT group with 17 mice of each group. In model control and SWT groups, the bone marrow injury were induced by using a single dose of 3.5 Gy radiation from a $^{60}$Co source. After radiation exposure, the mice of SWT group were orally administered with SWT decoction, and other groups were orally administered with equivalent volume of normal saline solution. The bone marrow of the mice was prepared after continuously administrating for 7 days. The extracted proteins of the bone marrow were analyzed by proteomic technology and the differential protein were identified. The results of the research showed that SWT recovered 10 up-regulated and 4 down-regulated proteins of the radiation injury mice marrow. According mass spectrum analysis, seven of the proteins may be lymphocyte specific protein 1, proteasome 265 ATPase subunit 4, Hematopoietic cell protein-tyrosine phosphatase, glyceraldehyde-3-phosphate dehydrogenase, Krox-6, growth factor receptor binding protein 14, and Lgals12 respectively. The result suggest that by regulating the expression of these proteins, SWT promote glycan metabolism, transcription, the signal transduction of hematopoietic growth factor and the growth and differentiation of hematopoietic cell and may thus exert its effects on blood enriching function.

5.16 Proteomic Analysis of Apoplastic Peptides Induce by Slat Stress in Rice Root

Yi Guo and Daye Sun
Institute of Molecular Cell Biology, Hebei Normal University, Shijiazhuang, China

To evaluate the role of apoplastic peptide in plant development especially in response for stress, we establish the methods for analysis apoplastic peptide based on 2-D gel-based protein separation method coupled with protein identification by mass spectrometry. In the present work, we purified the apoplastic soluble protein by vacuumed infiltration in rice root treated with salt stress (200 mM NaCl) for 1, 3, and 6 hours. The samples which treated by salt or not were analyzed by 2DE. The results show that the proteome of apoplastic protein has obviously changed to responded the salt stress, although the amount of the number of the proteins in apoplast is great less than proteins in intracellular. Fourteen proteins induced or increased by treatment were analyzed by mass spectrometry, and ten proteins were identified. These proteins include some well known stress response cell wall protein such as beta-1,3-glucanase and peroxidase. Most interestingly, we also identified some novel apoplastic peptides with unknown functions involved in salt stress response. These proteins include a putative receptor-like protein kinase, rab5B, and a pathogenesis-related protein. The subcellular location and further functional analysis of these proteins are in progress.

For the first time, these results reveal the proteome of apoplastic peptides changes in response for salt stress, and these results will enlarge our knowledge about the role of apoplastic peptides and plant stress resistance mechanism.

5.17 Specific Expression of Alpha Fetoprotein (AFP) During the Development of Embryonic Pancreas in Rat

J. Y. Zhou, J. J. Hu, Y. Zhong, L. Yuan, and W. De
Department of Biochemistry and Molecular Biology, Nanjing Medical University, Nanjing, P.R. China

In order to reveal proteins differentially expressed in the process of rat embryonic pancreas development, two-dimensional gel electrophoresis and mass spectrometry were performed on rat embryonic pancreas at different stages. Protein samples extracted from rat pancreas of four stages: 15.5 embryonic days (E15.5), E18.5, newborn and adult were separated by immobilized pH gradient two-dimensional gel electrophoresis. After silver staining, the 2D electrophoretogram was analyzed using ImageMaster 4.9 software. In the range of molecular weight 14−200 KD and pl 4.0−7.0, altogether (562 ± 13), (597 ± 10), (584 ± 12) and (583 ± 14) spots were detected on the 2-DE map of E15.5, E18.5, newborn and adult rat pancreas, respectively. 331 spots were matched between the two groups of E18.5 and adult maps. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were performed with 26 proteins which were found to be significantly different presented in these four maps, 21 of which were down-expressed at the adult stage and 5 of which were up-expressed. Among the 21 spots, 7 spots were identified as alpha fetoprotein (AFP), including two isoforms. From E15.5 to E18.5, the level of AFP increased to 1.4 times while decreased to 0.85 times when to the newborn stage. However, it almost disappeared at adult stage. The development of pancreas gave rise to the changes of the protein expression patterns. Altogether these 26 protein spots were identified and their possible roles are discussed. This helps to understand the molecular mechanisms of rat embryonic pancreatic development.

5.18 DDR1 Regulates Cell Morphology and Spreading Via Non-muscle Myosin II A

Y. Huang and W. F. Vogel
Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada

Discoidin Domain Receptor 1 (DDR1) is a receptor tyrosine kinase activated by the extracellular matrix protein collagen. DDR1 plays an important role in cell adhesion, migration and differentiation through an as yet unknown signal pathway. Here, we identified and characterized a 200kD protein co-immunoprecipitating with DDR1 in NIH3T3 cells overexpressing DDR1, particularly when cells were stimulated with type I collagen. The binding of this 200 kD protein to ligated-DDR1 was in time dependent manner. Using mass spectrometry, the 200 kD protein was identified as non-muscle myosin heavy chain IIA (NMHC-IIA). The formation of a molecular complex between DDR1 and NMHC-IIA was proven by reciprocal co-immunoprecipitation. Furthermore, DDR1 induced phosphorylation of NMHC-IIA was detected at the C-terminal residue Thr1939 of NMHC-IIA (the myosin tail) in a time dependent manner. When NIH3T3 cells overexpressed DDR1, cells formed longer protrusions than parental cells. Immunofluorescence analysis showed that NMHC-IIA was diffusely distributed in the cytoplasm of NIH3T3 cells, while some NMHC-IIA colocalized with actin stress fibers in cells overexpressing DDR1 and colocalization was enhanced in cells stimulated with collagen. In a cell spreading assay, DDR1 overexpressing NIH3T3 cells showed accelerated spreading compared to control cells. Blebbistatin, an inhibitor of myosin motor activity, did not reduce spreading of DDR1 overexpressing cells. In non-spreading NIH3T3 cells, NMHC-IIA colocalized with actin at the cell periphery, whereas in cells overexpressing DDR1, myosin II relocated to the thick cortical actin ring. Our data indicate that non-muscle myosin IIA is a new downstream target of DDR1 and that DDR1-dependent cell shape changes and cell spreading is mediated by myosin IIA heavy chain phosphorylation.
Small Peptides Blocking the Interaction Between SigH and RshA

