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Phosphoproteome Analysis of the Rat Brain

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Numerous stimuli are transduced in the mammalian brain via the reversible phosphorylation of the downstream effector proteins. Therefore, development of comprehensive and reproducible analytical methods for determination of protein phosphorylation are necessary proteins for the signal relay in neural circuits. We present an integrated analytical methods for fractionation, enrichment, detection and sequencing of phosphorylated peptides from rat brain by MALDI-TOF and LC-tandem MS/MS analyses. The brains from rats treated either with one of the selective serotonin reuptake inhibitors (SSRIs), Paroxetine-HCl, were dissected into cerebellum, cerebrum, and brain stem. Proteins were extracted by an SDS-sample buffer from the ground tissues in liquid nitrogen and separated by molecular weight on an SDS-PAGE gel. Phosphopeptides from the fractionated and trypsinized protein were enriched by immobilized metal affinity chromatography (IMAC) and further fractionated by a reverse phase column. Among over 200 phospho-proteins identified from all brain parts, 18 cerebellum specific, 9 cerebrum specific, and 13 brain stem specific proteins were localized to the tissues, and the rest of the proteins were found in common. With a quantitative mass spectrometric analysis technique, the information will be used for the profile changes of the phosphorylated proteins across the brain by various stimuli.

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Magnetic Bead-based Serum Proteome Profiling with MALDI-TOF MS for the Early Diagnosis of Ovarian Cancer

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Magnetic bead purification for the analysis of low-abundance protein in human serum facilitates the identification of potential new biomarkers with matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS). The aim of this study was to search for biomarkers that would distinguish ovarian cancer patients from matched controls. We performed comparative MALDI-TOF proteomic profiling of serum samples from twenty ovarian cancer patients and their age-, and gender-matched 24 disease-free control. The support vector machine algorithm identified three closely related spectra features of m/z value 2671, 3950, and 4962 from the weak cation exchange chromatography (WCX) magnetic bead purification that could distinguish ovarian cancer patients from disease-free individuals with 100% sensitivity and 100% specificity. The intensities of m/z value 2671 and 4962 peaks were considerably higher in ovarian cancer patients than in cancer-free individuals ($p < 0.05$). On the other hand, the intensity of m/z value 3950 peak was considerably lower in ovarian cancer patients than in disease-free individuals ($p < 0.05$). Validation of these proteomic biomarkers would have significant implications for early detection of ovarian cancer as well as for monitoring and better management of high risk patients much before the clinical diagnosis.

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Peptide Analysis Using a Planar Electrochromatography/Thin-layer Chromatography Separations Platform Coupled to Orthogonal MALDI-TOF Mass Spectrometry

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A new strategy is presented for the separation of complex peptide mixtures using a combination of electrically-driven planar chromatography (PEC) and thin-layer chromatography (TLC), followed by direct detection of analytes using mass spectrometry (MS). The separation of peptides is achieved by combination of electroosmotic and electrophoretic forces during the PEC separation and capillary forces during the TLC separation. Dependence of peptide mobility on peptide mass, charge, and peptide-surface/solvent interactions allows high-resolution separations of various populations of otherwise similar peptides (e.g., peptides with posttranslational modifications). The separated peptides were labeled with a fluorescent dye and visualized using a xenon-arc lamp-based charge-coupled device (CCD) camera system for fluorescent imaging. MALDI matrix was spotted on top of the imaged spots and peptide mass fingerprinting was performed by direct interrogation of the TLC plates in orthogonal MALDI-TOF MS. With this instrument, ionization and mass analysis are decoupled. Consequently, mass analysis is not affected by spatial variations and sample topography, allowing plates to serve as the sample target. Using the new 2D system, phosphorylated peptides and acidic peptides migrate slower than more basic peptides of similar mass in the first dimension and are further distinguished from adventitial peptides based upon hydrophilicity in the second dimension, as demonstrated with tryptic digests of ovalbumin, riboflavin-binding protein, heat shock protein 90, β-casein and α-casein. The newly developed phosphopeptide mapping approach should allow routine identification of peptides, without the use of radioisotopes or surrogate dyes. The cited multidimensional approach offers certain performance advantages relative to currently practiced on-line methods, including ease of use, ability to fractionate larger amounts of sample, ability to re-analyze fractions and the ability to use off-the-shelf equipment that requires little or no instrumental modification.

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Label-Free Quantitative Characterization of Cancer Proteomes by Multidimensional LC-MS/MS Combined with Integrated Ion Intensities and emPAI

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An important goal in proteomics is to compare protein expression levels in different biological samples to understand their functions. Relative expression levels of cellular proteins under different conditions have been precisely measured by mass spectrometry (MS) in cooperation with stable isotopic labeling strategy. Disadvantages are the cost of isotopic reagents and the requirement of labeling procedures. Label-free protein quantitation methods are promising simple alternatives. Exponentially modified protein abundance index (emPAI) was developed to estimate absolute protein contents in complex mixtures, and the integrated ion counts of peptides is the most direct parameter to compare protein expression in different samples. Discovery of cancer specific biomarkers has become an important challenge in clinical proteomics. Protein concentrations/relative expression levels are one of the most basic and important parameters to characterize different cancer types. Here we compared protein expression levels in five different cancer cell lines, which are of different tissue origins. After trypsinization of cell lysates, peptides were fractionated by SCX-HPLC and then analyzed by C18-LC/FT-MS. We identified several thousands proteins, and obtained quantitative data about relative protein expression levels by integration of ion intensities or absolute protein concentrations by emPAI. We elucidated that several hundred proteins were significantly different in abundance across the cell lines and several hundred proteins exhibited no significant differences in abundance among the cell lines. After using ion intensity-based quantitation and emPAI, we found that ion intensity-based approach was more sensitive than emPAI. We calculated pairwise-ratios between cancer cell-lines and then compared emPAI based values with ion intensity based ratios. Interestingly, there was no correlation between the two quantitative approaches. However, upon the removal of noisy datapoints, we found positive correlation between the two methods. We present pros and cons of two label-free quantitative approaches for a multiplexed comparison of protein abundance.

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Secretome Analysis of Olfactory Ensheathing Cells for Use in Tissue Engineering and Regenerative Medicine Applications

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Regeneration of olfactory receptor neurons is guided by olfactory ensheathing cells (OEC)[1], the specialised olfactory glial cells. OEC transplantation has been shown to enhance axonal regeneration and repair of nervous tissue[2–5], and their reconstitution following injury has been studied extensively6. These attributes make OECs the promising cell for spinal cord repair and regeneration.

However, little is known about the micro-environmental cues that influence and regulate OEC self-renewal though evidence suggests that regeneration is induced by soluble factors[3]. Since cell function and behaviour is determined by the repertoire of proteins they express, this study investigates the OEC micro-environmental secretome (secreted factors) and elucidates some of the factors that influence OEC regenerative capacity.

OEC conditioned medium proteins were labeled and quantified using stable isotope labeling using amino acids in cell culture (SILAC™) reagents to facilitate identification and expression profiling of OEC proteins. Furthermore, 2DE was used for OEC secretome differential expression profile mapping. Additionally, transmission electron microscopy was used to evaluate neurospherical morphology under defined conditions.

The study identified and provides a comprehensive profile of specific proteins that participate in OEC self-renewal such as the retinoic acid pathway. Collectively, it provides an understanding of the molecular cues, including neurotrophic factors and growth factors involved in and influencing OEC self-renewal and repair in spinal cord and will improve OEC regenerative potentiality for transplantation.

