Pulmonary Infection by PCR in Very Low Birth Weight Neonates with Bronchopulmonary Dysplasia or Mikity Wilson Syndrome

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Introduction. The Bronchopulmonary Dysplasia (BPD) or Mikity Wilson Syndrome (MWS) are chronic lung diseases with several pathogenic mechanisms. They can be associated with atypical infections by U. urealyticum, C. trachomatis, and Mycoplasma.

Objective. We researched pulmonary infections in 10 VLBWN with BPD or MWS and had negative cell cultures by PCR in tracheobronchial samples.

Methods. We studied VLBWN in our institution with BPD (n = 6) or WMS (n = 4) (with oxygen dependence and images lung radiographic of lung damage with fibrosis and cystic areas) as diagnosis and negative cell culture to atypical pathogens. We took new tracheobronchial samples at 28 days of life and send them Virology Department to PCR and infection determination.

First. Tracheobronchial aspirate was took with saline solution 0.9% and stored in aliquots at −20°C until needed.

Second. DNA isolation by proteinase K method and we used gel electrophoresis and densitometer to determine DNA concentration.

Third. Determine polymerase chain reaction (PCR). DNA concentration and extraction process were included as samples to identify background bacterial sequences present in reagents and supplies.

Results. We found 7/10 positive infections, 3/6 C. trachomatis infections in neonates with BPD and 4/4 with MWS: U. urealyticum (1), Mycoplasma genitalium (1), C. trachomatis (1) and M. pneumoniae (1).

Conclusions. We should researched atypical pulmonary infections by intracellular pathogens by another methods with more sensitivity as PCR to explain inflammatory response and lung damage.

Comparative Proteomic Analyses of Normal and Normal Pressure Hydrocephalus in Human Cerebrospinal Fluid

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Background: Cerebrospinal fluid (CSF) is an ultrafiltrate of plasma in which “brain specific” proteins are present. Changes in brain, due to any pathology or injury, can alter protein content of CSF. Normal pressure hydrocephalus (NPH) is the condition involving imbalance between its continuous production and efflux. As a result, pressure is exerted by the fluid filled ventricles on adjacent brain tissue leading to the clinical triad, abnormal gait, urinary incontinence and dementia. Imaging studies are commonly used for diagnosis, and surgery for CSF diversion is usually done to relieve excess pressure. In most of the cases, etiology of NPH is unknown, thereby lowering the success rate of treatment. Hence there is need to find specific disease markers to improve the success rate of diagnosis and treatment. Objective: In this presentation the comparison of ante mortem CSF protein profile between NPH patients and normal condition will be presented followed by characterization of unique proteins to NPH.

Methods: Comparative proteomic analyses of ante mortem CSF in 12 NPH patients and 12 normal subjects is carried out. To enhance sensitivity and detection, we have used two dimensional fluorescence difference gel electrophoresis (2-D-DIGE). For characterization of differentially expressed proteins in NPH, electrospray ionisation (ESI)-quadrupole (Q)-time of flight (TOF) mass spectrometry and tandem MS/MS is used.

Conclusions: Usage of these technologies have enabled us to identify differential expression of apolipoprotein J, haptoglobin α1, α1 microglobulin, leucine rich α2 glycoprotein associated to NPH condition in ante mortem CSF. These proteins if studied for their consistency over a large sample, will have potential use in developing differential diagnostic test for NPH.
Divide and Conquer; Fractionating Salivary Proteins

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Saliva contains a number of proteins that maintain homeostasis in the oral cavity. The availability of a complete catalogue of salivary proteins will be important in order to understand the genetic and biological mechanisms causing human disease. We have investigated a number of techniques to fractionate human salivary proteins coupled with LC-MS/MS to populate the salivary protein catalogue.

Three different pre-fractionation strategies were explored for whole saliva and parotid saliva:

1. C4-RPLC separation of whole saliva proteins. 35 fractions were collected, and the proteins in each fraction were trypically digested and analyzed by LC-MS/MS.

2. Proteins in human whole and parotid saliva were pre-fractionated by solution phase IEF (Zoom IEF, Invitrogen). This results in five fractions over a standard pH 3–10 range. The proteins in each fraction were trypically digested and analyzed by either 1D or 2D LC-MS/MS.

3. Glycoprotein capture enriches for the N-linked glycoproteins. The glycoproteins were coupled onto a hydrazide resin and were digested with trypsin. The formerly N-linked peptides were selectively released with PNGase and analyzed by LC-MS/MS.

By employing a combination of these fractionation methods, many low abundance proteins have been identified. This has increased our database of proteins from whole saliva to 1049. In addition, 46 unique N-glycoproteins were revealed from whole saliva, including 12 novel salivary glycoproteins.

A total of 692 non-redundant parotid proteins were found by using a combination of 1D-LC and 2D-LC analysis. Further, solution phase IEF separation was used to pre-fractionate and identify the differences between whole and parotid saliva proteins. 69 proteins were uniquely present in whole saliva and 91 proteins were uniquely present in parotid.

These fractionation strategies will be the basis for applying the salivary proteome to identify markers that can be used not only to identify a disease, but also to indicate disease progression.

On-chip Integration of Affinity Chromatography and Isoelectric Focusing for the Analysis of Post-translational Modifications

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Affinity chromatography (AC) is highly potent in specific enrichment of a minor protein component in a biological fluid. Its mode of operation is a batch-wise in most cases and not suitable for fine resolution of the proteins with subtle differences produced by post-translational modifications. In addition to this, nonspecific adsorption could occur more or less in most cases. For microscale analyses of post-translational modifications, we are developing a protein analysis chip by integrating AC and isoelectric focusing (IEF). IEF can accommodate a sample volume as large as that of a separation channel, allowing the sampling of most of the eluted proteins from an AC column, and can provide a fine separation of proteins according to their isoelectric points, in which some of the important post-translational modifications are reflected, e.g., phosphorylation, sialic acid addition, etc.

A microchannel was constructed in a Pyrex glass chip by wet etching. An affinity adsorbent was held in the channel by a constriction of the channel. Two branching channels for electrode solutions were added at the both sides of the IEF channel located downstream of the AC channel. For this preliminary work, labeled proteins and a fluorescence detection were used. The design and fabrication of the chip, and some results of separation will be reported.

Heterologous Production and Isolation of Selected GPCRs for Structural Analysis

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G protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors and approximately 1–5% of the vertebrate genome encode for GPCRs. In human genome there are 800–1000 genes which encode for these receptors. Despite large variations in their stimuli, all GPCRs share a common seven-α-helix transmembrane architecture and perform signal transduction by a common mechanism via heterotrimeric guanyl nucleotide binding proteins (G proteins). GPCRs play regulatory roles in many different physiological processes and they represent one of the most important classes of drug targets. However, due to the lack of three-dimensional structures, structure based drug design has not been possible. The major bottleneck in getting three-dimensional crystal structure of GPCRs is to obtain milligram quantities of pure, homogenous and stable protein. During this study, three different GPCRs namely, the human bradykinin receptor, the human angiotensin receptor and the human neumedin receptor, were produced in different heterologous expression hosts i.e. Pichia pastoris, Insect cells and Mammalian cells. The recombinant receptors were characterized in terms of ligand binding, glycosylation and their localization in host cells. Subsequently, the receptors were purified using affinity chromatography, which yielded milligram amounts of pure and stable receptors. Currently, structural analysis of these receptors (3-D crystallization and solid state NMR) is underway.
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Human saliva is a very attractive body fluid for disease diagnostics because saliva testing has several key advantages including minimum cost, non-invasiveness, and easy sample collection and processing. We have discovered and validated saliva protein markers for oral diseases including oral squamous cell carcinoma (OSCC) and primary Sjögren’s Syndrome (pSS).