Eun Hee Jeong and Deok Ryong Kim
Department Biochemistry, College of Medicine, Gyeongsang National University, JinJu, Korea

SigH, an alternative sigma factor of Mycobacterium tuberculosis, is a central regulator of the response to oxidative and heat stress. The activity of SigH is controlled by an anti-sigma factor, RshA. Under these conditions, SigH dissociates from RshA complexes and then interacts with RNA polymerase to initiate transcription. The interaction between SigH and factor RshA is crucial for the survival and pathogenesis of Mycobacterium tuberculosis. Using phage-display peptide library, some specific peptides blocking the interaction between SigH and RshA have been determined. Peptide library(7 mer) was applied to the purified SigH proteins, then phage particles were isolated by the panning four times. The binding affinity between isolated phage particles and SigH has been determined by ELIZA(Enzyme-Linked Immunosorbent Assay). From these analyses, we isolated three specific peptides HADH, EVWTL, TPETR. Their regulatory activities at the transcription in Mycobacterium Tuberculosis will be further determined. These findings might provide some information to develop new drugs for M. tuberculosis.

Proteomic Analysis of Sulindac Induced Protein Expression Profile Changes in Colon Carcinoma Cells

H. Ji, D. F. Frecklington, R. L. Moritz, and R. J. Simpson
Joint Proteomics Laboratory, Ludwig Institute for Cancer Research & the Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

It is well recognized that non-steroidal anti-inflammatory drugs (NSAIDs), such as sulindac and aspirin, have chemo-preventive effects on colorectal cancer (CRC) (1–4). Numerous experimental reports have demonstrated that NSAIDs induce apoptotic cell death in cancer cells in vitro (5, 6). In order to study the biological mechanism underlying this cancer preventive effect, and to study the early events which are involved in sulindac induced apoptosis, we have chosen the colon carcinoma cell line LIM 1215 (7, 8) as our experimental model. Both intra-cellular and secreted proteins (secretome) from sulindac-treated LIM 1215 cells were analyzed by 2D proteomic strategies, such as FFE/RP-HPLC (9) and conventional 2-DE employing DIGE technology. Selected proteins whose expression patterns were dysregulated by sulindac treatment over a 24 hour time period were analyzed using a combination of cytokine array, western blotting and mass spectrometry. A number of candidate proteins involved in a broad range of cellular functions, such as cell proliferation, differentiation, adhesion, invasion, angiogenesis, metastasis and apoptosis were identified. Our data may lead to a better understanding of sulindac-induced apoptosis and most importantly, provide information for the improvement of cancer preventive drugs and identifying novel therapeutic and/or preventive protein targets of CRC.

The term “preconditioning” refers to a phenomenon that pretreatment with potential noxious stress–stimulus can increase cellular tolerance to subsequent noxious stress-stimulus and is biological adaption to stress. However, the endogenous defence mechanism has not been fully identified. In this study, we use proteomic tools to try to find out the proteins involved in preconditioning in well established precondition/hypoxia model of human Type II Alveolar Epithelial Cells, A549 cell. We used 2-DE to show that compared with hypoxia group, several proteins were upregulated in precondition plus hypoxia group. Three proteins of which were identified by MALDI-TOF/MS. They were calreticulin, HSP27 and peroxiredoxin. The results of 2-DE were confirmed by western blot. In order to further study cytoprotection effect of calreticulin in A549 cell, we stable transfected human full length calreticulin gene into the cells. The results showed that A549 cells transfected with calreticulin gene had greater survival rate than the vector control when submitted to sustained hypoxia. Thus, our data suggest that proteomics are able to provide us a new strategy to investigate the proteins mediated preconditioning in the cells.

**C-Myc Cooperate with Cyclin/CDK/CKI in Modulating Proliferation of Liver Cancer Cell Line SMMC-7721 by Exposure to ATRA**

Department of Hemato-oncology, Institute of Basic Medicine, Shandong Academy of Medical Sciences, Jinan, Shandong, P.R. China

ATRA is able to trigger terminal differentiation and induce growth arrest of several established human myeloid cell lines and solid tumor cells. Although the biologic effects of ATRA so far are well characterized, the molecular mechanisms regulating these processes are largely unknown. The c-myc often play role in activating cell transcription and modulating cell proliferation and differentiation. To further study the role of c-Myc in modulating proliferation of SMMC-7721 liver cancer cell induced by ATRA, the proliferation of SMMC-7721 was detected by MTT assay, c-Myc, Mad1 and hTert gene expression were measured by RT-PCR, c-Myc, Cyclin/CDK/CKI protein were detected by Western blot assay, cell cycle profile was evaluated by flow cytometry, telomerase activity was assayed with TRAP, and the combination of c-Myc and Mad1 on target gene hTert promoter was assayed by CHIP. The results showed that C-Myc expression was down-regulated in differentiated SMMC-7721 cells with reduction of telomerase activity and down-regulation of hTert expression, which is contributed to the reduction of c-Myc and increase of Mad1 on target gene hTert. At the same time, the results also showed that up-regulation of P21 and P27 and down-regulation of CyclinA and CyclinE in differentiated cells, as compared with exponentially growth SMMC-7721 cells. It is concluded that down-regulation of c-Myc expression and inactivation of hTert play critical role in differentiation of SMMC-7721 cells after exposing to ATRA in vitro.
5.24
A Functional Study on a Novel KRAB Zinc Finger Protein—ZNF333
Zhe Jing, Haixin Yuan, and Shangzhi Huang
Department of Medical Genetics, Institute of Basic Medical Sciences; Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, P.R. China

To a large extent, transcription factors play an important role in gene regulation and cellular differentiation by interacting with specific DNA sequences present in the promoter region of target genes. As a novel and sole zinc finger protein containing two KRAB domains currently, ZNF333 was identified in the critical region of a polysyndactyly pedigree on chromosome 19p13.1. In order to understand the function of ZNF333, by using a random oligonucleotide selection assay, a specific DNA binding consensus sequence of ZNF333 was isolated and identified by electromobility gel shift assay, then a series of study of mutation analysis and competitive EMSA and luciferase assay all showed the consensus sequence of ZNF333 was conserved. EPD database analysis suggested its possible downstream target gene maybe Bcl-2—a gene involved in cell apoptosis.