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Mitochondrial Proliferation Inhibitor Prohibitin Was Strongly up-Regulated in Rats 24 Hours after Partial Hepatectomy

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Two thirds partial hepatectomy (2/3 PH) induces liver proliferation. The replicative phase of liver regeneration is regulated by growth factors or cytokines but the mechanisms underlying are still elusive. Mitochondria are directly involved in the process of liver regeneration. However, it is still not clear the mitochondria role in the replicative phase of liver regeneration after 2/3 PH. For exploring mitochondrial proteins which correlate with replicative phase of liver regeneration, we took two dimensional blue native electrophoresis (2D BN-PAGE) to separate mitochondrial proteins and protein complexes 24 hour after 2/3 PH, with focus on both differentially expressed proteins and their co-migrating partners. By comparing remnant liver tissue with that of sham-operated, we identified 32 up-regulated proteins from 13 protein spots and 24 down-regulated proteins from 9 protein spots on 2D BN-PAGE. Up-regulated proteins were involved in electron transport, redox regulation, oxidative phosphorylation, amino acid metabolism, urea cycle, fatty acid metabolism and several poorly characterized proteins, respectively. Most down-regulated proteins identified are ribosome proteins indicating the inhibition of protein synthesis at replicative phase. Among up-regulated proteins, we were interested in a proliferation regulating protein: prohibitin. It exists in a supper complexes in mitochondria which consistent with the knowledge of reported. ATP synthase α and β chains also co-migrate with prohibitin suggesting prohibitin possible regulating role in ATP synthesis. Prohibitin was strongly up-regulated at 24 hours after PH indicating its possible function in regulating over-proliferation of the liver tissue. This up-regulation was further confirmed by immuno-blotting. Taken together, our results suggest the role of mitochondrial in regulating reestablishment of homeostasis in liver regeneration, or on the other hand, in helping stabilizing correct protein folding state. Because prohibitin also function as chaperon to help protein folding in mitochondria. The further investigation will be carried out for the prohibitin function in liver regeneration.

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Comparative Studies of Skeletal Muscle Proteome and Transcriptome Profilings between Pig Breeds

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The purpose of this experiment is to compare proteome and transcriptome expressions of skeletal muscle for studying the differential growth performance and stress responsiveness between two pig breeds, Korean black pig (KBP) and Landrace. Landrace is more sensitive to stress than KBP breed, but in the respect of growth performance it is far superior than KBP. Therefore, we analyzed mitochondrial proteome and transcriptome levels in skeletal muscles of KBP and Landrace by two-dimensional electrophoresis and microarray analysis. In first, mitochondrial proteome database of pig skeletal muscle was constructed, which is the first 2-DE database of skeletal muscle mitochondrial proteins. This database is available at http://www.pigproteome.com. By the comparative study of the differentially expressed proteins between two pig breeds, we found metabolic-related and stress-related proteins in skeletal muscles. In microarray analysis, we confirmed that these protein-encoding genes also showed similar expression profilings with that observed at the proteome analysis. The metabolic-related genes (short-chain acyl-CoA dehydrogenase, peroxisome proliferative activated receptor α and glycerol 3-phosphate dehydrogenase) are involved in lipid metabolism. These genes were expressed highly in KBP than in Landrace breed at both levels of proteins and their mRNAs. The stress-related genes (α b crystallin, heat shock protein β-1, stress-70 protein and heat shock 70 kDa protein as indicators of cellular stress) were more greatly expressed in Landrace than in KBP breed at both levels of proteins and their mRNAs. Further investigation is necessary whether the different expressed levels of lipid-metabolic and stress-related genes between pig breeds are associated with the muscle growth and stress responsiveness of pig.

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Proteomic Analysis of Terfenadine-Treated Rat Cardiomyocytes

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Biomarker discovery and validation is an important part of most proteomics fields both in academic and industry. Biomarker allows early diagnosis and prediction of drug effect. Drug safety estimation with biomarker is necessary for drug evaluation as well as drug development. Our research goal is to elucidated the biomarker related with life-threatening drug adverse effect and to apply the biomarker for drug safety evaluation. Two-dimensional gel electrophoresis (2-DE) is currently the best method for separating and identifying protein biomarker and the application of 2-DE is gradually becoming more common in proteome analysis. In this study, we examined the protein profiles of terfenadine-treated rat cardiomyocytes through analyzing 2-DE results. Terfenadine, a nonsedating antihistamine, has been known that causes cardiac arrhythmia by prolong QT interval. We have tried to find out the biomarkers causing drug adverse effect using 2-DE to get more expansive arrhythmic references, because some electrophysiological method, such as telemetry or HERG assay, has only been performed to examine the terfenadine-induced arrhythmia. 2-DE was performed in terfenadine-treated groups and fexofenadine-treated groups, as a non-arrhythmic control. The gels were stained by silver, and analyzed with image analysis software. The results will provide a new way for evaluation the cardiac risk of antihistamine drug with high throughput 2-DE screening.

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Characterization of Regulatory DNA Elements

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Cells differentiate along distinct developmental pathways in response to signalling through activation of transcription factors. Despite significant progress in identifying the mechanisms that lead to activation of these transcription factors, the precise understanding of the mechanisms underlying transcription factor activation in a given developmental state is incomplete.

Microarray techniques were used to characterize the interaction between transcription factors and their target region in more detail. DNA fragments were immobilized onto modified substrates and incubated with purified protein or cell extract. Binding of the proteins to their target regions were monitored either with protein specific antibodies or with a label free system. For the data evaluation either end-point values were chosen or the interactions classified by a kinetic profiling. The typical working range for the analytes is nM–μM concentration.

Both types of arrays are used with the same set of proteins or target DNA, respectively. Results obtained with those techniques will help us to reveal a better understanding of transcriptional regulation and signalling in eucaryotic cells.

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Correlation between Genome Activation and Some Changes in the Composition of Nuclear Components

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Most eukaryotic genomes are packaged into two types of chromatin: euchromatin and heterochromatin. Heterochromatin represents significant fraction of most eukaryotic genome and its function remain unknown. Nuclear pore complexes (NPC), including in nuclear membrane also appear to control the spatial orientation and transcriptional activity of chromatin.

Our investigation have been shown changes in some physicochemical characteristics of cereal seeds DNA, chromatin and nuclear membrane during genome activation. We have suggested that the high methylated region of repeated DNA commonly lies in heterochromatin region adjacent to the nuclear envelope (NE).

Recent studies have support that the NPC through association with the underlying chromatin regulates gene expression as well as phospholipid's content of nuclear membrane. Particularly have been obtained the changes in the DNA, RNA, protein and phospholipid content of the NE, soluble nuclear fraction and chromatin during germination of cereal seeds embryos under influence of exogenous gibberellin A3 and Extremely High Frequency Electromagnetic Irradiation. The characteristic trait for growing seed nucleus is a rising of protein and phosphatidic acid in nuclear membrane content to the third day of growing. The chromatin separated on the euchromatin and heterochromatin constitutive regions, and subsequently obtained parameters of thermal denaturation and the level of DNA methylation. Have been revealing changes in all above mentioned parameters during seed germination under influence of gibberellic acid and Extremely High Frequency Electromagnetic Irradiation.

The goal of this work is to show correlation between genome activation and the change in content of the nuclear membrane during genome expression. We would share with our suggested model of nuclear membrane transport. Nevertheless much about the relationship between chromatin organization and the NE remains to be discovered.

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Generation of Training Data Sets for Improved Isoelectric Point Prediction Algorithm

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Isoelectric focusing of peptides is a recently developed and promising separation method for complex proteome samples. Among the advantages high resolving power, sensitivity and loading capacity can be mentioned, as well as the possibility of using peptide isoelectric point (pI) to filter the results and to minimize the number of false positive and false negative identifications. To make the most of the pI filtering, the pI prediction algorithm used needs to deliver values with high accuracy. The classical algorithm (Bjellqvist et al, *Electrophoresis* 1993, 14, 1023–1031) deliver pI-values with a precision of ±0.2 pH-units (3s = ±0.6 pH-units). Observations were made that this algorithm could be improved by taking the effect of charged neighbouring amino acids into account. To enable training of the improved pI prediction algorithm and determination of the new introduced constants, training data sets were generated. The data sets contained identified peptides and their precise experimental pI-values.