Many of the putative markers for OSCC identified by shotgun proteomics are regulatory proteins or glycoproteins, including Myc binding protein 2, angiomotin like 2, Ras-related protein Rab-7, Mac-2-binding protein, Rho GDP-dissociation inhibitor 2, CD59 glycoprotein precursor, involucrin, KRAB box family protein, hematopoietic lineage cell specific protein, peroxisome biogenesis factor 1, nuclear mitotic apparatus protein 1, swiprosin-2, PHD finger protein 3, histone H1.2 & H1.3 and calgranulin C. We further validated Mac-2 binding protein (M2BP) on a new group of patients using ELISA. The average level of M2BP was determined as $17.93 \pm 7.56$ (OSCC, $n=20$) and $5.35 \pm 3.75$ (control, $n=20$) ng/mL, respectively ($p=0.00000011$). Receiver operating characteristic (ROC) analysis indicated that the sensitivity and specificity for this single glycoprotein was 94.4% and 90.0%, respectively.

The candidate markers for pSS we identified in saliva using 2-D gel electrophoresis/mass spectrometry (2-DGE/MS) include $\alpha$-enolase, fructose bisphosphate aldolase A, caspase 14, S100 calcium binding protein A, fatty acid-binding protein, and $\beta$-2-microglobulin. $\alpha$-enolase, a potential autoantigen for pSS, was further validated using western blotting, suggesting it is a promising saliva marker for pSS detection.

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Identification of the Interaction between TRAF6 and V-ATPase for Osteoclast Function by a Proteomics Approach


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Osteoclasts are cells specialized for bone resorption. For osteoclast activation, tumor necrosis factor receptor-associated factor 6 (TRAF6) plays a pivotal role. To find new molecules that bind TRAF6 and have function in osteoclast activation, we employed a proteomic approach. TRAF6 binding proteins were purified from osteoclast cell lysates by affinity chromatography and their identity was disclosed by mass spectrometry. The identified proteins included several heat shock proteins, actin and actin binding proteins, and vacuolar ATPase (V-ATPase). V-ATPase, documented for a great increase in expression during osteoclast differentiation, is an important enzyme for osteoclast function; it transports proton to resorption lacunae for hydroxypatite dissolution. The binding of V-ATPase with TRAF6 increased osteoclasts by coimmunoprecipitation and confocal microscopy experiments. In addition, the V-ATPase activity associated with TRAF6 increased in osteoclasts stimulated with receptor activator of nuclear factor-κB ligand (RANKL). Furthermore, a dominant-negative form of TRAF6 abrogated the RANKL stimulation of V-ATPase activity. Taken together, our study identified V-ATPase as a TRAF6 binding protein by proteomics strategy and proved a direct link between these two important molecules for osteoclast function.

Differential Profiling of Breast Cancer Plasma Proteome by Isotope-coded Affinity Tagging Method

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Background: Isotope-coded affinity tag (ICAT) tandem mass spectrometry (MS) allows for qualitative and quantitative analysis of paired protein samples. We sought to determine whether ICAT technology could quantify and identify differential expression of tumor-specific proteins in plasma samples.

Material and methods: First, plasmas from 7 breast cancer patients and 6 healthy women were gathered and pooled separately. After depletion of six abundant plasma proteins using multiple affinity removal system, the plasma samples were using ICAT labeling, liquid chromatography, and MS, and analyzed by LC-MS/MS.

Results: A total of 114 proteins were identified and quantified, with 24 proteins exhibiting statistically significant abundance change. Among these, 12 were up-regulated by more than 1.5-fold and 12 were down-regulated by the same fold in breast cancer plasma. The proteins could be categorized by their biological functions; the main groups correspond to immune response, metabolism, cell adhesion and cytoskeleton, proteolysis, coagulation, transport and signaling.

Conclusion: ICAT tandem MS was able to identify and quantify differences in specific protein expression between plasma samples from breast cancer patients and healthy women. Proteomic screening techniques using ICAT may be useful method to find markers for diagnosis of breast cancer. And Our results provide candidates for potential biomarkers that are useful in diagnosis of breast cancer.

A New Approach to Apolipoprotein Profile Analysis for Clinical Studies

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Cardiovascular diseases are disorders of lipid transport, storage, and metabolism - processes mediated largely by apolipoproteins which associate directly with lipids and facilitate interactions among lipid particles and other molecules. Several apolipoproteins exist in multiple forms due to post-translational modifications such as glycosylation at specific sites, many of which have been identified. These modifications directly impact the rates of reaction and binding capacities of the proteins. Growing evidence suggests that apolipoprotein levels are associated with a variety of chronic diseases—disorders, including stroke, diabetes, hypertension, metabolic syndrome, and obesity, but the difficulty of isolating and measuring these levels prohibits major studies of the implications for human health. We have developed a novel, rapid, high through-put technique for detecting and measuring multiple apolipoproteins and their modifications. This technique uses surface-enhanced laser desorption ionization mass spectrometry to detect apolipoproteins extracted from plasma with antibodies bound to protein G-coated biochips. These surfaces retain proteins to be detected rather than elute them, concentrate rather than dilute them, and allow unbound proteins, salts, and other interfering substances to be washed away. This approach simplifies spectra, reduces noise, and increases sensitivity and specificity. Our investigation revealed the known isoforms of human apolipoproteins CIII and CI along with peaks characteristic of CII, AI, and E directly in human plasma within three hours and multiple forms of the CIII0 isoform, not previously reported. Preliminary analysis of samples from type II diabetics revealed elevated levels of CIII0 isoforms compared to non diabetics. The process is rapid and simple; it provides an enhanced apolipoprotein profile that is more comprehensive than any currently available for research and testing. Added advantages include capabilities for quantitative analysis of multiple apolipoproteins, direct sequencing, and the study of complex interactions of lipoproteins and processes associated with CVD including oxidation, inflammation, coagulation, and platelet activation.
A Big Picture of the Big Protease


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Proteasome is a key protease of the ubiquitin-proteasome pathway involved in the regulation of majority of intracellular biochemical processes. The enzyme core is a heteromultimer of subunits arranged into a tube-like structure. Additional protein complexes can control the enzymatic capabilities of the core. Proteasome is in the center of clinicians and pharmacologists attention as a promising drug target against cancer and inflammation. We propose here that proteasome, in concert with other proteases participating in the controlled degradation of regulatory proteins in the cell, constitutes a functional entity. Together they form multibranch degradation pathways enabling a tight regulation of the protein cleavage. We postulate that this proteolytic web is often dysfunctional in pathological processes like cancer, inflammation or neurodegeneration and in aged organisms, leading to a “proteolytic instability.” Proteomics methods help to dissect the instability and identify the most vulnerable points of UBP, appropriate for pharmacological intervention, as we show on the example of breast cancer and aging. To complement the proteomics approaches, we are using nanobiology methods to get insight into the structure-function relationship governing the proteasomal assemblies. We created an allosteric model of molecular actions of the proteasome. The model is critical to reach our goal of total control over the proteasomal actions via small-molecule noncompetitive regulators. We are promoting the use of allosteric regulators of the proteasome as an alternative to less precise competitive drugs. The power of noncompetitive regulators is shown using anti-inflammatory proline and arginine-rich peptides as an example. In our promotion of the nanomedicine approach, we are proposing to combine microarraying technique with atomic force microscopy to test in a high throughput manner the ligand-induced dynamic structural changes in proteins and nucleic acids. Such screen will establish AFM as a unique tool in drug design and drug testing.