Further, a yeast two-hybrid system was used in human lymphocytes cDNA libraries to screen the potential cofactors that can interact with ZNF333 protein. A serine/threonine kinase—pim-2 was identified by GST-pull down and co-immunoprecipitation technique, which can contribute to the cell survive and confer resistance to many apoptotic stimuli. The study strongly suggested that the function of ZNF333 may be related to cell apoptosis.

5.25
HMGB1 Overexpression Patterns in Colorectal Carcinoma Cell Lines and Hepatocellular Carcinoma Cell Lines

Department of Pathology, Cancer Metastasis Research Center and Brain Korea 21 Projects for Medical Sciences, Yonsei University College of Medicine, Seoul, Korea

Altered expression of high mobility group box chromosomal protein 1 (HMGB1), an intranuclear protein that interacts with several transcription factors and plays a role in tumor metastasis after its secretion, is reported in several tumors, however the significance of overexpression and its regulatory forms are essentially unknown. We examined expression of HMGB1 by two dimensional electrophoresis (2DE) and Western blotting, and the specific localization of HMGB1 was also evaluated by nuclear and cytoplasmic fraction in 13 colorectal carcinoma (CRC) and 5 hepatocellular carcinoma (HCC) cell lines. Modifications of HMGB1 were evaluated by cytoplasm fraction in 13 colorectal carcinoma (CRC) and 5 hepatocellular and the specific localization of HMGB1 was also evaluated by nuclear and secretory forms are essentially unknown. We examined expression of HMGB1 in 18 cell lines, however the expression of 13 colon cancer cells were greater than 5 HCC cells (average relative ratio: 2.83 versus 1.5). Moreover, the cytoplasmic HMGB1 expression was higher in colon cancer cells than liver cancer cells. Immuno-2DE revealed 4 different spots in CRC cell lines (Colo205, LS174T, NClH508) and 3 different spots in HCC cell lines (Hep3B, HepG2, SNU182). Additionally, the spot patterns and intensity were remarkably different between two types of cancer cells. Our finding suggests that HMGB1 play different roles in the tumor growth and invasion, because HMGB1 expression and modifications are remarkably different between CRC and HCC cell lines.

5.26
Caspase-3-mediated Cleavage of Caveolin-2 in Drug-induced Apoptotic v-Src Rat-1 Cell

Dong Joon Kim, Eun Jin Lim, and Choong Won Kim
Department of Biochemistry, College of Medicine, Gyeongsang National University, Republic of Korea

Caveolin-1 and caveolin-2 are the major coat proteins found in plasma membrane caveolae of most of cell type. Caveolin-2 is a member of the caveolin gene family with no known function. It is believed that caveolin-2 is “accessory protein” that functions in conjunction with caveolin-1. The potential roles of caveolin-1 and caveolin-2 in apoptosis remain controversial. But disruption of caveolar structure alters the profile of drug-induced caspase-3 activity suggesting a role for caveolae in the regulation of cell apoptosis. We expect that caveolin-2 will have the normal physiological role for the mechanism with caveolin-1. Here we demonstrate that endogenous caveolin-2 is caspase-3 substrates that are cleaved in v-Src Rat-1 cell and Rat-1 cell during staurosporine, etoposide-induced apoptosis. But endogenous caveolin-1 is not observed that phenomenon. The drug-induced proteolytic cleavage of caveolin-2 are completely inhibited by pretreatment with 100 µM concentration of the caspase inhibitor benzoyloxycarbonyl-Asp-Glu-Val-Asp-fluorometylethylone (Z-DEVD-FMK) indicating that the proteolytic activation of caveolin-2 is caspase-3-dependent. Thus our results suggest that caveolin-2 may act as an important factor in signaling apoptotic cell death in both v-Src Rat-1 cell and Rat-1 cells.

5.27
Proteomic Analysis of Caveolar-localized Proteins Purified from HIRc B Cells Treated with Insulin

Yun Hee Kim, Young-Sool Hah, and Choong Won Kim
Department of Biochemistry, College of Medicine, Gyeongsang National University Biochemistry, Jinju, Korea

Caveolae are specialized structures of the plasma membrane as flask shaped cell surface invaginations with a diameter of 50–100nm. Functionally, caveolae plays a role in many important cellular processes, including transcytosis, endocytosis, and signal transduction. Many functions of caveolae are mediated by caveolin. Caveolin, a 21–24kDa integral membrane protein, is a principal structural component of caveolae. Caveolin interacts with a number of signaling proteins, insulin receptor, EGFR, Src, G-protein coupled receptor. Especially, insulin receptor interacts with caveolin-1 scaffolding domain and this interaction between caveolin-1 and insulin receptor enhances or diminishes insulin signaling in various cell types. To investigate caveolar-localized proteins after insulin treatment, HIRc B cells, which overexpress the human insulin receptor cDNA, were culture in DMEM supplemented with 10% FBS, treated with insulin (100 nM) for indicated time, and caveolin-rich fractions were isolated from these cells using the 5%-35% sucrose gradient and percoll density gra-dient centrifugation. These caveolin-rich fractions were subjected to 2D SDS-PAGE. Several spots were appeared or disappeared after insulin stimulation, resulting in redistribution of these proteins consistent with the lapse of the insulin treated time. Several proteins, containing signaling molecules involved in insulin signaling, were identified by MALDI-TOF mass spectrometry. These results suggest that the caveolae may regulate insulin signal pathway through the functional interaction between caveolae and signaling proteins.
Recently, a large number of chemicals that are biologically interesting but with largely unknown mechanism have been isolated from natural products and chemical libraries. Identification of direct binding receptors of such chemicals can provide tremendous benefits in functional genomics and proteomics toward disease-related new drug development. We previously developed several novel curcumin derivatives and evaluated their biological activities. Among them, 4-(3,5-bis-[2-(4-hydroxy-3-methoxy-phenyl)-ethyl]-4,5-dihydro-pyrazol-1-yl)-benzoic acid (referred as HBC) showed potent inhibitory activities against the proliferation of several human cancer cells. However, the mechanisms underlying how HBC inhibits tumor cell growth are entirely unknown. We utilized cDNA-phage display biopanning to identify the cellular receptor protein of HBC from the phage libraries expressing most of human genome-wide human cDNA libraries. As a result, we isolated a major binding protein of HBC from the phage libraries expressing most of human proteome with native folding structure, and subsequently identified Ca²⁺/CaM as a putative receptor protein of HBC. Direct interaction between HBC and Ca²⁺/CaM was confirmed using both phage display binding assay and surface plasmon resonance analysis. Flexible docking modeling of the binding between HBC and Ca²⁺/CaM suggests a possible binding mode of the new Ca²⁺/CaM antagonist. In biological systems, HBC induces sustained phosphorylation of ERK1/2 and activates p21WAF1 expression resulting in the suppression of the cell cycle progression of HCT15 colon cancer cells. These biological activities of HBC are similar to those of other Ca²⁺/CaM antagonists, suggesting that Ca²⁺/CaM is a biologically relevant receptor of HBC. The present study demonstrates that HBC is a new Ca²⁺/CaM antagonist with a unique structure and offers a new lead compound for the development of more potent Ca²⁺/CaM antagonists. Moreover, this study support the idea that Ca²⁺/CaM is an emerging target for antitumor drug development.