Tryptic digest of whole cell lysate from *S. cerevisiae* was used to generate training data sets for optimizing the new algorithm. The tryptic digest was focused and separated in narrow range Immobilized pH gradient strips (0.01–0.02 pH units/cm), calibrated with pI-markers, i.e. fluorescently labelled peptides with known isoelectric points. The pI-markers positions were determined by fluorescence scanning of the strip after focusing and the experimental pH-gradient could be determined with high precision. Following scanning, the IPG-strips were fractionated, achieved fractions extracted, analysed with LC-MS/MS, and identified by searching the yeast proteome using X!Tandem. A first resulting training data set of more than 20 000 peptide sequences in the pH-region 3.4–4.9 was used to optimize the improved algorithm and define values for the new introduced constants. The new improved algorithm has an expected precision that falls in the range of ±0.020–0.040 pH-units.

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Discovery and Verification of Candidate Biomarkers of Metastatic Progression in Lewis Lung Carcinoma

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Despite advances in diagnosis and treatment, cancer mortality rates have not declined appreciably over the last decade. Mortality is mainly attributed to cancer metastases, for which no effective treatment is currently available. Thus, there is a critical need for early detection of biomarkers of cancer, especially biomarkers that would enable the differentiation between localized cancers and more aggressive forms of the disease that are prone to metastases. The goal of this study is to discover proteins involved in metastasis, specifically those related to two pathways that are important in metastasis (the ErbB2 cell proliferation and the integrin activation pathways). The high-throughput provided by the iTRAQ™ reagent strategy and ESI-QqTOF technology allows for simultaneous analysis of these two pathways by studying two different breast cancer cell lines, grown under two different conditions.

The MCF7-AP2 (control) and MCF7-ErbB2 (highly metastatic) cell lines were lysed, digested, labeled with iTRAQ reagents, mixed and then fractionated in 40 fractions by strong cation exchange. Each fraction was analyzed by reverse phase LC/MS/MS on a QSTAR® Elite system. Identification and relative quantitation analysis was performed using a novel new database searching tool, ProteinPilot™ Software.

More than 1000 proteins have been identified with a high degree of confidence (>95%) in the sample. A number of proteins have been found with altered expression between non-metastatic and metastatic cancer cell lines and are candidates for markers of progression. Numerous cytoskeletal proteins and proteins involved in cell motility and structure are differentially expressed. Several proteins, such as filamin B, vinculin and focal adhesion kinase, are involved in cytoskeletal and integrin signaling, and are currently being validated using biological assays. Using a targeted quantitative MRM approach and the MIDAS™ workflow, the expression of these proteins will be monitored in multiple cell lines to verify these candidates.

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The Proteomic Alterations of Rat Mitochondria Caused by Chronic Alcoholism

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It is well known that chronic alcoholism causes a high incidence for many diseases. Recently a number of reports have revealed the increase of reactive oxygen species (ROS) is a biochemical feature for alcoholism. Since many major metabolic events occur in mitochondria with high oxidative stress, we inquire of whether their proteomes are affected by the activated alcohol metabolism. The experimental rats were fed with the water containing 6% alcohol over a period of 3 months. Three rat organs, heart, kidney and liver, were collected for mitochondrial preparation using density gradient centrifuge. The mitochondrial proteins were separated by 2DE followed by silver staining and image analysis. These 2DE spots responding to alcohol fed were further identified by MALDI-TOF MS. As compared with the control group, the liver mitochondria of alcohol treatment exhibited the significant changes of 2DE images, whereas the 2DE images obtained from the alcoholic kidney and heart mitochondria were quite comparable with the control groups. In the liver mitochondria, 31 differential spots with infinite changes in spot volume were defined carefully, and 12 were identified as unique mitochondrial proteins. Surprisingly, over 50% of the alcohol-responsive proteins in liver mitochondria were the members of aldo-keto reductase family, which were down-regulated due to alcohol treatment. Normally, these enzymes exist in mitochondria with low abundance, however, the current evidence suggests that they may translocate from mitochondria to cytoplasm corresponding with chronic alcoholisms. Aldo-keto reductases actively participate in a series of detoxifications for aldehyde compounds, thus their translocation implies that the anti-oxidative capacity in liver would be declined due to the activated alcohol metabolism.

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ECL Plex, Now with Improved Possibilities for Fluorescence Based Multiplex Western Blotting

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Detection of proteins using chemiluminescence based Western blotting is a well known technique. Although it is a sensitive method for single protein detection, the dynamic range is limited and only qualitative or semiquantitative analysis can be performed. ECL Plex* is a new fluorescence based Western blotting system comprising CyDye conjugated antibodies and low fluorescent membranes. This system enables direct detection of two proteins simultaneously on the same membrane with high sensitivity, linear dynamic range and minimal unspecific detection. The ECL Plex fluorescence based Western blotting system for multiplex detection has now been complemented with new antibody conjugates. The existing ECL Plex Cy3 goat-anti-mouse conjugate has been improved, in addition to a new ECL Plex Cy3 goat-anti-rabbit conjugate. The main advantage of the new Cy3 conjugates is an increased signal to noise ratio and higher sensitivity. Also, new ECL Plex Cy2 goat-anti-mouse and goat-anti-rabbit conjugates have been developed. This enables ECL Plex multiplexing to be performed on the Storm system. The new conjugates have been tested using a model system with actin and transferrin in single- and multiplex protein analyses. They have also been evaluated for the specific detection of endogenous proteins using several different cell lysates. Direct fluorescence has been detected using the Ettan DIGE Imager, the Typhoon scanner or the Storm system. The new and improved CyDye conjugated antibodies demonstrate good performance and are compatible with many scanners and CCD cameras on the market.

*Disclaimer: ECL Plex, CyDye, Storm, Ettan, and Typhoon are trademarks of GE Healthcare companies.

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Pancreatic Endocrine Tumours, a Proteomic Investigation of the Trichostatin-A Antitumoral Effect

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Pancreatic endocrine tumours (PETs) are rare neoplasms arising from pancreatic islet cells, and are classified as "functioning" (F) or "non-functioning" (NF), depending on the presence/absence of an associated syndrome due to excessive hormone secretion from cancer cells. Although considered to be "indolent" tumours, at the time of diagnosis almost two/thirds of NF-PETs present with liver metastases, which is the main factor determining patients' outcome. The molecular pathways underlying the development and progression of PETs are poorly understood, as well as the epigenetic events involved. It has been demonstrated that histone deacetylases inhibitors, such as trichostatin A (TSA), lead to an increase in histone acetylation and induce an enhancement of the expression of genes that elicit growth arrest and apoptosis [1].

The present study is therefore aimed at investigating the proteomic expression profiles of endocrine human pancreatic cancer cell lines treated with TSA. Three human neuroendocrine tumour (NET) cell lines were used: QGP-1 (metastatic pancreatic somatostatinoma), BON (metastatic pancreatic carcinoid) and CM (metastatic pancreatic insulinoma). We report the modulation of the proteins which is consistent with the observation that TSA inhibited cell growth by cell cycle arrest at the G2 phase and apoptotic cell death. Among the major changes observed in TSA-treated cells: heat shock protein 27, nucleophosmin, peroxiredoxin II were down-regulated in CM cell line; hnRNP C, oncoprotein Dek and endoplasmic reticulum protein 29 were downregulated in BON cell line; tumor rejection antigen 1 and diablo isoform 1 (mitochondria-derived activator of caspase) were up-regulated in QGP1. The significance of some of these major changes is discussed.

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Anticancer Activity of a Novel Histone Deacetylase Inhibitor against Human Colorectal Cancer Cells

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Colorectal cancer (CRC) is one of the most common malignant tumors, accounting for at least 1,000,000 new cases worldwide and leading to more than 500,000 deaths each year. About half of the patients with CRC develop distant metastases during the course of their disease. Genetically, CRC tumorigenesis appears to be the result of a progressive transformation of colorectal epithelial cells due primarily to the accumulation of mutations in a number of oncogenes as well as tumor suppressor genes. This kind of tumor presents microsatellite instability progress along a genetic pathway with a high rate of insertion and deletion mutations in mononucleotide repeats. It has been demonstrated that histone deacetylases inhibitors, lead to an hyperacetylation of histones that permits the transcription of genes that, once expressed, stop differentiation and induce apoptosis of cancer cells.