Achievement of Optimal MS and Data Processing Conditions on an LTQ-Orbitrap towards Investigations of CSF Profiling in Neurodegenerative Disorders

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Neurological disorders such as Migraine, Alzheimer’s and Parkinson’s disease affect more than half the population and treatments, except for infections, are inadequate because of limited understanding of pathophysiology. Cerebrospinal fluid (CSF) is the most accessible biological sample that closely reflects brain composition. Many hundreds of CSF proteins have been identified, but a relatively small number have contributed to our understanding of disease. It is desired to corroborate suspected/reported low and high abundance protein biomarkers achieved through gel-based comparative proteomics and recent LC/MS-based methodology, such as label-free mass spectrometry quantification. The goal is to exploit an approach amenable to large data sets required to identify changes within biological heterogeneity.

Much work is done to reduce the complexity of samples prior to MS analysis—including various sample fractionation techniques and depletion of abundant proteins. Yet, there remains a requirement for increased detection capabilities of components in that “reduced complexity” sample. This work attempts to profit from the optimization of performance capabilities of a hybrid high capacity linear ion trap/novel orbitrap mass spectrometer to achieve maximal sample information for positive identification of peptides/proteins. The effects of flexible scan functions (utilizing MS/MS and MS3), sensitivity (lower LTQ ion populations injected for faster cycle times), and automated intelligent acquisition of tandem MS data (data-dependent on-the-fly monoisotopic precursor selection, selection of precursor at the apex of chromatographic elution, and dynamic exclusion) will be investigated. In addition, an investigation of false discovery rates within acquired data sets exploiting high mass accuracy filtering of forward/reverse database hits will be employed to address the rising challenge of false positive identifications from large data sets/databases. Preliminary data suggests a dramatically reduced instance of false positive rates, and increased identification of large peptides utilizing the increased resolution and mass accuracy performance of the Orbitrap over standard ion trap technology.
Comparative Proteome Analysis to Study the Blood Platelet Storage Lesion

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Platelet transfusion is a very common life-saving medical procedure for patients with platelet-deficient diseases like leukemia. In contrast to other blood components, the availability of platelets is restricted since they have a limited shelf-life of 5 days for transfusion purposes. This is due to storage-related deterioration in product quality resulting in the clearance from circulation. To overcome this problem, it is important to understand the molecular mechanisms leading to blood platelet lesion during storage.

We were using two different proteomic approaches combined with functional biochemistry to investigate time-dependent changes in the blood platelet proteome. One type of analysis consisted of the separation of the platelet proteome at two different time points of storage, day 1 and day 8, using 2-dimensional (2D) gel electrophoresis for qualitative and DIGE technology for quantitative analysis. The second method was based on stable isotope labeling with ITRAQ/ICAT reagents in combination with protease treatment, equivalent mixing, separation of the resulting peptides and quantitative analysis by mass spectrometry. Taken together, for the 2D/DIGE approach we analyzed 977 spots corresponding to 103 different proteins and for the ITRAQ approach 1428 peptides corresponding to 355 proteins, resulting in 37 proteins significantly changing both quantitatively due to protein synthesis or degradation and qualitatively due to post-translational modification and enzymatic activity. The high degree of correlation between the two approaches validates the experimental set-up and confirmed the requirement for complementary tools to enhance proteome coverage. Among others, increased amounts of integrins and other proteins known to form receptor signaling complexes with these integrins as well as proteins observed in platelet activation were detected. This proves, for the first time, that there is an apparent link between blood platelet storage lesion and cell signaling.

Playing “Tag” with the Receptor; an Efficient Rapid Screening Method for G-protein Coupled Receptors

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G-protein coupled receptors (GPCRs) represent a large group of membrane proteins targeted for drug discovery. Despite extensive research in the field, only one high resolution structure exists for this family of proteins. Structural studies are challenged by the intrinsic difficulty involved in purifying these receptors in high quality and quantity. In addition to structural information, detailed molecular information is essential for the development of tools to study the modulation of the receptor and for the development of novel therapeutics. Here, we propose a mass spectrometric, sequence-based approach to characterize GPCRs allowing for the rapid molecular characterization of ectopically and endogenously expressed receptor. Our purification system employs an in-house tag to the receptor which does not significantly alter receptor activity or membrane expression. The sensitivity of the approach allows for the implementation of a mammalian expression system with physiologically relevant post-translational modifications (PTMs). We have mapped receptor sites of PTMs and identified receptor isoforms and novel interacting proteins. To date, we have characterized the model GPCRs CXCR4 and CCR5 with sequence coverage comparable to naturally abundant bovine rhodopsin and other membrane proteins such as ATP-binding cassette (ABC) transporters. Furthermore, we have used tagged CXCR4 as a positive control for screening commercial as well as in-house produced monoclonal antibodies to the wild type receptor. As proof of principle, we have developed a monoclonal CXCR4 antibody that has wide-ranging applications and implications for the study of this receptor on primary and diseased tissues.
Characterization of Nuclear Matrix from Drosophila Embryo

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The nucleus is an intricate structure containing many functional domains whose complex spatial organization is believed to be maintained by non-chromatin scaffolding, the nuclear matrix. We have standardized procedure to prepare nuclear matrix of Drosophila embryos by introducing several modifications in the known protocols and setting up several quality control checks. With such preparations, we have established 2D protein profile of the nuclear matrix of Drosophila embryos.

We have identified more than 100 proteins from these preparations and begun to study selected ones in detail. During the comparison of the 2D profile of matrix prepared from different developmental stages, we notice remarkable difference with spots emerging or disappearing during development. We are also in the process of studying the dynamics of matrix proteome in the context of embryonic development.

In the NuMat proteome, we identified BEAF 32B, a protein known to interact with SCS’ boundary element. 25% of the total nuclear BEAF exists in the matrix. A region of the protein extending from 140 to 224 amino acids are needed for nuclear as well as matrix localization of this protein. This region has many potential sites for glycosylation and phosphorylation. We further find that BEAF is O-glycosylated as well as phosphorylated at its Ser/Thr residues and that the phosphorylated form of BEAF exists in nuclear matrix. Upon dephosphorylation of matrix, some of them are lost. This along with earlier report that BEAF makes trimer and our observation that only the non-phosphorylated form of BEAF binds to DNA, indicates that the phosphorylated and non-phosphorylated forms of the protein might coexist in the nuclear matrix and that each form may have a specific function. This provides a molecular insight into the mechanism by which boundaries might link to nuclear matrix.

Temporal Profile of Human Brain Endothelial Cells in Oxidative Stress; a Bench-side Model for Endothelial Response to Ischemic Injury

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Background: Human brain endothelial cells (HBEC) constitute a major component of the blood brain barrier and have important roles in cell-matrix signaling in the neurovascular unit, which becomes damaged during cerebral ischemia.

We conducted proteomic screening of HBEC culture media over time in an in vitro model of ischemic injury. Our goal is to better understand ischemic progression and to develop future screening tools for the discovery of neuroprotective factors for stroke therapy.

Methods: HBEC were treated with nitric oxide donor (SNP) to trigger oxidative stress. Cell culture media were sampled pre SNP treatment, and at 6 hours, 12 hours and 24 hours post SNP treatment. Samples were separated using gel-based methods followed by MS/MS. To assess reproducibility, experiments were performed in triplicate.