Major function of sebaceous gland includes secretion of lipids, triglycerides, fatty acids, cholesterol and its ester, which appear to be important in the pathogenesis of acne vulgaris. As therapeutic agent for acne, 13-cis-retinoic acid (RA) has been known to decrease sebaceous gland size and inhibit growth of P. acnes associated inflammation. To determine what proteins may be involved in mediation of the effects of 13-cis-RA on cellular proliferation and lipid synthesis of human sebaceous gland in vitro, proteomic analyses of cultured S295 cells that had been treated with this agent were performed. HPTLC analysis of lipid component shows that 13-cis RA treatments markedly decreased the synthesis of triglycerides, wax/stearyl esters, and free fatty acids, whereas squalene or cholesterol synthesis remained unchanged or slightly increased. Using a standard proteomic analysis system, we found that levels of keratins K19, K10, lanoster synthase and steroid 5α-reductase were decreased but androgen receptor increased over time in cultured S295 cells, which were further confirmed by Western blot analysis. In addition, 13-cis-RA treatment resulted in decrease of sebaceous gland proliferation in a dose- and time-dependent manner by XTT-assay. These results suggest that 13-cis RA may act through these proteins and their associated proteins in modulation of cell proliferation rate and lipid synthesis, resulting in suppression of acne.

Treatment of GA completely inhibited HSP90 stimulated significant phosphorylation of HSP90 and the phosphorylation was diminished by administration of HSP90 inhibitor, geldanamycin (GA). Treatment of GA completely inhibited H. pylori-induced IL-8 production due to inactivation of AP-1 and NF-κB. These results subsequently lead to inactivation of AP-1 and NF-κB, which are known to be major transcriptional factors of IL-8. Our data provides important insights that HSP90 involved as a crucial regulator in H. pylori-induced IL-8 production and its inhibitor could be potentially used for the inhibition of H. pylori-provoked inflammation.
Identification of Angiogenic Signaling Pathways by Proteomic Approach for Protein Modifications Including Phosphorylation, Oxidation and Acetylation

Kong-Joo Lee

From the Center for Cell Signaling Research, Division of Molecular Life Sciences and College of Pharmacy, Ewha Womans University, Seoul, Korea

Angiogenesis is a key process generating new capillary blood vessels for solid tumor growth and metastasis. Vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) are known to play significant roles in angiogenesis. In endothelial cells, the regulating molecular machineries of VEGF and Ang-1 signaling pathways are not well understood. In this study, we have identified the tyrosine phosphorylated proteins and acetylated proteins modified in VEGF, hydrogen peroxide and Ang-1 signaling pathways by using 2D-gel separation, western analysis and mass spectrometric (MALDI-TOF MS and LC-ESI-q-TOF MS) analysis. We could identify the modified proteins in pathway-dependent manner. Also the combination of 2D-gel separation and mass spectrometry makes it possible to exhibit the heterogeneous populations of same protein which was responded in different ways and the modification was varied depending on the pathways. The results analyzed by bioinformatic tools were used to extract the biological meaning from proteomic data. The combined results provide the insight for new VEGF, Ang-1 and H2O2-induced signaling pathways, and give us the direction of functional studies of target proteins involved in angiogenesis.

Supported by MOST FP03B3-04-110, by KOSEF through the CCSR, by IMT2000 project, and by BK21 program.

Proteome Analysis Associated with Cadmium Adaptation in U937 Cells: Identification of Calbindin-D28k as a Secondary Cadmium Responsive Protein That Confers Resistance to Cadmium-induced Apoptosis

TaeHo Lee, Hye-Kyung Jeon, Hyung-Seung Jin, and Dong-Hee Lee

Department of Biology and Protein Network Research Center, Yonsei University, Seoul, Korea

Cadmium is a well-known environmental toxicant and carcinogen. To identify proteins involved in cellular adaptive responses to cadmium, we established cadmium-adapted U937 cells that exhibit resistance to cadmium-induced apoptosis, and performed comparative proteome analysis of these cells with parental cells that were either untreated or treated with cadmium. Newly identified proteins that were changed in expression level in both adapted cells and cadmium-treated parental cells included proteins implicated in cell proliferation and malignant transformation. Interestingly, a calcium binding protein calbindin-D28k was increased only in the adapted cells, but not in cadmium-exposed parental cells. The level of calbindin-D28k increased by the degree of cadmium adaptation and was stably maintained without selective pressure of cadmium. Cadmium-adapted U937 cells were resistant to the toxic effects of cytosolic calcium rise by cadmium treatment and by depletion of intracellular calcium stores, suggesting that enhanced calcium buffering by up-regulated calbindin-D28k may be responsible for acquiring resistance to cadmium-induced apoptosis. We demonstrated that over-expression of calbindin-D28k in an MN9D neuronal cells resulted in reduced cadmium-induced apoptosis. Our study documents for the first time that cells respond to long-term cadmium exposure by increasing calbindin-D28k expression, thereby attenuating cadmium-induced apoptosis.