The aim of the present study was to investigate the molecular effects of a novel histone deacetylase inhibitor on proteomic profiling of a CRC cell lines, HCT116, in which microsatellite mutations occur at a remarkably high rate (4×10^{-2} mutations per cell per generation).

In an effort at identifying differentially expressed proteins the proteomic expression has been studied by 2D-PAGE comparing untreated cells with cells treated with the novel histone deacetylase inhibitor. 2D patterns were matched by PDQuest software, thus enabling the identification of 48 differentially expressed proteins after the drug treatment.

These spots were identified via MALDI-TOF-TOF and/or HPLC-ION TRAP MS analyses. Most of the regulated proteins are involved in two major biological processes, namely apoptotic cell death and proliferation. Among these some are of particular interest: HSP 90 a, cytokeratin type II CK8, annexin II and glutathione S-transferase.

These findings may contribute to improve the understanding of histone deacetylase inhibitors action and the molecular effect of this antitumoral drug treatment.

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Sensitive and Specific Protein Detection via Triple-specific Proximity Ligation Assays (3PLA)

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The detection of weakly expressed proteins and protein complexes in the presence of abundantly expressed proteins is currently a major challenge. We have developed a novel proximity ligation strategy named 3PLA that utilizes three recognition events for highly specific detection of biomolecules. Proximity ligation assay (PLA) has added increased specificity to the detection of proteins by requiring two independent recognition events per protein and by allowing the protein to be monitored via a DNA based signal. However, the proximity ligation mechanism has been limited in sensitivity and specificity due to unspecific background signal. The 3PLA assay greatly reduces background due a design which uses blocking oligonucleotides and it detects three different determinants on one target molecules which makes this assay highly specific. The 3PLA is especially interesting for the detection of complexes of proteins and of proteins with posttranslational modification. We have applied the assay to detect different classes of proteins such as the growth factor VEGF, the biomarkers Troponin I and PSA and the protein complex PSA-ACT, demonstrating the versatility of 3PLA. Due to the low background signal based on the design and the high specificity of the assay the 3PLA enables the detection of as little as hundreds of molecules.

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Evaluation of IMAC-based Enriching Methods for Phosphoproteins

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In this study, we evaluated several IMAC (immobilized metal affinity chromatography) based enrichment methods for phosphoproteins. Protein mixtures containing one phosphoprotein (ovalbumin) and five non-phosphoproteins (phosphorylase b, bovine serum albumin, carbonic anhydrase, trypsin inhibitor and lactalbumin) were used as testing materials. We have compared the enriching effect for phosphoproteins of three trivalent metal IDA-Sepharose (M^{3+} = Fe^{3+}, Ga^{3+}, Al^{3+}). It was found that the specificity of phosphoprotein enrichment is dependent on the pH of system buffer for IMAC based chromatography. A Low pH environment may cause binding of non-phosphoproteins to the matrix through assumed charge-charge interaction. On the other hand, pH value higher than 7.2 may sharply vanish all interactions between phosphoproteins and the matrix. Mild acidic environment was found to be ideal for IMAC based enrichment of phosphoproteins. Among the three M^{3+} IDA-Sepharose resins, only Fe^{3+} charged resin showed good specificity to phosphoproteins and moderate non-specificity to non-phosphoproteins. This unexpected adsorption of non-phosphoproteins was found to be significantly reduced by inclusion of two different counter ions, acetate and arginine. After optimization, the in-lab constructed Fe^{3+}-IMAC displayed a better separation between phosphoproteins and non-phosphoproteins than other reported methods or the commercial kits.

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Protective Mechanism of aFGF on Spinal Cord Injury: a Proteomic Approach

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Spinal cord injury (SCI) causes damage to neurons and triggers processes that are destructive to ascending sensory and descending motor conduction and eventually results in permanent behavioral dysfunction. Acidic fibroblast growth factor (aFGF) acts as a potent neurotrophic factor and has shown to be a stimulant of neuronal survival in spinal cord injury. However, the neuroprotective attributes of aFGF in SCI is still elusive. In this study, we aim to reveal the protective mechanism of aFGF on female adult rats by proteomic approach. We systematically investigated the temporal protein expression profiles of the contusive injured spinal cord tissue with or without aFGF treatment by using 2-DE and mass spectrometry (MALDI-TOF MS and LC-MS/MS). Animals receiving aFGF treatment showed significant functional recovery over phosphate-buffered saline (PBS)-treated controls on the Basso Beattie Bresnahan (BBB) locomotor rating scale ($P < 0.01–0.001$). The temporal protein expression profiles of spinal cord tissue and their respective cell body in the brain of sham and SCI with or without aFGF treatment at 1, 7, 28 days after surgery had been established. As a result, more than 1,200 protein spots resolved on each gel, 56 protein spots showed significant regulation during at last one time point. These identified proteins might have specific role during the injury and regeneration process and could be functionally categorized as several different classes, i.e. cytoskeleton related proteins, proteins involved in glycolysis and lipid metabolism, antioxidant proteins, heat shock proteins, signaling transduction related proteins, Apoptosis, transporters and unknown function proteins. In addition, the generated adeno-associated virus (AAV) carried with human aFGF (AAV-aFGF) cDNA also promote functional recovery after spinal cord injury. In conclusion, this study might contribute to the understanding of the widespread contribution of aFGF in neuroprotection and an effective aFGF gene transfer approach for the future medication application.

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Quantitative Profile of Differential Expression between L6 Myoblasts and Myotubes by Stable Isotope Labeling by Amino Acids in Cell Culture

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Stable Isotope Labeling by Amino acids in Cell culture (SILAC) is an accurate method for relative protein quantitation in proteomics.

In this study, we analyzed the differential expression between L6 myoblasts and myotubes using SILAC to understand the molecular mechanism of the myoblast differentiation.

L6 myotube cells were grown in DMEM media supplemented with normal arginine ($^{12}C^{14}N$-Arg) and L6 myoblast cells with isotopically labeled ($^{13}C^{15}N$-Arg). Both populations were harvested respectively. The two cell lysates were combined in equal protein amounts. The protein mixture was then separated with 12% SDS-PAGE. Protein bands were excised into 15 aliquots followed by in-gel digestion with trypsin. And then tryptic peptide mixtures were analyzed by LC-ESI-MS/MS. Protein qualitative analysis was carried out by SEQUEST against NCBI rat nonredundant protein Database and quantitative ratio was determined by the XPRESS algorithm in Bioworks™ 3.2. software suite. An accepted SEQUEST result had to have a ΔCn score of 0.1 at least. Peptides with +1, +2, or +3 charge states were accepted if they had a cross correlation (Xcorr) of = 1.5, >2.0, and >2.5 respectively. 1383 proteins were identified. 552 of them are quantified. In L6 myotube cells, 243 proteins showed significant (>1.5-fold) increases, and 112 proteins showed a significant (<0.66) decrease. Some up-regulated proteins in the L6 myotube cells are the molecular marker or typical proteins of skeletal muscle cells such as desmin, tropomyosin 1, myosin light chain regulatory B, myosin heavy polypeptide 3, tubulin α 4, tubulin β 2 and tubulin β 5. Some down-regulated proteins, such as testis-specific histone 2a and transcription factor EB, are related to cell proliferation. The result is mostly compliance to the normal regularity of myoblast differentiation.