Results: We found over 800 proteins expressed over time in cell culture media. The number of proteins peaked at 12 hours post oxidative insult. Principal component analysis demonstrated reproducibility among triplicates, with the greatest variance in the data coming from the difference in treatment state. Extracellular secreted factors are among the proteins differentially expressed in the SNP-treated samples in comparison to the controls. Since SNP treatment did not alter markers of cell lysis such as LDH, the differences may be attributable to endothelial response to oxidative stress, rather than cell death.

Conclusions: In our in vitro model of ischemic injury, we report a temporally distinct profile of protein expression in HBEC media post oxidative insult. These results advance research under way to understand ischemic progression and to validate specific secreted novel factors associated with endothelial response to oxidative stress. Our long-term goal is to find candidate therapeutic targets post cerebral ischemia. Studies are ongoing to assess our in vitro findings in clinical stroke settings.
Reverse Phase Protein Arrays for Protein Quantification in Biological Samples

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The measurement of protein abundance in biological samples is a standard procedure in molecular biology. However, currently available methods are not sufficient for precise measurements in high throughput.

To overcome this limitation, we have established the Reverse Phase Protein Array (RPPA) technology for relative and absolute protein quantification in a high throughput format. RPPAs carry serial dilutions of complex biological samples (e.g., cell lysates, body fluids), which are printed by piezo-spotting onto nitrocellulose-coated glass slides. Currently, approx. 600 protein spots can be delivered on each chip; the spot number can easily be extended to several thousand. Less than 10 μl sample volume are required to produce a batch of 25 arrays. The detection of a protein of interest is performed by incubation with a specific antibody. After incubation with a secondary (near-infrared (NIR)-dye labeled) antibody directed against the Fc terminus of the primary antibody, the arrays are scanned with an infrared scanner, and signals are quantified.

We developed a NIR-based total protein quantification assay performed directly on the slide, detecting spotted proteins in a concentration range of 0.1–5 ng/μl. With specific antibodies, we established the determination of protein abundance and protein modifications on the arrays. Currently, the lowest amount of detectable protein is in the fg range, depending on the antibody used. Intra- and inter-chip signal variances are <2% and 3.3%, respectively. In summary, the reverse phase protein array technology is a useful tool for large-scale determination of protein and protein modifications.

We show that those properties can be used to predict a proteins’ properties for each proteomic platform that differentiate proteotypic from other peptides. In fact, only a small number of so-called ‘proteotypic’ peptides are repeatedly and consistently identified for any given protein present in a mixture. Using >600,000 peptide identifications generated from four large proteomic studies in yeast employing common proteomic platforms, we have empirically identified >16,000 proteotypic peptides for >3600 distinct yeast proteins. Using computational approaches, we have discovered physico-chemical properties for each proteomic platform that differentiate proteotypic from other peptides. We show that those properties can be used to predict a proteins’ proteotypic peptides with >85% cumulative accuracy. The algorithm also accurately predicts proteotypic peptides for human proteins, suggesting that it might be generally applied across all species. Possible applications of proteotypic peptides include validation of protein identification, provision of reliable synthetic peptide standards for absolute quantification experiments and further characterization of the physical principles governing mass spectrometric and proteomic experiments.

Development of Quantitative Protein Detection Arrays for Systems Biology

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A reliable method to quantify proteins is mandatory to monitor the subtle changes that occur on the protein level in health and disease. The method should be sensitive and robust so that protein abundance can be monitored in a high-throughput format to allow the generation of time resolved quantitative data for systems biology. Ideally, this approach should also provide information on dynamic events such as regulation by posttranslational modification (PTM).

In order to establish a protein microarray-based method for the quantitative detection we immobilized monoclonal antibodies on slides. Detection of captured proteins was performed with the help of protein-specific or PTM-specific antibodies. Protein complexes were visualized with secondary antibodies labeled with near infrared-dyes. The strength of specific signals will be determined by NIR-imaging and the results were compared to data generated by quantitative immunoblotting. The quality control is based on statistical analysis.

Data from the activation of ERK/MAPK and from STAT signaling will be presented. In the future we plan to label antibodies with quantum dots to facilitate simultaneous detection of multiple proteins/posttranslational modifications.
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Expression and Functional Study of Proteins Involved in the Accumulation of Intramuscular Fat within the Bovine Longissimus Dorsi Muscle
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Once the marbling (accumulation of intramuscular fat) begins in bovine longissimus dorsi muscle (BLDM), the muscle may undergo characteristic changes in the expression of specific proteins. Therefore, the aim of this study was to investigate protein profiles related to the induction of adipogenesis (determination of multipotent stem cell) within BLDM by proteomic analysis. The expressions of twenty-two spots were differed in high marble group with a minimal 5-fold change compared to the low marble group in forty Korean native steers. Among these, three spots showed an increase of expression in BLDM tissues during the fat development stage in test steers. In a cell culture study of BMFS (spontaneously immortal bovine muscle fibroblast), we observed the abundance of eight proteins when the cell was induced to differentiate into adipocyte with inducing factors of adipogenesis. Also in 3T3L1 cell, we found an increase in four specific spots upon differentiation into adipocyte. Our results suggest that these proteins may be related to the induction of transdifferentiation and adipogenesis within bovine longissimus dorsi muscle.

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Expression Profiling in Neuronal Differentiation Cells
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Most of cells proliferate rapidly at the early stage of CNS development. These cells are differentiated into three cell types: neuron, astrocyte and oligodendrocyte. It is known that activation and (or) suppression of genes is deeply involved in the neuronal differentiation. In this study, we investigated the mRNA and protein expression of embryo stem (ES) and PC 12 cells differentiated by retinoid and nerve growth factor (NGF), respectively. cDNA microarray is for mRNA and 2-dimentional electrophoresis (2-DE) for protein. Twenty genes were showed to change the intensity of mRNA expression in cDNA microarray, which was confirmed using RT-PCR. Results of 2-DE experiment were agreement in that of cDNA microarray. We are investigating the how these genes contribute to neuronal differentiation and hope that this study help to unveil the secret of CNS development.

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Potential Roles of Gadd45a in MMS-induced Base Excision Repair (BER)
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Gadd45a is a ubiquitously expressed mammalian gene that is induced by DNA damage and certain other stresses. As one of many p53-regulated genes, Gadd45a has been shown to delay carcinogenesis. Gadd45a is known to regulate nucleotide excision repair (NER) in response to UV-radiation. Here we report an emerging role for Gadd45a in base excision repair (BER). Gadd45a-null mouse embryoblast fibroblasts (MEFs) and human colon cells exhibited slow BER after treatment with methyl methanesulfonate (MMS) a pure base-damaging agent. In addition, the localization of apurinic/apyrimidinic endonuclease 1/redox factor 1 (APE1/Ref1) within the nucleus was observed in gadd45a wild-type cells. Inasmuch as p53 has been shown to regulate BER, our data suggest that p53-regulated gene Gadd45a contribute to the BER response by affecting the localization of APE1/Ref1 with PCNA on nucleus. In addition, using structural proteomics and IP western, we suggest potent protein-protein interactions among Gadd45a, Ape/ref-1, and PCNA proteins as a possible mechanism of Gadd45a-dependent BER. Gadd45a might be a key component gene of the p53 pathway involved in protection from carcinogenic base damage and maintenance of genomic stability although the downstream mechanism including APE1/Ref1 was needed in future study.