Up-regulation and Tyrosine-phosphorylation of Heterogeneous Nuclear Ribonucleoprotein K Proteins (hnRNP K) During BCR Signaling: A Proteomic Approach

TaeHo Lee and Hye-Kyung Jeon

Department of Biology and Protein Network Research Center, Yonsei University, Seoul, Korea

hnRNP K has diverse molecular partners implicated in signal transduction pathways, and is tyrosine-phosphorylated in response to growth factors and oxidative stress. Among the structurally distinct domains of hnRNP K, a SH3-binding domain (SH3BD) has been known to promote the association of SH3-containing tyrosine kinases and protooncoprotein Vav which are involved in BCR signaling. In this study, we employed a functional proteomic approach to identify proteins associated with BCR signaling pathway using human Burkitt lymphoma B cell line, Ramos. This cell line has been shown to undergo apoptosis upon cross-linking of BCR with anti-IgM antibody. With this approach, we found that a certain hnRNP K isoform is up-regulated within a short time treatment of anti-IgM (10 min) and its level is continuously increased until 2 hr treatment. Subsequent analysis showed that hnRNP K is tyrosine-phosphorylated in response to BCR ligation. Analysis of phosphorylated proteins which are co-immunoprecipitated with hnRNP K revealed that hnRNP K binds phosphorylated Vav transiently after treatment of anti-IgM, and this interaction is mediated by the SH3 binding domain. Furthermore, Ramos cells expressing the mutant protein lacking the SH3 binding domain are less susceptible to anti-IgM-induced cell death, indicating a functional involvement of hnRNP K during BCR-mediated signaling.
The regulation of eIF5A signaling to p53 for apoptosis.

For p53-dependent apoptosis, and syntenin might regulate p53 by balancing the protein level was significantly inhibited. Therefore, eIF5A seems to be a previously un-recognized regulator of p53 that may define a new pathway of eIF5A led to a p53-dependent apoptosis, or sensitized cells to induction of apoptosis by chemotherapeutic agents. However, when eIF5A interacted with its novel partner, syntenin, the eIF5A-induced increase in p53 of p53 transcriptional activity was further demonstrated by the additional functional studies were made on several proteins, based on the protein interaction map for the validation of the expected connection between proteins found in the map and the TGFbeta pathway.

In a second part, I will discuss the importance of correctly annotated databases for protein function and protein interactions in order to build biological networks on solid grounds.

Large scale yeast two-hybrid (LS-Y2H) technology has been developed to explore protein-protein interactions networks. I will present the general outlines of the methodology and discuss its assets and limitations. Related technologies will also be compared to LS-Y2H. Genetics studies and functional assays can then validate protein involvement in a pathway or a complex. I will present studies made in the TGFbeta pathway. Key features will be given on the protein interaction map that was built in this pathway. Additional functional studies were made on several proteins, based on the protein interaction map for the validation of the expected connection between proteins found in the map and the TGFbeta pathway.

In a second part, I will discuss the importance of correctly annotated databases for protein function and protein interactions in order to build biological networks on solid grounds.

The angiogenic switch of endothelial cells from a quiescent state to a highly active state plays an important role in angiogenesis. Clarification of the key factors regulating this process and its signal pathway should greatly help us in understanding the mechanism of angiogenesis. This study was designed to comprehensively analyze the differential expression of proteins from human umbilical vein endothelial cells (HUVEC) exposed to tumor conditioned medium (TCM) and to identify the key regulators of the cell cycle progression which is closely related with the cellular proliferation. The HUVECs were exposed to TCM from breast carcinoma cell line MDA-MB-231, then their proliferating activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Their cell cycle distribution was measured by flow cytometer (FCM), and the differentially expressed proteins were detected by 2-Dimensional Electrophoresis (2-DE). Following the stimulation of TCM, HUVECs showed higher pro-mitogenic ability and more cells in S phase than did the negative control group (ECGF-free medium with 20% FBS), but a similar ability to the positive control group (medium with ECGF and FBS). After 2-DE/Matrix Assisted Laser Desorption-Time of Flight (MALDI -TOF), 8 up-regulated and 3 down-regulated proteins were significantly visualized on the protein expression profile compared with control group. Nine proteins from these 11 proteins are involved in important activities in the cell cycle such as DNA synthesis, transcription, cell proliferation and migration. The result of our investigation has proved that the breast carcinoma cell line MDA-MB-231 may secret soluble pro-angiogenic factors and induce HUVEC to produce some key proteins that could promote the HUVEC angiogenic switch and proliferation which is involved in the formation of angiogenesis.

Although elf5A was originally designated as an “initiation factor”, recent data have shown it to be also involved in apoptosis. However, the actual function of elf5A in apoptosis is still unknown. In this study, we performed yeast two-hybrid screens to identify elf5A-interacting proteins to help us understand its mechanisms. Our results demonstrated that elf5A and syntenin could engage in a specific interaction both in vitro and in vivo, and functioned collaboratively to regulate p53 activity. Our findings, for the first time, revealed a new biological activity for elf5A as the regulator of p53. Overexpression of elf5A or its EFP domain resulted in up-regulation of p53, and silencing elf5A by siRNA reduced p53 protein level. Further analysis by RT-PCR showed elf5A activated p53 transcription. The effect of elf5A on p53 transcriptional activity was further demonstrated by the increasing expressions of p21 and Bax, well-known target genes of p53. In contrast, a point mutant of elf5A, hypusination being abolished, was revealed to be functionally defective in p53 up-regulation. Overexpression of elf5A led to a p53-dependent apoptosis, or sensitized cells to induction of apoptosis by chemotherapeutic agents. However, when elf5A interacted with its novel partner, syntenin, the elf5A-induced increase in p53 protein level was significantly inhibited. Therefore, elf5A seems to be a previously un-recognized regulator of p53 that may define a new pathway for p53-dependent apoptosis, and syntenin might regulate p53 by balancing the regulation of elf5A signaling to p53 for apoptosis.
High-throughput Construction of Recombinant Adenovirus via Directional Cloning, Direct Ligation and Self-Rescue of Circular Adenovirus Vector