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Dissect the Plasma Protein Markers for Parkinson's Disease

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In this study, we employed proteomic techniques to dissect the potential plasma protein markers for Parkinson's disease, the second most common neurodegenerative disease without diagnostic molecules. Two groups of plasma samples from 16 healthy individuals (normal) and 37 patients (PD) clinically diagnosed with Parkinson's disease were first collected in EDTA tubes and quantitated for protein concentration. Preliminary analysis by 1D SDS-PAGE (1-DE) revealed no significant variation of protein profile for all samples. The observed abundant proteins showed nearly identical distribution in normal individuals and PD samples. In order to enrich minor plasma proteins, samples from two groups were pooled and further depleted for major proteins by either (1) Cibacron Blue resin and Protein A resin to remove albumin and IgG, or by (2) multiple affinity protein column (MAPC, Agilent Technologies) to remove six most abundant plasma proteins, including serotransferrin, albumin, IgG, α 1-antitrypsin, IgA and haptoglobin. The processed samples were then analyzed by 2D electrophoresis (2-DE). It is found that depletion of albumin and IgG was sufficient to enrich some minor proteins for their appearance on 2-DE gels. Two proteins, IgG κ light chain and serum amyloid precursor P (SAP), was found dominantly present in the pooled PD samples, but scarcely detectable in the pooled normal samples. Interestingly, we observed that although MAPC strategy removed more abundant plasma proteins and delivered 2-DE pattern with more spots, the processed plasma for pooled normal and PD samples showed insignificant difference in their 2-DE gel images. Furthermore, we examined the applicability of identified Parkinson's disease markers by using western blot to investigate all individual samples. At the time being, although the pathological consequence of SAP on Parkinson's disease remains unclear, our study may demonstrate the possibility of using proteomic techniques for target identification in the current epidemics.

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Identification of Candidate Biomarkers in Human Nasopharyngeal Carcinoma Serum Using a Proteomics Approach

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Nasopharyngeal carcinoma (NPC) is one of the most common cancers in southeast Asia as well as in southern China, it is difficult to diagnosis of this disease at an early stage. Therefore, searching highly sensitive and specific biomarkers for NPC is very vital to early diagnose and improve the patient prognosis. The purpose of this study was to investigate the candidate biomarkers of serum proteins from patients with NPC by using a proteomic approach. As the high abundance of proteins such as albumin and immunoglobulin impedes the investigation of lower abundance proteins that may be more suitable as biomarkers of disease, affinity chromatography was applied to removal of 95% of albumin and 90% of immunoglobulin from human serum form NPC patients and health volunteers, and the subsequent improvement in the number of spots detected and their resolution following two-dimensional gel electrophoresis (2-DE). After staining, the differential expression proteins were analyzed using PDQuest software, and identified using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS). At the same time, SDS-PAGE electrophoresis (1-DE) was applied to separate the total proteins in the intact serum from NPC patients and health volunteers, differential expression protein bands were cut from the gel, in-gel digested and identified by LC-ESI-MS/MS. Serum electrophoresis figures of removed highly abundant protein were obtained with high resolution and reproducibility. In all, twelve differential expression proteins were found. There are haptoglobin, serum amyloid A1 protein, ceruloplasmin Cp, Cytokeratin 1, et al. The validation of these candidate biomarkers in human NPC is in process. It is hopeful to find the biomarkers for diagnosis and prognosis of NPC using proteomics analysis.

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Cryptomics: Identification of Novel Bioactive Peptides from Human Tissue Extracts

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There is increasing evidence that proteolytic cleavage gives rise to "hidden" peptides with bioactivities that are often unpredicted and totally distinct to the parent protein. The liberation of these cryptic peptides or crypteins has so far been shown to be prevalent in proteins associated with endocrine signaling, extracellular matrix, the complement cascade and milk. A broad spectrum of proteases has been implicated in the generation of natural crypteins that appear to play a role in modulating diverse biological processes such as angiogenesis, immune function and cell growth. Cryptomics is a new systematic and integrated approach of finding crypteins in vitro. It involves a reiterative proteomic based process of systematic proteolytic fragmentation, chromatographic fractionation, screening for bioactivity and then proteomic identification of functional crypteins focussing on (but not limited to) the human cryptome.

Using this approach we have discovered novel crypteins with potent anti-coagulation and anti-proliferative properties derived from circulating human plasma proteins. Furthermore, we have shown that synthetic peptides based on the sequences of these crypteins also have potent in vivo activity.

We believe that the human cryptome could contain a plethora of undiscovered crypteins involved in regulating disease processes yet to be discovered and representing potential human therapeutics and/or possible biomarkers. It is also possible that additional crypteins in search of function or have not yet been subjected to the forces of natural selection reside in the cryptome.

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Lectin Affinity Chromatography with Preparative Liquid Phase IEF and MALDI TOF MS as Tool to Study the Human Serum Glycoproteome

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Due to their high specificity, plant lectins are more and more frequently used for affinity based purification of glycoconjugates. Appropriate profiling and isolation of glycoproteins is a key factor in proteomics, as changes in glycosylation patterns usually characteristic of disease states and environmental conditions. In the present study, human serum is applied to various lectin affinity columns (Concanavalin A, Jacalin and Lentil) to profile and isolate glycoproteins of interest. After the lectin partitioning process, the purified glycoprotein mixture is further resolved by preparative liquid phase isoelectric focusing in the pH range of 3–10. The lower complexity of the resulting fractions allows simpler interrogation by MALDI TOF mass spectrometry.

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Proteomic Analysis of Cyclin-dependent Kinase Inhibitor (CDKI)-regulated Protein Expression in Tumour Cells via SELDI-TOF-MS and 2D-Gel Electrophoresis/Peptide Mass Fingerprinting

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The cyclin-dependent kinase inhibitors (CDKIs) p16Ink4A, p21Waf1/Cip1/Sdi1 and p27Kip1 are key regulators of cell cycle progression, especially in response to genotoxic insult. They have also been shown to positively influence the expression of genes associated with several age-related disorders including cancer and Alzheimer's disease. Previous work by our collaborators has shown that expression of p21 in tumour cells mediates an anti-apoptotic and mitogenic paracrine effect, which is in contrast with the arrested state of p21-expressing cells. We have previously examined changes in secreted proteins from HT-1080 human fibrosarcoma cells displaying inducible p21 expression by analysis of conditioned culture media using (SELDI-TOF-MS) and identified candidate mediators of the observed anti-apoptotic paracrine effect (Currid et al., *Proteomics*, 2006). Here, we present preliminary results of SELDI-TOF-MS analysis of cell extracts from HT-1080 human fibrosarcoma cells displaying inducible p21 expression. In addition, we have also profiled altered protein expression in HT-1080 cells displaying inducible expression of other CDKIs, namely p16 and p27. A set of differentially regulated candidate peaks have been nominated for purification, identification and validation. In addition, preliminary work has been performed analysing the differential regulation of protein expression by p21, p16, and p27 using 2-D gel electrophoresis and peptide mass fingerprinting. From this, 17 proteins displaying altered expression in CDKI-expressing cells have been identified so far. The proteins identified as differentially expressed are associated with functions including cytoskeletal rearrangement, calcium signaling, exocytosis, apoptosis and differentiation, oxidative stress and ATP synthesis. Elucidation of mechanistic pathways that control tumour cell senescence and response to genotoxic insult should make it possible to design more effective and less toxic therapeutic approaches to diseases such as cancer.

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Stable Isotopic Labeling (SILAC) in the MS Analysis of Cortactin Binding Partners

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The Protein Discovery initiative of the Cell Migration Consortium is investigating the binding partners of focal adhesion molecules and their phosphorylation states during various steps in the migration process. Recently we have focused on determining the binding partners of cortactin, a key protein in focal adhesions. The experimental approach chosen was SILAC coupled with mass spectrometric analysis of binding complexes using FT ICR MS/MS instrumentation. From these experiments we have identified 33 putative intracellular binding partners of cortactin, some of which are described for the first time. For example, we have identified RCC2 (a likely Ras-GEF), Rec8, septin and TABP as cortactin binding partners which suggest a possible interaction of cortactin with the cytokinetic apparatus of the cell. In summary, our results lend support to a recent hypothesis that links mitosis with cell migration.