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Proteomic Analysis in the Pancreas of Diabetic ZDF Rats Using Two-Dimensional Difference Gel Electrophoresis
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Type 2 diabetes is a serious metabolic disease accompanied with various type of vascular disease. Therefore, the identification of proteins that contribute to type 2 diabetes is important to elucidate the molecular pathogenesis of the disease. We performed proteomic analysis on pancreas of Zucker diabetic fatty (ZDF) rats, a well characterized type 2 diabetes animal model, using 2-D DIGE to identify deferentially expressed proteins in this disease. Proteomic analysis from ZDF rats at 7 weeks (pre-diabetes) and 16 weeks (diabetes) can show the differences by progression of diabetes. Comparative analysis from 16 weeks ZDF rats (diabetes) and 16 weeks Zucker lean control(normal) allowed us to identify differential expression by pathogenesis of type 2 diabetes. We found 8 deferentially expressed known proteins from 7 weeks (pre-diabetes) and 16 weeks (diabetes), 5 proteins from 16 weeks ZDF rats (diabetes) and 16 weeks Zucker lean control(normal). Proteins commonly expressed in 3group were also observed. These proteomic data provide better fundamental understanding of development of type 2 diabetes.
The Progress of Human Liver Proteome Project

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As the first proteome initiative on human tissues/organs, the Human Liver Proteome Project (HLPP) aims to: 1) generate an integrative approach leading to a comprehensive protein atlas of the liver; 2) expand the liver proteome to its physiome and pathome to dramatically accelerate the development of diagnostics and therapeutics toward liver diseases; 3) develop standard operating procedures (SOPs) as references for other HUPO initiatives.

In the light of the consensus SOPs, we collected Chinese liver samples and analyzed their proteins expression profile with 6788 unique proteins. The data have been collected by Beijing Proteome Research Center (BPRC), which serves as the HLPP Headquarters. The further analysis is still in progress and some related biological significance has been disclosed.

Meantime, other subprojects of the HLPP have also made lots of progress, such as: 1) the Human Liver ORF Bank was established and more than 4500 ORFs have been collected; 2) 1293 protein-protein interactions were discovered, 20% of which would be validated by Co-IP or GST pull-down assay; 3) 1910 hybridoma cell lines were established and 1492 mAbs were characterized for 150 different proteins; 4) some high-throughput technologies have been set up for protein post-translational modifications and protein localizations; 5) Tens of specific protein biomarkers were discovered or validated in the hepatocellular carcinoma (HCC) by proteomic analysis. In addition, the proteomic dataset of French human liver was delivered to EBI and comprehensively analyzed by the international bioinformatics team.

Proteomic Analysis of Liver in Miniature Pigs According to Developmental Stages Using 2DE and MALDI-TOF

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Since there have been problems of serious shortage of human organ in transplantation, various studies for xenotransplantation which is unlimited animal organ instead of one of human have been performed actively. The organs of porcine are thought to be more safe and size available has been used rather than nonhuman primate for xenotransplantation. Although understanding the levels of expression of proteins and their post-translational regulation would be very practical between different species and among developing stages, the molecular profiling for xenotransplantation is well known for human and rodent while that for porcine has been rarely studied. Here, in this present study, we reported protein regulation of the developing stages of liver (4-day old neonate miniature pig, 19-day old miniature piglet, 14-month old miniature adult pig) using 2-DE gel electrophoresis and MALDI-TOF. From pictures of the three different stages, totally 8 spots which differently regulated were detected and 5 spots (aldehyde dehydrogenase mitochondrial precursor, serum albumin precursor, keratin type II cytoskeletal-8, proliferation-associated protein 2G4, lambda-cristallin homolog) were identified with MALDI-TOF MS. The data shown in this study can be for the first reference direction of the developmental livers of miniature pigs that will help the way for further proteome analysis of liver in the future.

Increasing the Potential of Proteome Analysis via Comparison of Proteomic Data with Protein Interaction Databases; Discovery of Lymphocyte Pathways

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Signal transduction is an important biological process conducted by a variety of protein-protein interactions (PPIs) which transduce external signals into cellular responses. Proteome analysis has the potential to uncover these mechanisms but has limitations. Membrane receptors, kinases and transcription factors are normally underrepresented in a typical proteome study but are most interesting targets for drug development. To get access to these protein classes high-confidence PPI datasets can be used to predict potentially involved proteins on the basis of proteome data.

We created a non-redundant and manually curated PPI database comprising HPRD, BIND, and DIP data according to XML-PSI data format of the Human Proteome Organization (HUPO). Additionally, protein data from a well characterized non-redundant T cell specific expression library was implemented to simulate T cell specific networks. The reliability of this PPI data was proven by mapping differentially expressed proteins involved in development and activation of CD4+CD25+ regulatory T cells (Tregs) to this pathways. Proteins were obtained from proteome analyses of Tregs as well as from literature.

The validation was focused on TGFBR1 and Integrin signalling pathways. By mining PubMed it could be shown that several proteins in the predicted pathways are reported to be functionally involved in TGFBR1/Integrin-signaling. Furthermore, the mapping of mainly cytosolic proteins identified by proteome comparison of activated T cells and Tregs to the network resulted in predicted and partially validated pathways linked to well known T cell specific receptors and transcription factors, e.g. responsible for IL-2 production.

We conclude that proteome analysis in combination with high quality PPI data assists to uncover new pathways from receptors to transcription factors on the basis of regulated proteins identified in typical proteome studies. Furthermore, it has the potential to identify proteins that can be targeted for therapeutic and diagnostic applications.
Circulating Human Monocytes in the Acute Coronary Syndrome Express a Characteristic Proteomic Profile

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Objectives: To disclose new potential pathways leading to acute coronary syndromes (ACS).

Background: Monocyte-macrophages participate in the genesis of ACS. Assessment of monocyte protein expression during this disorder may disclose new pathways in its pathophysiology. Novel proteomic techniques allow to analyze the expression of multiple proteins at once.

Methods: Protein expression was assessed in circulating monocytes from 18 patients with ACS (on admission, at 4 days, 2 months and 6 months), 10 patients with stable coronary artery disease (CAD) and 12 healthy controls.

Results: In ACS monocytes, the expression of several proteins were upregulated (Vimentin, mature Cathepsin D, and -Enolase) or downregulated (Nuclease, chain A Triosephosphate isomerase, chain A Endonuclease, MY032, Protein disulide isomerase ER60 and Uracil DNA A glycosylase). However the most relevant finding was the total absence of 14 proteins. These include RNA-binding regulatory subunit oncogene DJ1, Protein disulide isomerase ER60, Chain A nuclease DNase, Paraoxonase I, Heat-shock protein-60 and -70, Mannose-6-phosphate receptor binding-protein, Thymidine phosphorylase precursor, Albumin precursor, Glutathione transferase, S100 calcium-binding protein A8 calgranulin, Cathepsin D proform, Chain A triosephosphate isomerase and pyruvate kinase M2. The number of proteins differentially expressed decreased progressively. At 6 months, only five proteins were altered, this pattern being identical to that of stable CAD patients, suggesting a stabilization of protein expression by circulating monocytes.

Conclusion: Monocyte expression of twenty-one proteins changes in ACS. Further studies are required to explore if they are involved in the pathophysiology of ACS and whether variations in serum levels of any of these proteins have prognostic implications.