C. H. Li, J. Zhang, R. Tao, and L. X. Ma
Institute of Biochemistry and Molecular Biology, Hubei University, P.R. China

High-throughput creation of recombinant adenovirus is a critical path to the expanding utility of adenovirus vector techniques. Here we report a novel vector that makes use of directional cloning & direct ligation for the generation of replication-defective recombinant adenovirus constructs and I-SceI-mediated rescue of circular adenovirus plasmids for high efficient transfection in HEK293 cell. Firstly, we eliminated two kinds of restriction endonuclease sites Ascl and Clal in pAdEasy by site-directed mutation and recombination in Escherichia coli producing vector pAdEasy-1, and cloned into pShuttle a stuffer with unique Ascl and Clal at each end under control of a CMV promoter, upstream of a SV40 polyadenylation signal resulting in a plasmid designated as pShuttle-2. Then the recombinant adenovirus plasmid with unique Ascl and Clal, denominated pAd-3, was generated by recombination of pAdEasy-1 and pShuttle-2 in E. coli BJ5183. Next, without considering the restriction endonuclease sites in the inserted genes, we only need to treat PCR products, amplified by forward and reverse primers with CGA and CGCGA at both ends respectively, with T4 DNA Polymerase and dTTP to produce 5’CGG 3’ overhang at one end and 5’CG 3’ overhang at the other, and then ligate them with vector pAd-3 digested with Ascl and Clal, followed by the transfection of ligation into HEK293 directly. Moreover, we cloned an I-SceI expression cassette into vector pAd-3. Owing to enzymatic rescue of circular virus vector by an intron-encoding rare endonuclease I-SceI in vivo, the efficiency of transfected construct was increased at least 10-fold compared with linearized one.

This system is high-throughput and labour saving. It does not rely on recombinant either in mammalian or in bacterial cells, and allows the generation of recombinant adenovirus within two weeks, even lets ligation products be straight transfected. Based on this vector, we can develop a series of powerful vehicles, such as adenovirus expression vectors with various affinity tags, adenovirus-based RNAi vectors. This system should be extensively used in constructing cDNA library, expressing genes of interest, assessing gene function, etc, and believed to facilitate the researches in proteomics.

EDAG, an Anti-apoptotic Protein, Also Regulates the Proliferation, Differentiation, and Lineage Commitment of Hematopoietic Cells

ChangYan Li¹, YiQun Zhan¹, Wei Li¹, ChengWang Xu¹, WangXiang Xu¹, SiYing Wang¹,², Jun Lv¹, Ying Zhou¹,², PeiBin Yue¹, Bing Chen¹,², and XiaoMing Yang¹
¹Beijing Institute of Radiation Medicine, Beijing, and ²Department of Pathology of Anhui Medical University, Hefei, P.R. China

EDAG is identified in human fetal liver by PCR-based subtractive hybridization. It is preferentially expressed in human fetal liver and adult bone marrow and is increased in lymphoma and PMBCs of patients with leukemia. Down-regulation of EDAG protein in K562 cells resulted in inhibition of growth and colony formation, and enhancement of sensitivity to erythroid differentiation induced by hemin. Overexpression of EDAG in HL-60 cells significantly blocked the expression of monocyte/macrophage differentiation marker CD11b after PMA induction. Moreover, overexpression of EDAG in pro-B Ba/F3 cells prolonged survival and increased the expression of c-Myc, Bcl-2 and Bcl-XL in absence of IL-3. Furthermore, we showed that EDAG enhanced the transcriptional activity of NF-κB, and high DNA binding activity of NF-κB was sustained in Ba/F3 EDAG cells after IL-3 withdrawn. Inhibition of NF-κB activity resulted in by a NF-κB-specific inhibitor or overexpression of a nongraded form of IkBα promoting Ba/F3 EDAG cells death. These results suggest that EDAG regulates the proliferation and differentiation of hematopoietic cells and resists cell apoptosis through the activation of NF-κB.

To further investigate the function of EDAG in hematopoiesis in vivo, we generate EDAG transgenic mice in which EDAG is overexpressed in hematopoietic tissue under the control of CD11a promoter. No leukemia developed, but blood analysis showed a significant increase of granulocytes and reduction of lymphocytes in transgenic mice. In the bone marrow, we found an increase of hematopoietic stem cells (HSC) but decreased number of CFU. Further statistics showed that CFU-GM formation was enhanced but the CFU-ML formation was reduced compared to the littermates. FACs analysis suggested an augment of Mac-1/Gr-1 cells and a decrease of B220/CD11b cells in transgenic bone marrow. The spleen was much enlarged and the livers exhibited a disrupted morphology and an infiltration with granulocytes in transgenic mice. Transgenic mice also exhibited an arrest of thymocytes within the CD4/CD8 double-negative (DN) population, specifically at the DN-I stage. These results suggest that overexpression of EDAG inhibits the differentiation of HSC and breaks the homeostasis of lineage commitment by promoting the myeloid especially the granulocytic lineage and inhibiting the development of lymphoid lineage.
5.39  
Protein Interaction Networks in Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster: Large-scale Organization and Robustness

Dong Li1,2, Jianqi Li2, Songfeng Wu2, Ping Wan2, Tinggui Chen2, Chunjuan Du2, Jian Wang2, Yunping Zhu2, Fuchu He2, and Xiaojie Xu1

1College of Chemistry and Molecular Engineering, Peking University, P.R. China; and 2Department of Genomics and Proteomics, Beijing Institute of Radiation Medicine, Beijing, P.R. China

High-throughput screens have begun to reveal the protein interaction networks that underpin most cellular function in several organisms. To uncover the general properties of these protein interaction networks, systematic topology structure and robustness analyses are performed on the protein interaction networks of Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster, which shows that the protein interaction networks have scale-free and high degree clustering nature as the consequence of the hierarchical organization. These networks are found to have the small-world property with almost similar diameter at 4–5. Simulation of the node removal shows the high error tolerance and attack vulnerability of these networks. Under the attack removal, critical point appears in three networks with different values, which may reflect the relationship between the networks’ scale-free property and their robustness. These fundamental analyses of the networks may serve as a starting point for further exploration of complex biological networks and the coming research of “systems biology.”