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Quantitative Analysis of Complex Protein Mixtures Using a Peptide-based Isobaric Mass Tagging Technology

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Proteomics analysis has proved as a key to determining drug mechanisms and assessing toxicological potential during preclinical screening studies. Up until now proteomics has largely been a binary black-versus-white method, where one looks at "healthy" versus "diseased," or "before" versus "after" states. A major goal in proteomics is to accurately measure changes in the relative abundance of large sets of proteins in complex biological systems as a function of some experimental parameter, such as drug dose or exposure time. When measuring quantitative changes in protein expression, consistency and reproducibility of the measurements are critical to success. This study focuses on accurately assessing the quantitative consistency of a peptide-based Isobaric Mass Tagging (IMT) technology without introducing the confounding issue of biological variability. Complex samples were prepared consisting of different defined ratios of human serum and *Escherichia coli* bacterial cellular lysate. The protein mixtures were then labeled with different cysteine-reactive tags in order to compare protein expression levels among the different samples all at once. The ability of the IMT technology and tandem mass spectrometry to identify and quantify large sets of proteins from the two proteomes was demonstrated using a combination of public domain and proprietary software analysis tools. Proteins were identified using the MASCOT search engine and their relative abundances were quantified using an internally developed IMT quantification routine. The measured differences in protein levels correlated well with the known input ratios of the two complex samples. The described technology provides a powerful method for enhancing proteomic analysis by mass spectrometry. The highly multiplexed and quantitative nature of the new technology should herald in new opportunities to provide diagnostic and functional insights into the proteomics discovery process.

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Affinity Capture/ Mass Spectrometry-based Methods for Multiplexed Quantification of Protein Kinases

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Precise targeting of specific aspects of kinase cascades is now known to provide previously unattainable breakthroughs for disease therapies. Protein kinases represent only 2% of the human genome but about 22% of the "druggable" portion and their importance is underscored by the numerous disease states that arise due to disregulation of kinase activity. Aberrant cell signaling by many of these protein and lipid kinases can lead to diseases, such as cancer, Alzheimer's disease, and type II diabetes. Overall 400 human diseases are thought to be linked to defects in protein kinase signaling pathways. We describe a new technology for selectively capturing a population of kinases directly from detergent-solubilized cells and tissues or from bodily fluids using either polymeric beads or high-capacity streptavidin-coated 96-well microplates. Once captured, the kinases are proteolytically digested to constituent peptides, supplemented with rationally designed peptides relating to particular native protein kinase peptide sequences that contain sessile aspartate-proline (DP) bonds, and the native peptides derived from the kinase population are then quantified by tandem mass spectrometry. Strategies for profiling the relative abundance of protein kinases in multiple samples using isobaric peptide tags containing sessile DP bonds are also described. The technologies described herein provide a multiplexed quantification strategy for the precise determination of protein kinase levels. The simplicity and sensitivity of the methods, coupled with the widespread availability of tandem mass spectrometers, make the strategy highly useful for simultaneously measuring the levels of multiple kinases directly from the human kinome.

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Towards Improved Diagnosis of Neuromuscular Disorders by Serum Protein Profiling

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The muscular dystrophies are heterogeneous in their molecular basis. With current methodologies, it is difficult to discriminate between certain sub-types, and invasive intervention for a muscle biopsy is often required. We are applying protein profiling of serum with MALDI-TOF mass spectrometry to this group of diseases as a less invasive, fast and economical alternative. To develop and optimize the technology, serum from different mouse models for muscular dystrophies as well as from a cohort of 450 Duchenne and Becker muscular dystrophy patients was first fractionated in an automated manner using a range of functionalized magnetic beads. Mass spectra of the peptides and proteins present between 1 and 10 kDa were generated. A double-cross validatory approach to linear discriminant analysis was taken to generate diagnostic classifiers for the different disease groups. Both richness of spectra and strength of the classifiers varied per bead type. Interestingly, combining complementary data from different bead types resulted in much improved classification rates. We are currently elucidating the identity of discriminating peaks by tandem MS, which would be vital for their validation as a true disease marker. Combining controlled and automated fractionation of serum with careful statistical analysis improves classification capabilities and has great potential for application in the diagnosis of these diseases.

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Identification of Proteomic Patterns Using Ion Distance Ratios to Classify Disease States

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The classification of serum samples through the use of computer algorithms has been done using a wide variety of tools including genetic algorithms, decision trees and support vector machines (SVM). We analyzed serum samples from two different cancer conditions (ovarian cancer and cutaneous T-cell lymphoma) and matching normal samples using the BioExpression kits enriching for peptides carried on albumin. Mass spectra were generated using the PerkinElmer prOTOF orthogonal mass spectrometer. The data was analyzed using the CPRL routine method (feature selection) and this was compared with classification using the ratio of peak pairs at m/z distances corresponding to phosphorylation (80), deamidation (1) or disulfide bond formation (2). For both data sets, classification sensitivity and specificity of the ion distance ratios method was equivalent to or superior to those of the routine classification. These results demonstrate that classification is possible using ion distances correlating with possible disease induced protein modifications.

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A Study of Different Sample Preparation Methods for the Non-label Quantification of the Human Serum Proteome

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This work describes the investigation and outcome of different sample preparation methods for label-free quantitative proteomic study of human serum proteins. All analyses were preformed on in-solution trypsin digestion of human serum. MS analysis was on a Q-TOF instrument with a nanoflow split-free LC system interfaced to the ion-source (75 μ id LC columns). Data was acquired using data dependent analysis (DDA) or a high/low energy strategy. The high/low method is a "protein expression" method were a mass window of data is acquired in low energy (intact peptide ions) followed by a high energy acquisition on all ions. This fast duty cycle yielded extensive qualitative and quantitative information. Samples were prepared by a verity of methods including, top-6 or top-20 abundant protein depletion and enhancement (reproducibility) of digestion with trypsin-friendly surfactants. Fractionation of intact proteins by 1D SDS-PAGE and chromatography was investigated. In addition, peptide mixtures from digestion were separated by multi-dimensional chromatography. The 2D methods included SCX/RP and RP/RP. The objective was to understand the most effective sample preparation method for studying the human serum proteome. A summary of the results will be presented.

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High sensitivity, Multiplexed Quantitative Analysis of Biomarkers Using Novel Tandem Mass Tags

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The comprehensive and quantitative analysis of complex proteomic samples remains a very demanding task in spite of the recent advances in mass spectrometric instrumentation. Quantitative analysis of large set of samples (e.g. biomarker evaluation) is often rate limited by the instrument accessibility. In that respect the iTRAQ reagents alleviates this problem by enabling concomitant analysis of several samples. The development of a second generation of iTRAQ reagents with eight different isotopically labeled forms increases the analysis throughput dramatically.

Quantitative analyses based on the tandem mass tagging strategy are performed in the MS/MS mode by measuring the ratio of the reporter ions in the low mass region. Analyses were performed either on tandem mass spectrometers operated either in MALDI mode on a ToF-ToF instrument or electrospray mode using a quadrupole time-of-flight or a quadrupole linear ion-trap instrument. With the latter platform, high throughput is achieved while maintaining high selectivity and sensitivity using the multi-reaction monitoring mode.

One requirement for the quantitative analysis of peptides in complex samples such as serum is an effective sample preparation protocol that reduces sample complexity while retaining the integrity of the remaining components. In this study we have combined the glycopeptide sample preparation (i.e. selective isolation of N-linked glycopeptides), iTRAQ labeling, prior to the multiplexed quantitative mass spectrometric analysis of serum samples.

Furthermore, derivatization of peptides with either non-labeled or heavy labeled reagent (mCAT reagent) has been used to screen for discriminating peptides; i.e. by rapidly detect in one single LC/MS run peptide with different abundances between normal and disease serum samples.

The combination of an effective sample preparation, isotope labeling enabling multiplexed analysis, and a robust, selective and sensitive mass spectrometry platform allows rapid screening of large number of samples for biomarker evaluation.