Lipid Cartography of Atherosclerotic Plaque; Use of Cluster TOF-SIMS Mass Spectrometry as a Molecular Imaging Tool in Vascular Pathology

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Secondary Ion Mass Spectrometry (SIMS) has proven to be a very useful tool for the imaging of low-molecular weight molecules on biological tissues. Particularly, hydrophobic molecules as lipids display a very high intensity when illuminated with novel bismuth cluster ion source. Thus, we use several frozen vessels bearing atherosclerotic lesion to test if using this mass spectrometers we can regain information on their lipid composition. Quasimolecular ions from multiple metabolites present on the lesion were characterized, including several inorganic ions, fatty acids, cholesterol, vitamin E, triglycerides, phosphatidic acid or fragments from complex lipids, as phosphatidylinositol. Spatial distribution throughout the tissue of the most intense ions were displayed as three-dimensional images, susceptible to be used with image analysis packages and compared with images from the same tissue obtained by optical microscopy. Despite, its limitation on the mass range that can be detected, SIMS Imaging becomes a complementary technique bridging the gap between histology and “metabolomics”, being able to characterize unknown molecules as well as being able to show their relative abundance across the tissues, with a high sensitivity and a sub-optical resolution.

Kinase Inhibitors; Chemical Genomics Based Screening, Selectivity Profiling, and Mode-of-Action Studies

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To aid in the assessment of efficacy and the elucidation of potential side effects of kinase inhibitors, the determination of the target profile of an inhibitor directly within the target cell or tissue can be more predictive than the more conventional in-vitro assays using recombinant enzymes. We have developed a range of Chemical Genomics approaches that allow the study of interactions of compounds with their targets directly in tissue culture, model organisms or tissue samples from patients. The drug or lead compound is applied at a range of concentrations to living cells or to cell lysates, and the lysate is subsequently captured on an affinity matrix which specifically binds hundreds of protein kinases and kinase-associated proteins. The target affinity profiles of the compound are then quantitatively determined by differential analysis of the captured proteins from treated and untreated samples, using stable isotope labeling and mass spectrometry. The application of this technology to compound library screening, selectivity profiling, and mode-of-action studies on compounds will be presented.
Clinical Biomarker Number and Discovery: Examples in Cerebral, Infectious, and Renal Diseases

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The number of specific biomarkers available in routine clinical practice is small compared to the number of diseased tissue or cells. From available transcriptomic data provided by MPSS or other techniques and known physio-pathological conditions, it could be anticipated that many single specific protein biomarkers or simple panel exist in multiple disease conditions. To discover and establish them as practical and reliable biomarkers in clinical practice, two investigation phases should be carefully designed and conducted: a discovery phase using most likely tissue or their surrounding fluids and a validation phase done with easily accessible bio-fluids such as plasma, serum or urine. Post mortem tissue should be considered as a valuable source of potential biomarkers in the discovery phase.

Mass spectrometry is an essential tool in the discovery phase. Among several methodological possibilities, "Shotgun" IEF LC-MS/MS analysis, combined with iTRAQ labelling, provides the most comprehensive list of polypeptides present in the biological sample with reliable differential quantification. Multiple software with proper statistic evaluation should be used to identify a large number of peptides. For the validation phase, the most practical approach is the development of immunoassays. The limiting factor is by far the establishment and conduction of clinical trials to validate the newly discovered biomarkers.

The presentation will show available transcriptomic data demonstrating the potential existence of multiple single specific biomarkers or limited panels. It will highlight successful biomarker discovery and application in a clinical setting using multiple protocols in the fields of cerebral, infectious and kidney diseases.

The Albuminome as a Tool for Biomarker Discovery; Application for Differentiation of Stable Angina and Myocardial Infarction

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The removal of albumin and other high abundance proteins is a routine first step in the analysis of serum and plasma proteomes. Our work has focused on identifying the proteins and peptides removed from serum during albumin depletion and identifying which of these are bound to albumin (rather than co-purified) and whether the bound proteins are intact proteins or peptide fragments. Sequential, independent analyses including both anti-albumin antibody affinity chromatography and size exclusion chromatography (SEC) were used to isolate albumin bound proteins. Of the 35 albumin-associated proteins discovered, 26 have been previously reported to be potential biomarkers, with 9 of these being found exclusively in the albuminome and not in the albumin-depleted fraction of human serum. Combining these observations with the knowledge that the concentration of albumin and metal binding properties of albumin in serum are known to change with disease, it is therefore predicted that the albuminome could provide access to a unique group of biomarkers.

In the Emergency Department, there is an urgent clinical need to quickly differentiate between patients experiencing chest pain who have non life-threatening/stable angina (SA) from those with acute myocardial infarction (AMI). To assess the possibility of whether the albuminome could aid in this differentiation, the albuminome from a pooled healthy control, individual healthy controls (n = 7), patients with AMI (n = 7), and patients with SA (n = 7) were analyzed. The albuminome of these comparative groups were captured by two independent methods including (1) antibody immunoaffinity capture (IgY-anti-HSA) and (2) isolating the high MW complexes in serum that contain albumin by SEC. The high MW complexes eluting from SEC above 66 KDa (albumin’s MW) changed in ratio and quantity between ischemia and angina patients. Downstream analyses including 1D SDS-PAGE and RP-HPLC followed by protein identification by LC-MS/MS and revealed differences among diseased and control samples.
The Utility of Multiple Dissociation Techniques in a Linear Ion Trap in the Characterization of the Phosphorylation Status of Kinases

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The standard mass spectrometric technique for the analysis of phosphopeptides on a linear ion trap mass spectrometer is to perform data dependent MS/MS followed by MS3 of the putative neutral loss peak. This is termed Data Dependent Neutral Loss MS3 (DDNLMS3). This neutral loss ion would typically be the base peak in the MS/MS spectrum. The strength of this approach is the sensitivity in full scan MSn of segmented linear ion trap technology and the characteristically strong neutral loss peak which serves to flag the MS/MS spectrum. Indeed this approach has been much applied to phosphoproteomic analyses, often with some form of up-front phosphopeptide enrichment. However, there are also weaknesses, such as the fact that phosphotyrosine residues rarely produce a neutral loss from the precursor during MS/MS.

Recently, a new dissociation technique, electron transfer dissociation (ETD), has been invented in the lab of Don Hunt at the University of Virginia. ETD has quite different and complementary characteristics to that of CID. For example, (i) ETD works best on multiply charged ions of 3+ and above, whereas typically CID works best on 2+ and 3+ ions, (ii) amino acid side chains and important modifications such as phosphorylated amino acid residues are left intact by ETD, producing a rich ladder of c and z sequence ions. CID produces mostly b and y type ions by collision with gases such as helium, nitrogen or argon.

Work reported here will compare DDNLMS3 data (and another neutral loss based technique referred to as multi-stage activation) generated on a series of human kinases, such as PKB/Akt, with that created by data dependent ETD. The limitations of automated acquisition and analysis will also be discussed. A number of novel phosphorylation sites have been observed for the kinases studied.