5.40  
Tec Is Involved in HGF Signaling Pathway and Regulates the Proliferation of WB-F344 Cells

Feifei Li,1,2 Peibin Yue, Yonghui Li1, Shiyong Wang1,2, Wangxian Xu1, Yiqun Zhan1, and Xiaoming Yang1,2

1Beijing Institute of Radiation Medicine, Beijing, and 2Department of Pathology of Anhui Medical University, Hefei, P.R. China

Tec is a non-receptor tyrosine kinase and is characterized by N-terminal pleckstrin homology (PH) domain and Tec homology domain downstream of PH domain. Tec has been identified as a Src-related cytoplasmic protein tyrosine kinase expressed in liver and is highly expressed in hepatocarcinogenesis. However, Tec functions are remained to be understood. Hepatocyte growth factor (HGF) can significantly induce the proliferation of rat hepatic stem-like cell line WB-F344 cells, which is mediated by MAPK signaling pathway. HGF can also activate transcription factor Elk-1, one of the substrates for Erk, up-regulating the expression of immediate early response genes, including c-Fos. In this report, we demonstrate that TEC is expressed in WB-F344 cells and stimulation of the cells with HGF led to tyrosine phosphorylation of TEC. In vitro kinase assay confirmed that auto-phosphorylation activity of TEC was enhanced with the phosphorylation of TEC. Further studies showed that HGF-induced phosphorylation of TEC was blocked by treatment of WB-F344 cells with specific inhibitor U0126 for MAPK/ERK pathway, but not for p38 MAPK and Akt pathways. Luciferase assay showed that the activity of SRE induced by HGF was significantly increased by over-expression of wild-type Tec in WB-F344 cells but inhibited by over-expression of kinase-dead mutants of Tec. Moreover, using PathDetect in vivo signal transduction pathway trans-reporting system, we also found that introduction of Tec markedly increased the transcriptional activity of Elk stimulated by HGF, and this activity was suppressed by kinase-dead mutants of Tec. Furthermore, treatment of WB-F344 cells with U0126 suppressed Tec-driven Elk activation induced by HGF. In addition, the proliferation of WB-F344 cells induced by HGF was inhibited by the kinase-dead mutants of Tec. Interestingly, expression of TEC kinase-dead mutants did not affect the proliferation of the cells induced by serum, suggesting that TEC is specifically required for HGF signaling.

In conclusion, we demonstrate that TEC kinase is involved in the HGF/c-met signaling pathway and regulates the proliferation of WB-F344 cells via mediating Elk activation.
Proteomics to Study the Aging Related Proteins in Human Normal Colorectal Epithelial Tissue

M. Li1,2, Z. Q. Xiao1, X. P. Feng1, P. F. Zhang1, F. Li1,2, and Z. C. Chen1,2

1Key Laboratory of Cancer Proteomics, Ministry of Health of China, Xiangya Hospital, Central South University, Changsha, Hunan, P.R. China; 2Cancer Research Institute, Central South University, Changsha, Hunan, P.R. China

The aging of the human colorectal epithelia is a complex process involving multiple events and steps. Many studies have been done on gene level to elucidate the molecular mechanism of this process, however, little is known about the effect of aging on protein expression levels of genes involved in the regulation of cell proliferation and apoptosis. The purpose of this study is the establishment of two-dimensional electrophoresis (2-DE) profiles with high resolution and reproducibility from normal colorectal epithelial tissue between young people and paired old people, and identification of differential expression proteins. The total proteins of normal colorectal epithelial tissue of young and old people were separated by means of 2-DE. The differential expression proteins were analyzed and then identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Our study showed that well-resolved, reproducible 2-DE patterns of normal colorectal epithelial tissue of young people and paired old people were obtained. A total of 1072 ± 45 spots were matched between the electrophoretic maps of normal colorectal epithelial tissue of young people and paired old people. Among them, twenty-one differential proteins were identified by PMF, these proteins were involved in the cell proliferation, differentiation and signal transduction. These data will be used to screen the biomarker to further study human colorectal carcinoma.

Genome Class Prediction Based on Amino Acid Composition (AAC) from Proteomes

Wuju Li, Tao Liu, Xiaomin Ying, and Ming Fan
Beijing Institute of Basic Medical Sciences, Beijing, P.R. China

With genomic sequences from three domains of life become increasingly available, the relationships between the AAC and the genome classes (organisms’ phenotype) have been widely studied in the following two aspects. The first aspect is to concentrate on the difference of AAC of proteins from particular type or whole proteomes in different genome classes. The second aspect is to study the issue of genome class prediction based on the AAC. The purpose of the above two aspects is to explain why certain organisms can live in extreme conditions of temperature, salinity, or pressure. Here we want to emphasize whether there is a possibility to predict the genome classes as accurately as possible using small subsets of amino acids. In order to investigate the issues systematically, the Fisher linear discriminate analysis (FLDA) was applied to the following four data sets DOMAIN, LIFE, HTHAB, and ARCHAEA. The DOMAIN is about the three domains of life (16 archaea, 75 bacteria, and 6 eukaryotic genomes). The LIFE is about the three lifestyles (13 HTH, 4 TH, and 79 MES). The HTHAB includes 10 HTH in archaea and 3 HTH in bacteria. The ARCHAEA is about the three lifestyles in archaea (10 HTH, 3 TH, and 3 MES). By using the feature selection method of all possible combinations of features (amino acids), we found that the cross-validation accuracies for above four data sets could reach 94.8%, 97.9%, 100.0%, and 100.0% by only using the compositions of four (A, I, K, and Q), five (I, K, P, V, and Y), two (E and Q), and two (M and Q) amino acids respectively. The average cross-validation accuracy reaches 98.2%. Therefore, AAC from the proteomes provides an alternative way to determine the genome classes such as the lifestyle or the domains of life. According to what we know, the correspondence analysis, principal component analysis (PCA), and hierarchical cluster analysis have been applied to study the distinction of different genome classes using the AAC, but the classification methods have not been used. Therefore, our work represents a first attempt on this effort in this field.
The Function of Variant IL-13 Protein in Asthma

Hongyan Liang, Xiaofeng Jiang, Yongchen Xu, and Yuwen Wang

Department of Clinical Laboratory, The 2nd Affiliated Hospital of Harbin Medical University, Harbin, P.R. China

Airway inflammation and airway hyperresponsiveness (AHR) are hallmarks of asthma. In asthma, cytokines produced by activated Th2 lymphocytes are believed to play critical roles in regulating the inflammatory process. Interleukin-13 (IL-13) in particular have been suggested to be key factors contributing to the chronic inflammatory state characterizing asthma.