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A Bioinformatics Analysis Scheme for Protein Identification and Quantification using Peptide-based Isobaric Mass Tagging (IMT) Technology

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Proteomic differential display analysis has proved indispensable to determining drug mechanisms and for assessing toxicological potential during preclinical screening studies. Peptide-based Isobaric Mass Tagging (IMT) technology is a powerful platform to simultaneously analyze relative protein levels from multiple complex samples. The technology provides a superior approach for determining the relative expression level of individual proteins by mass spectrometry, with high precision (low coefficients of variation). Since multiple samples are tagged and mixed before processing steps, sample-to-sample recovery differences are completely eliminated. A bioinformatics approach has been developed to enable fast and accurate protein identification and quantification through analyzing IMT labeled peptides from MS/MS spectra. The software has parallel functions for processing and data analysis. Workflow-wise, it generates a list with predicted masses for the in-silico-digested IMT-labeled peptides, based upon proteins identified to be present in the samples using the unlabeled peptides identified by popular protein search engines such as Mascot and Sequest. Next, it extracts a list of precursor masses for the labeled peptides from raw tandem MS data by scanning for particular signals generated by IMT. By matching these lists, the software provides data on IMT-labeled peptides with associated quantitative information, organized according to the protein identifications. The software allows users to store all the chemistry-related parameters (post-translational modifications, protein cleavage agent) into individual protocols and retrieve them for the quantification process. It provides a menu of visualization tools for analyzing and interpreting the results. Output results are in Microsoft Excel spreadsheet format and statistical analysis, including data normalization, can be performed on the quantitative results. The software provides an intuitive graphical interface to allow users to get started quickly with minimum training and allows analysis of MS/MS spectra from IMT labeled peptides generated by the most commonly used tandem mass spectrometers.

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Arabidopsis Proteomics; from Proteome Back to the Genome

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Arabidopsis thaliana was the first higher plant for which the entire genome sequence was available. Based on gene predicting algorithms, a well-annotated protein database is available for the community and is being used for protein identification using mass spectrometry. As with all software-based predictions, gene prediction may be erroneous and without additional information it remains unclear which predicted gene structure reflects the in vivo situation. This is especially problematic in cases where gene predictions by different algorithms are contradictory.

In order to tackle this question, we analyzed the proteome of an *Arabidopsis thaliana* cell line with LTQ mass spectrometry. Although many proteins were identified with this standard experimental setup (in total more than 6370 distinct proteins), this approach has limitations due to potentially wrong gene prediction, unspecified amino acid modifications or unpredicted splice sites. To circumvent these limitations, we implemented an alternative MS/MS-data analysis strategy that uses as one of its integral components a spectrum quality-scoring tool (QUALSCORE). It assigns a database-independent quality-score to each MS/MS spectrum in order to extract high quality spectra for which no reliable database result could be found. So far, we detected approximately 100000 out of 3 million spectra that fall into this category. These spectra are then subjected to cascading database searches with different search algorithms that include genome-level searches and an in-house developed algorithm that is able to detect spliced peptides.

We report here our first results that indicate the depth of our analysis and how many additional identifications such as posttranslational modifications and unpredicted splice sites or even unpredicted open reading frames can be expected when protein database-independent analytical scenarios are used for *Arabidopsis thaliana*.

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Dynamic Quantitative Analysis of the Nucleolar Proteome Using an Isobaric Mass Tagging (IMT) Approach

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The nucleolus represents a large, highly dynamic multifunctional nuclear organelle. It plays a key role in ribosome biogenesis and participates directly or indirectly in cell-cycle regulation, senescence, proliferation, differentiation and maturation states. Recently, several studies have dramatically increased the scientific community's knowledge of nucleolar proteome regulation using mass-spectrometry-based quantitative analysis. Here, we present a dynamic analysis on the nucleolar proteome using Isotopic Mass Tagging technology (IMT), a novel quantitative system based on a class of isobaric reagents and tandem mass spectrometry (MS). The properties of this technology allow analytes modified by the IMT reagents, to be separated as a group from other molecules and distinguished from each other through tandem MS. Six nucleolar protein extracts, isolated from HeLa cells that were treated with the metabolic inhibitor actinomycin D for different time periods, were modified with a unique IMT reagent on the free cysteine residues. Based on the MS results, a total of 542 proteins were qualitatively identified, and 232 proteins were unambiguously quantified. The quantification data demonstrate that the nucleolar proteome significantly changes over time in response to differences in growth conditions, which is consistence with the previous observations from several groups. The IMT approach should provide a widely applicable multiplexing tool in the quantitative proteomics field.

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Toward a Comprehensive Quantitative Phosphoproteome Analysis of Hyperphosphorylated PHF Tau in Early Alzheimer's Disease

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The accumulation of paired helical filaments (PHF) of hyperphosphorylated microtubule-associated protein tau is a key pathological hallmark of Alzheimer's disease (AD). Recently, we demonstrated a robust mass spectrometry-based phosphoprotemic platform to identify PHF tau phosphorylation sites (*J. Biol. Chem.* 2006 281, 10825–10838). As in most large-scale proteomics projects, a large number of MS/MS spectra were generated in these studies. To fully utilize such data, it is necessary to develop a reliable computational and bioinformatics platform. Therefore, in order to objectively evaluate and annotate the degrees of tau phosphorylation in various biological samples, such as brain tissue and CSF samples, new computational tools have been utilized to extend our previous findings so as to provide a comprehensive quantitation of the tau phosphoproteome. In addition, an in-house database search program based on hidden Markov model (PepHMM) was developed that makes it possible to identify peptides not normally identifiable with commercial proteomics database search packages such as SEQUEST and MASCOT (*Anal. Chem.* 2006 78, 432–437). Analysis of various PHF tau proteomics datasets indicates that PepHMM identifies 43% and 31% more correct spectra than SEQUEST and MASCOT, respectively. These PHF tau proteomics datasets have also been coupled to a series of visualization tools to allow rapid evaluation of the performance of the experimental platform (HPLC and mass spectrometer). The integration of this mass spectrometry and bioinformatics approach has enabled us to simultaneously quantify and monitor PHF phosphorylation and other post-translational modifications in various AD samples and biological fluids.

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Proteomic Characterization of White Adipose Tissue in a Diet-Induced Obesity Study

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The individual susceptibility to high fat diet-induced obesity is unclear. Consistent with literature, our present study shows that about 30% of the ICR mice were diet resistant (DR) to obesity development in response to a chronic feeding of 30% high fat (on weight basis). These diet resistant (DR) mice had significantly lower feed efficiency, percentage of body weight gain and white adipose tissue (WAT) weight compared to diet-induced obese (DIO) mice. Since WAT plays an active role in the development of obesity, protein expression in WAT between DIO and DR mice would provide new insight to their different metabolic profiles. The objective of this study was to search for protein biomarkers which could be used to study the development and resistance of obesity to a high fat diet. A proteomics approach combining 2-DE and MS was used to compare the proteomes between DIO and DR mice and a total of 400 protein spots were identified. Protein spots were analyzed by MALDI-TOF MS and further identified by MASCOT. Proteins that are involved in carbohydrate metabolism (carbonic anhydrase 1, enolase 1&α), for lipid metabolism (glycerol-3-phosphate dehydrogenase 1), in electron transport chain (cytochrome b-5), antioxidation (glutathione transferase ζ 1) and lipid peroxidation (peroxiredoxin 6) were identified in WAT. Glycerol-3-phosphate dehydrogenase 1 and peroxiredoxin 6 showed a decreasing trend in DR group. The results suggest that both lipid metabolism and lipid peroxidation in WAT may differ between DIO and DR groups. These findings give new insight for the study of the etiology of obesity and its resistance.