Enriching for Mitochondrial Proteins to Identify Modifications Initiated during Myocardial Ischemic Preconditioning; Preferential Methodologies to Identify Protein Subclasses

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Cardiac muscle damage caused by ischemia and reperfusion (I/R) involves impairment of the electrophysiological, contractile, and energy producing systems of the heart, and the mitochondria play a key role in cell survival and recovery of function. Myocardial ischemic preconditioning (IPC), a series of short sub-lethal ischemic events, is able to protect the heart against cell death after severe ischemia, triggering a powerful cellular defence. The protective mechanisms of IPC have been linked to major changes in the mitochondria. The focus of this work is to characterize how IPC affects the mitochondrial proteome using multiple proteomic technologies. Isolated rabbit hearts were subjected to 1 cycle of ischemic preconditioning (IPC = 5I/5R), 2 cycles IPC (5I/5R + 5I/5R) and constant perfusion (control) and prepared by differential centrifugation to enrich for mitochondrial proteins. To improve the resolution of the mitochondrial proteome, numerous protein separation techniques, including gel based (2-DE and 1-DE with reverse phase LC (RP-LC)), and gel free (2 dimensional liquid chromatography (2D-LC) and size exclusion chromatography (SEC) coupled with RP-LC) methodologies, have been utilised prior to protein identification by mass spectrometry. Optimisation of these techniques showed that the gel based methodologies enhanced visualisation of proteins not associated with the mitochondrial membranes, whilst the gel-free methodologies provided improved detection of these membrane associated proteins. By combining these techniques, there is improved coverage of the mitochondrial proteome, numerous protein separation techniques, including gel based (2-DE and 1-DE with reverse phase LC (RP-LC)), and gel free (2 dimensional liquid chromatography (2D-LC) and size exclusion chromatography (SEC) coupled with RP-LC) methodologies, have been utilised prior to protein identification by mass spectrometry. Optimisation of these techniques showed that the gel based methodologies enhanced visualisation of proteins not associated with the mitochondrial membranes, whilst the gel-free methodologies provided improved detection of these membrane associated proteins. By combining these techniques, there is improved coverage of the mitochondrial proteome content as less than 10% proteome coverage occurs between all three methods. These divergent techniques provide the opportunity to identify discrete subclasses of proteins, based on the individual localisation in the mitochondria and the discrete protein chemistries. By complementing these techniques, there is improved coverage of the proteins comprising the mitochondria. As such, the modifications to mitochondrial proteins in response to IPC, that aid in the conferred myocardial protection, are observed.

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Post-translational modifications (PTMs) of proteins play key roles in regulation of various biological functions of protein. Due to the dynamic complexities in vivo and low abundance of PTMs, however, comprehensive identification of PTMs remains a highly challenging problem in proteomics. Using existing approach, limited PTMs were identified by combining affinity-based enrichment and mass spectrometry analysis handling a few types of PTMs. We describe a new PTM-specific strategy, a selectively excluded mass screening analysis (SEMSA) of unmodified peptides in LC-ESI-q-TOF MS/MS in replicated runs of a purified protein on 2-dimensional gel. Precursor ion list of unmodified peptides having high mass intensity were obtained in first run, and the exclusion of unmodified peptides in subsequent runs enables the identifications of modified peptides having low intensity of precursor ion by MS/MS sequencing. This strategy yields a rapid and efficient finding of comprehensive PTMs occurred in vivo. Application of this approach on GAPDH and Hsp27 protein expressed in oxidized mammalian cells, give us the information on multiple protein modifications (more than 10 chemical modification species on 27 sites) in >93% peptide coverage. The results show that in vivo PTMs in mammalian system are very complicated and heterogeneous than previously reported, and this novel strategy have a great potential for examining the systematic characterization of multiple PTMs for functional proteomics. [Supported by KOSEF grant FPR05A2–480, CCSR, CCS & DDR and Brain Korea 21].

Proteomes Involved in the Biosynthetic Pathway of Actinorhodin in Streptomyces coelicolor A3(2)

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One of them, Streptomyces coelicolor A3(2) produces at least four antibiotics including actinorhodin which is known as a pH indicator because its color depends upon the pH condition. Actinorhodin functions killing microorganisms surrounding S. coelicolor A3(2). The increment of actinorhodin production enhances its antibiotic effect. The biosynthetic gene clusters for actinorhodin have been isolated and characterized. Because in the usual growth condition, actinorhodin is not produced, even though the gene clusters for actinorhodin production are known, actinorhodin biosynthesis is interested. The accumulation of S-adenosyl-L-methionine (SAM) causes actinorhodin production in S. coelicolor A3(2) as well as S. lividans. Exogenous addition of SAM on S. coelicolor A3(2) enhances actinorhodin production through AfsK, AfsR, AfsS, and ACTII-ORF system. A comparison of proteomes produced without an addition of SAM and those with SAM may help us discover proteins involved in the biosynthetic pathway of actinorhodin. Authors carried out 2-dimensional gel electrophoresis and LC/MS/MS for the proteome analysis. [Supported by KRF2004-F00019 (KRF) and the second BK21 (MOE)].
Heart failure (HF) is the pathological condition in which the heart is unable to meet the physiological demands of the body. Myosin, which exists as two isoforms (α/β), is a major protein responsible for contraction in the heart. An increase in β-myosin occurs during HF which is characterized by contractile dysfunction. Recently, transgenic rabbits over-expressing α-myosin have been produced which are cardioprotected following pacing of the heart at a high rate until failure. We hypothesized that these rabbits would exhibit a different proteome compared to normal following pacing. Methods: Normal (NTG; n = 14) and transgenic rabbits (TG; n = 14) were used with each group separated into either a sham (n = 7) or paced (n = 7) at 300–380 bpm for 30 days group. Protein separation used 2D gels of cyanine dye labeled extracts from frozen left ventricular tissue in a DIGE style experiment. DeCyder software was used to analyze protein changes but manual confirmation was also performed. In order to validate protein changes, a unique experimental strategy was used in which 2D gels were run of both individual tissue samples as well as pooled samples of each group. Protein spots were excised and identified by MALDI-TOF MS respectively (n = 3). To examine the protein composition of each fraction a 2DE analysis of protein spots was undertaken. The majority of proteins identified by MALDI-TOF MS in each fraction were mitochondrial or cytoplasmic and we were unable to assign any of the identifications to the nucleus. Subcellular assignments were made based on UniProt annotations or, if unavailable, by consensus of three prediction programs. 58% of the spots identified in the Cytosolic fraction were cytoplasmic proteins and 61% of the identified in the Membrane/Organelle fraction were found to be mitochondrial. High levels of mitochondrial proteins were also observed in the Nuclear and Cytoskeletal fractions where 74% and 52% of the proteins identified had mitochondrial association, respectively. Additionally, the Cytoskeletal fraction displayed a clear enrichment of the myofilament proteins spots, many of which were only identified in the final fraction. The ProteoExtract kit yielded variable separation efficiency, largely sequestering and enriching cytoskeletal proteins to the final fraction but leaving cytoplasmic and mitochondrial proteins distributed across multiple fractions.
Combined Proteomic and Metabolomic Analysis of ApoE−/− Smooth Muscle Cells and Their Vascular Progenitors

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Apolipoprotein E (apoE)-deficient mice are the preferred animal model in vascular research. We demonstrated previously that apoE−/− vein grafts show increased neointima formation even if grafted to normolipidemic animals and that adventitial Sca-1+ progenitors contribute to vein graft atherosclerosis. To clarify which changes persist in the absence of hyperlipidemia, we compared apoE−/− and −/− SMCs using a combined proteomic and metabolomic approach. Among the differentially expressed proteins were key enzymes in glucose metabolism, resulting in increased glycolytic activity and a corresponding rise in lactate concentrations. Cell type differences became even more pronounced in hypoxia: While the concurrent response in both cell types encompassed an increase of adenylate kinase and glycolytic enzymes and a reduction of pyruvate dehydrogenase and enzymes of the tricarboxylic acid cycle, key enzymes in the sorbitol and the pentose phosphate pathway were predominantly upregulated in hypoxic apoE−/− SMCs. Their faster glucose consumption resulted in a depletion of the adenosine nucleotide pool in the presence of normal (5 mM), but not high glucose concentrations (25 mM). Strikingly, these alterations in glucose metabolism were also apparent in SMCs derived from Sca-1+ progenitor cells that were isolated from the adventitia of apoE-/- mice and differentiated in vitro. Analysis of their cell culture supernatants revealed an upregulation of insulin-growth factor binding proteins (IGFBP) and a marked reduction in baseline interleukin 6 (IL6) secretion. Notably, reconstitution of IL6 to levels measured in wild-type controls, attenuated the elevated IGFBP expression in apoE−/− SMCs and their vascular progenitors. Thus, the simultaneous assessment of protein and metabolite changes proved to be highly complementary and translated proteomic and metabolomic data into a functional context.