The study began with the level of Interleukin-13 in serum and sputum of patient with asthma. The Interleukin-13 level is higher than which from normal controls. Then, to discuss the roles of the IL-13 gene exon4 A2044G single nucleotide polymorphism (SNP) in the pathogenesis of bronchial asthma. The IL-13 exon4 was amplified by PCR. Then separating the amplification with capillary electrophoresis followed by hybridization of molecular beacons into the PCR product which were sequenced in the end. There was significant difference in the distribution of A/G in IL-13 exon2044. A allele frequency was higher in asthma compared with normal controls, same as sequencing. It is proved that IL-13 A2044G SNP is important in the asthmatic mechanism.

The variant of the IL13 gene (Arg130Gln) is genetically associated with bronchial asthma. To address whether the Gln130 variant of IL13 influences IL-13 function, contributing to the pathogenesis of bronchial asthma, we studied the functional properties of the variant. We generated 2 types of recombinant IL-13 proteins, the amino acids of which at 130 were arginine or glutamine, and analyzed the function that 2 types of recombinant IL-13 proteins influence bronchial epithelial cell to secrete MCP-1, eotaxin and sICAM-1. These results suggested that the variant might act as a functional genetic factor of bronchial asthma with a unique mechanism to upregulate local and systemic inflammatory factor concentration.

Functional and Structural Proteomics of the Venoms of the Chinese Bird Spiders

Songping Liang1, Yucheng Xiao1, Chunhua Yuan1, Quanyuan He1, Dongling Li1, Jianbo Diao2, Shanyun Lu2, Xiaocheng Gu2, Zhonghua Liu1, Meichi Wang1, and Jinyun Xie

1College of Life Sciences, Hunan Normal University, Changsha, P.R. China; and 2College of Life Sciences, Peking University, Beijing, P.R. China

All spiders are predators and have venom glands. The primary purpose of spider venom is to kill or paralyze prey. Spider venoms are known to contain multitude components with different biological activities and are of interests as tools for studying neuroscience and physiology; and as potential lead structures for insecticides and pharmaceuticals. The venoms of three Chinese bird spiders, O. huwena, O. hainana and C. Jingzhao, have been investigated for their functional and structural proteomics. The venom of each species was fractionated first into two parts of the molecular weight below or over 10,000D by gel filtration. The fraction with high molecular weight (>10,000D) was then further separated by 2D-PAGE. The fraction with low molecular weight (<10,000D) was further separated by 2D HPLC. About 400–500 protein and peptide components from each venom can be separated and detected by this separation strategy. Of these, about 50 toxins have been analyzed by Edman degradation or mass spectrometry to get their complete amino acid sequences. These toxins possess quite different biological activities including inhibition of voltage-gated calcium, sodium or potassium channels, insecticidal activity, lectin-like agglutination, analgesic effect and inhibition of proteases etc. Most of these toxins contain 30–50 amino acids with three disulfide bonds. Three disulfide bond patterns have been found among these toxins: I-IV, II-V, III-VI, I-III, II-V, IV-VI and I-VI, II-IV, III-V. By means of 2D-NMR and molecular modeling, three types of 3-D structural motifs were found to be adopted by these toxins. Most of the toxins adopt an “inhibitor cystine-knot” (ICK) motif which contains a triple-stranded anti-parallel β-sheet and a cystine knot. Several toxins adopt a motif as that of HWTX-II, which is different from that of ICK motif. HWTX-XI adopts the third structural motif which is composed of a double-stranded β-sheet and a C-terminal α-helix. The cDNAs analysis revealed that these toxins can be classified into three different cDNA superfamilies according to the “pre-pro” region of their cDNA sequences.
A Novel Tumor Suppressor Gene ARHI Interacts with and Inhibits Stat3 Activity in Breast and Ovarian Cancer

Arata Nishimoto, Yinhua Yu, Zhen Lu, Warren S.-L. Liao, Robert C. Bast Jr., and Robert Z. Luo
The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA

ARHI is a novel imprinted tumor suppressor gene whose expression is downregulated in a majority of breast and ovarian cancers. ARHI is a member of the Ras superfamily with high homology to both Ras and Rap. ARHI structurally differentiates from other Ras family members in its unique N-terminal extension. Unlike the Ras oncogene, ARHI functions to inhibit tumor cell growth.

To elucidate the mechanisms by which ARHI inhibits cancer growth, we identified that ARHI interacts with Stat3, a potent transcription factor and an oncogene frequently detected in breast and ovarian cancers. This association was subsequently confirmed by immunoprecipitation. To determine the significance of this interaction between ARHI and Stat3, we re-expressed ARHI in SKBr3 and SKOV3 cells and examined the effects of ARHI on IL-6-dependent Stat3 activation. Immunofluorescent staining indicated that ARHI colocalized with Stat3 and trapped a majority of Stat3 in the cytoplasm, markedly inhibited the translocation of Stat3 into nucleus. We also demonstrated that ARHI markedly inhibited the binding of Stat3 to its promoter elements and Stat3-dependent promoter activity, whereas ARHI mutant that lacks the N-terminus had no such inhibitory effects. Interestingly, the N-terminal region of ARHI contains an LXXLL signature motif that is conserved in the N-terminal region of all PIAS (protein inhibitor of activated Stat family) proteins. These results suggest the LXXLL motif in the N-terminal region of ARHI may play an additional important role in the inhibitory activity of ARHI for repressing the transcriptional activity of Stat3.

Thus, the physical association between ARHI and Stat3 and the functional inhibition of Stat3 activity by ARHI reveal a novel pathway in which a tumor suppressor gene, and also a Ras family member, inhibits oncogene Stat3 activity in breast cancer.