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Proteomic Approaches for Biomarkers Discovery in Gastric Cancer

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Gastric cancer is the second leading cause of cancer mortality worldwide. Most patients are presented at advanced stages due to lack of specific and sensitive biomarkers for early detection of the disease, resulting in poor prognosis and limited treatment options. In this study, we analyze the protein profiles of tumor and its adjacent non-tumor gastric tissues from the same patient, aiming to identify potential clinical biomarkers. Stomach protein extracts were separated by 2D gel electrophoresis using 24 cm IPG strips of pH 4–7 (GE Healthcare, Biosciences) and focused on an Ettan IPGphor IEF system. In our preliminary analysis of silver-stained gels, 73 proteins spots were found to be differentially expressed. Of these, 17 protein spots have been identified by MALDI-TOF/TOF. Identification of the remaining protein spots are in progress. We are currently comparing the gastric tissue proteomes using narrow-range IPG strips to improve the resolution of our gels and to identify more differentially expressed proteins in the gastric cancer proteome. Identification to these proteins may lead to useful biomarkers and provide clues for gastric carcinogenesis.

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Genome-wide Characterization of Promoter Occupancy Using ChIP-GLAS, a Novel Tool for Studying Transcription Regulation and Disease Pathways

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Recent advance in genome-wide location analysis has made it possible to systematically identify target genes for sequence specific transcription factors. However, the requirement for 108 cells in a standard ChIP-on-chip assay precludes high throughput analysis and biological applications where sample quantity is limited. To address this technical challenge we developed ChIP-GLAS (Guided Ligation And Selection) technology, based on a unique DNA biotination and template DNA mediated oligo ligation process. To implement ChIP-GLAS, we designed 40 bp oligos, which representing 20,000 human promoters and fabricated on glass slide as promoter array to be used together with chromatin immunoprecipitation. Subsequently we proved that ChIP-GLAS technology permits detection of DNA-protein interactions in vivo using two-orders of magnitude less material than that required by the standard ChIP-on-chip procedure. A series of reconstitution experiments demonstrated that $\sim 3 \times 10^6$ cultured mammalian cells (~one 100 mm plate) was sufficient to produce detectable signals. Furthermore, we have successfully applied the ChIP-GLAS technology to various applications including studying transcription factors such as YY1, NFkb, and DNA methylation in the context of understanding human diseases. In combination with gene expression profiling data, the technology provides a powerful filtering method to gain insight into transcriptional mechanisms involved in disease pathways, thus providing a novel tool for biomarker discovery and assisting other drug discovery efforts.

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Mining Urinary Proteome Using an Alternative MS Data Acquisition Approach and a Novel Database Search Algorithm

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Human urine is a highly complex mixture of proteins produced by the filtration of the blood within the glomerules, proteins secreted from the kidneys and the urogenital tract. Thus far, in clinical laboratories, the identification and the measurement of known disease-associated proteins or hormonal markers have relied highly on immunological analyses, such as RIA or ELISA, in a one-marker-at-a time-manner. In this work we investigate the human urine proteome by liquid chromatography (LC) with mass spectrometry (MS) detection. The breast cancer female patient urine have been examined and compared to control. The mix-mode cation-exchange reverse-phase sorbent (MCX) has been applied to sample cleanup prior to LC-MS. The sample pretreatment is critical for successful analysis. In order to eliminate run to run peptide/protein identification variability usually observed for tandem mass spectrometry (MS/MS), an alternative low and elevated energy scanning modes were used for parallel monitoring of precursors and their fragment ions. The precursors and their corresponding high-energy ions were linked via dedicated software, using an accurate mass and retention time parameters. Three different database search algorithms were employed for peptide identification, including MASCOT, Protein Lynx Global Server (PLGS) and Ion Accounting (IA) software. IA, a novel database search approach, matches the theoretical tryptic peptide b/y ions with acquired spectrum. The rules for confident identification included the parameters such as the number of matched ions, corresponding precursor/fragment ion intensity, and robustness of the match in the repeated experiments. Only peptides detected at least in two out of three replicate LC-MS experiments were considered as confident hits and counted towards protein identification. The novel MS scanning approach in conjunction with IA database search algorithms improved the repeatability of LC-MS proteomic experiment, and provided a more representative protein coverage. List of confidently identified peptides/proteins in normal and cancer patients urine was compiled.

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Distinguishing Progressive Fatty Liver Disease from the Non-progressive Form Using Reverse Phase Phosphoproteomic Array Analysis of Intracellular Signaling Pathways

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Non-alcoholic Fatty Liver Disease (NAFLD) is the most common cause of abnormal liver enzymes in the United States. The role of omental adipose tissue, secreting adipokines and cytokines, in the pathogenesis and progression of NAFLD remains unknown. We have developed a new protein microarray tool, called Reverse Phase Protein Microarrays, for multiplexed cell signaling analysis from human clinical specimens. This ultra sensitive and quantitative tool was used to distinguish subtypes of NAFLD. We employed an Aushon Biosystems 2470 Arrayer to spot 30 nL containing 0.25–4 μg of protein from lysates derived from adipose tissue taken from patients with varying degrees of fatty liver disease. Proteins were extracted using a novel hydrostatic pressure cycling technology (The Barocycler NEP3229). We analyzed 66 different phosphorylation events at once, focusing on insulin signaling, apoptosis, pro-survival and cytokine pathways. Image analysis was performed using MicroVigene (VigeneTech) software and network analysis was performed to uncover which phosphorylation levels of signaling molecules in the adipose tissue can distinguish NAFLD subtypes. Our data shows that by measuring discreet components of the insulin receptor-mediated signaling, it is possible to differentiate subtypes of liver disease by investigating signaling in adipose tissue. Specifically, components of the AKT/mTOR pathway were significantly altered and correlated with disease. We conclude that omental adipose tissue can be effectively analyzed for a highly multiplexed cell signaling analysis using Reverse Phase Protein Microarrays and that signal pathway profiling of omental adipose specimens from patients with NAFLD can differentiate patients with NASH from those with other forms of non-progressive NAFLD. This would suggest that NAFLD is a disease in which omental fat tissue plays an active role, possible through autocrine and paracrine cytokine and adipokine-mediated signaling.

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A Strategy for Determining Protein Phosphorylation Sites by Using LC-MS Data

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Knowing phosphorylation sites in proteins provides essential information of the molecular basis of the regulatory mechanisms. We propose here a strategy for determining phosphorylation sites of phosphoproteins using liquid chromatography-mass spectrometry (LC-MS) data derived from a high-resolution quadrupole time-of-flight mass spectrometer (Q-TOF-MS). Because a LC-MS experiment usually results a large dataset, a computational algorithm is required to facilitate data processing.

Immobilized metal affinity chromatography (IMAC) was utilized to enrich phosphorylated peptides from a tryptic digest of proteins. One aliquot of the IMAC-enriched peptides was dephosphorylated by adding alkaline phosphatase. Both IMAC-enriched peptides and phosphatase-dephosphorylated peptides were analyzed by LC-ESI-MS respectively. A computer program, DeltaFinder, was written using Matlab (MathWorks, Inc.) for finding out pairs of mass spectral signals with an 80-Da mass difference (or multiples of 80 Da) in these two LC-MS data. After a list of potential phosphopeptides was generated, LC-ESI-MS/MS of the same sample was performed for sequencing and confirmation of these phosphopeptides and also their non-phosphorylated forms. The m/z values in the potential phosphopeptide list were utilized to program subsequent LC-ESI-MS/MS experiments.

In order to demonstrate this strategy, a mixture containing 50 ng tryptic digest of a-casein and β-casein was used. Total 60 peptide masses existed in LC-MS run of IMAC-eluted sample. An aliquot of the IMAC-eluted sample was incubated with 0.25 U AP at 37 for 1 hr, converting into their non-phosphorylated forms. Eventually, 7 masses in the IMAC-eluted sample can find their corresponding 80 or 160-Da shift in the LC-MS run of AP-treated sample. The m/z of these 7 pairs of potential phosphopeptides and their nonphosphorylated forms were subjected to subsequent LC-MS/MS analysis. Five phosphopeptides were identified along with their counterparts.

A strategy for phosphorylation site determination has been developed on LC-MS system coupling a computational algorithm to facilitate data processing.