Speed and Reliability; the Past, Present and Future of 2-D Gel Image Analysis

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Since the 1980s computer assisted imaging systems have been used for the analysis of 2-D protein gels. Until the end of the 1990s, the “traditional approach”—spot detection with subsequent quantitation and spot pattern matching based on spot outlines—evolved. Though adequate for the computer hardware of the time, this approach required tedious manual corrections in virtually all of its steps. Reliable spot quantification together with unique spot correspondences was realized in 2003 by using a spot mask which is valid for a whole experiment that may contain a variety of gel images. Based on the proteome map, the spot mask describes each spot of the whole experiment by its coordinates and its general shape. Because the same spot mask is applied to all gel members of an experiment, unique and 100% spot matching is guaranteed. Furthermore, the resulting expression profiles do not contain gaps which makes them suitable for further analysis with software tools that were developed for microarrays. Overall, the spot matching problems that were substantially decreasing the power of statistical analysis in earlier approaches can now be considered a thing of the past.

In order to generate more reliable quantitative results on each gel, the spot mask is refitted to reflect the individual gray level distribution of any spot. We compare this to the traditional approach that uses gel by gel spot detection, and to using the unmodified spot mask. In our benchmark, the refitting alone decreases the variation between replicates’ spot quantities by 20%. By combining these improvements with an automatic warping algorithm, we were able to decrease the hands-on time for a gel to less than 7 minutes with simultaneous increases of reliability and reproducibility never reached before.

Applications from cancer and bacterial proteomics will illustrate the technological improvements.
Specific and Efficient Enrichment of Phosphopeptides for Phospho-proteome Analysis by Mass Spectrometry

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In eukaryotic cells, post-translational modifications of proteins like phosphorylation and dephosphorylation are involved in numerous metabolic pathways and the transmission of signals to control processes like proliferation, differentiation and apoptosis. However, if the tight control and the balance between phosphorylation and dephosphorylation is disturbed, it may lead to serious damage, degeneration or illness. Therefore a phosphoproteome analysis is very important for the understanding of the underlying mechanisms. Drawbacks in the analysis of phosphorylation and dephosphorylation are that phosphorylated signal-proteins are of rather low abundance in cells and that phosphorylation is only in rare cases stoichiometric. Thus a large portion of potentially phosphorylated proteins may remain non-phosphorylated even in case a signalling event occurred.

Mass spectrometry of phosphopeptides has become a powerful tool for phosphorylation site identification. However, there is a general need for specific and efficient enrichment strategies of phosphorylated peptides in order to compensate for the rather low abundance of phosphopeptides. Titanium dioxide and immobilized metal ion affinity chromatography (IMAC) alone or in combination with other methods are described to be able to enrich phosphorylated polypeptides without bias towards a specific phosphorylated amino acid.

In this context we recently developed and optimized specific enrichment protocols based on proprietary materials and compared them with different commercially available methods. In our comparison we enriched phosphopeptides from a digest of four different proteins and also from complex biological samples like complete tissue extracts and extracts from transfected cells in the presence of high abundant non-phosphorylated peptides. Aspects like specificity, efficiency, sensitivity, robustness etc have been analysed using ESI- and also MALDI-MS.

Combined Proteomic and Metabolomic Analysis of PKC [epsilon] Transgenic Hearts

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Activation of protein kinase C (PKC) [epsilon] and inhibition of PKC alpha are known to be cardioprotective against ischemia/reperfusion injury, but the link between the two PKC isoforms remains unknown. Using a combined proteomic and metabolomic approach, we demonstrated previously that loss of PKC alpha results in a shift from glucose to lipid metabolism in murine hearts. In the present study, we evaluated the effect of activated PKC [epsilon] on cardiac metabolism.

Mitochondrial and cytosolic proteins from control and transgenic hearts with constitutive active or dominant negative PKC [epsilon] were analyzed using difference in-gel electrophoresis (DIGE) and identified by nano-LC MS/MS. Among the differentially expressed proteins was mitofilin, an inner mitochondrial membrane protein and key regulator of metabolic flux. Moreover, transgenic activation of PKC [epsilon] resulted in a concomitant downregulation of creatine kinase, pyruvate kinase, lactate dehydrogenase and cytosolic malate dehydrogenase, which is required for the import of reducing equivalents from glycolysis across the inner mitochondrial membrane. These enzymatic changes appeared to be dependent of PKC [epsilon] activity, as they were not observed in mice expressing inactive PKC [epsilon]. High-resolution nuclear magnetic resonance (NMR) spectroscopy confirmed a pronounced effect of PKC [epsilon] on cardiac metabolism: normoxic hearts with constitutive active PKC [epsilon] had significantly lower concentrations of glucose, lactate, glutamine and creatine, but higher levels of choline, glutamate and total adenosine nucleotides. During ischemia and reperfusion injury, glucose depletion was reduced, energy metabolites were better preserved and the rise in amino acids was higher in PKC [epsilon] transgenics compared to control hearts.

Taken together, our findings provide the first evidence that PKC [epsilon] activity modulates cardiac glucose metabolism and provides a possible explanation for the synergistic effect of PKC alpha inhibition and PKC [epsilon] activation in cardioprotection.
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An Extensive Heart Tissue Bank for Proteome Research

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We have collected a very large heart tissue bank. All samples are preserved and stored in liquid nitrogen in <60 min of the loss of coronary circulation. In the LV we collect transmural sections of LV free wall, sub-auricular and sub-atrial papillary muscles, endocardial and epicardial regions of LV free wall. We harvest right ventricle, left and right atria, and coronary arteries (left and right coronary aa, circumflex and LAD) free of fat and muscle tissues. We collect 50–100 samples from each heart. The bank contains >300 failing explanted hearts obtained in the cardiac transplantation theaters. Patients were diagnosed as: idiopathic dilated cardiomyopathy (CM) (82 hearts), ischemic heart disease (66), viral-induced CM (11), familial dilated cardiomyopathy (10), hypertrophic CM (10), Adriamycin-induced CM (9), alcohol-induced CM (8), peripartum CM (8), rheumatic heart disease (7), Eisenmenger’s syndrome (7), restrictive CM (4), sarcoid CM (4), myocarditis (4), Marfan syndrome (2), cystic fibrosis (2), and many conditions for which only 1 heart was available. Clinical parameters (LVEF, NYHA class, FS, CO, CI LVEDD, LVESD) are recorded and while the drug histories of many patients are known, we are in the process of putting the entire clinical histories on a closed database that will be accessible to approved users. We have 58 non-failing donor hearts aged 7 weeks through 65 years. Donor hearts are removed by surgeons, flushed with cardioplegia chilled under the control of the Australian Red Cross Service. They are the benchmarks for measuring changes in failing hearts. They also provide the material for examining changes as a function of age. Potential users must be approved by the St Vincent’s Hospital HREC before samples are shipped in a liquid nitrogen dry shipper. Interested researchers should contact the author on crisdos@anatomy.usyd.edu.au. All users agree to share their data, normally prior to publication.