A Differential Proteomic Approach to Study Stimulus-induced Plasticity of Olfactory Receptors in Mice

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The olfactory epithelium (OE) lines the interior surface of the nasal cavity and consists of olfactory sensory neurons (OSNs), basal cells, and supporting cells. When odorants enter the nasal cavity, they dissolve in the mucus covering the luminal surface of the OE and bind to specific olfactory receptors (ORs) located on the ciliary membrane of the dendrites of OSNs. ORs are G-protein-coupled membrane receptors that encode the largest vertebrate multigene family (~1,000 ORs in the mouse). To investigate how OR mediate odor perception and how they influence short- and long-term neuronal responses, a differential proteomic strategy using Fluorescence 2-D Difference Gel Electrophoresis (DIGE) was employed. Test mice were exposed to odorants at defined times and their OE was compared to the OE from control mice using DIGE followed by nano HPLC/ESI-MS/MS peptide analysis for protein identification via database searches with SEQUESTTM. Complementary to the differential analysis of soluble proteins, membrane proteins were extracted and enriched by phase separation with Triton X-114. SDS-PAGE of the obtained OE membrane fraction was performed and immunoblots were probed for the presence of components involved in olfactory signal transduction: adenyl cyclase type III and G-protein subunit GuoII. To reveal stimuli-induced changes, olfactory membrane protein fractions from odorant-exposed mice and control mice were labelled with different fluorescent dyes and jointly analyzed by 16-BAC/SDS-PAGE. By analyzing the mouse OE from single individuals in 5 independent experiments, we could determine a biological relevant set of differentially expressed proteins, which provides greater insight into OR plasticity.

Identification of Highly Expressed Proteins by IL-3 Stimulation in Lymphocytes

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Interleukin 3 (IL-3), a glycoprotein hormone, has an important role for growth of hematopoietic progenitor cells. IL-3 exerts its biological function through a specific receptor (IL-3R) that is expressed on its target cells. The IL-3R activates multiple signal transduction pathways including ubiquitin-mediated proteolysis resulting in the induction of immediate early genes. How these immediate early proteins couple IL-3R activation to the biochemical machinery of cell growth and cell cycle progression is poorly understood. To verify proteins over-expressed by IL-3 stimulation on an IL-3-dependent murine pro-B cell line (Ba/F3), two-dimensional polyacrylamide gel electrophoresis coupled with mass spectrometry was performed using IL-3-stimulated and -depleted Ba/F3. Spot detection was accompanied by using ImageMaster™ 2D Platinum software. More than 100 candidate proteins were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALD-TOF-MS) or peptide sequencing. The expression levels of the individual proteins were examined by Western blotting with the respective antibodies to assess the protein values and by comparing mRNA expression levels in the same samples. It shows that the comprehensive proteomics is powerful approach to reveal the IL-3-stimulated protein profile and these results provide the insight into elucidating the effects of IL-3 on the growth and differentiation of hematopoietic progenitor cells.

A novel strategy was demonstrated in the comparison of control and activated cells, where differential protein expression was sequenced using a protein microarray. This approach enabled the targeted validation of multiple protein candidates, specifically involving the IL-3 receptor (IL-3R) and its downstream signaling pathways. The study provides new insights into the molecular mechanisms governing lymphocyte responsiveness to IL-3 stimulation, which could be further utilized for therapeutic interventions.
Quantitative Proteomics of EGF and PDGF Signaling Networks Reveals Controlling Mechanism of Mesenchymal Stem Cell Differentiation

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Closely related signaling events initiated by Receptor Tyrosine Kinases (RTKs) often lead to different cellular outcomes such as proliferation or differentiation. Although RTKs have been a main focus of signaling research for decades, little is known about the mechanisms controlling differential growth factor effects. A major challenge for systems biology is to reveal from such largely overlapping and dynamic protein networks the critical differences that influence cell fate. We found that the differentiation of human mesenchymal stem cells (hMSC) into bone-forming cells is enhanced by epidermal growth factor (EGF) but not platelet-derived growth factor (PDGF), despite the ability of both to induce tyrosine phosphorylation. Using mass spectrometry-based proteomics with triple-encoding SILAC, we determined, and quantitatively compared, the EGF and PDGF tyrosine phosphoproteomes. Three populations of hMSC cells were grown in medium containing distinct forms of arginine - either the normal Arg0 or Arg6, or Arg10 isotopic variants. Arg0 cells were left untreated and served as a control; Arg6 cells were exposed to EGF and Arg10 cells to PDGF. The combined cellular lysates were immunoprecipitated with anti-phosphotyrosine antibodies, the precipitated complexes were proteolytically digested and the resulting peptide mixture analyzed by LC-MS/MS. We identified 113 proteins that were specifically utilized by at least one of the growth factors. More than 90 percent of these signaling proteins were found in both RTK networks, while the phosphatidylinositol 3-kinase (PI3K) pathway was exclusively activated by PDGF, implicating it as a possible control point. Indeed, chemical inhibition of PI3K in PDGF-stimulated cells removed the differential effect of the two growth factors, bestowing full differentiation effect onto PDGF. Thus, quantitative proteomics can be used to directly compare entire signaling networks and discover critical differences capable of changing cell fate.

Impaired Functionality of Mutated CDC48P in the ER-associated Protein Degradation Pathway Triggers Mitochondria-mediated Cell Death

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Valosin-containing protein (VCP) is a pathological effector for expanded polyglutamine-induced neurodegeneration. Point mutations in VCP associate with inclusion body myopathy associated with Paget disease of bone and frontotemporal dementia (IBMPFD). However, the mechanism underlying VCP-mediated cell death in these disorders is poorly understood. In Saccharomyces cerevisiae, the exchange of a single serine residue in Cdc48p (cdc48S565G), the highly conserved orthologue of VCP, confers yeast cells with high susceptibility to undergo apoptosis.

Methods: The functional state of yeast cells and mitochondria and their impairment after applying physiological stress conditions was analysed using plating assays, TUNEL staining, staining of reactive oxygen species (ROS), enzymatic assays and in vivo fluorescence microscopy. Yeast mitochondria were fractionated by differential centrifugation steps as well as by zone-electrophoresis (ZE) using a free-flow electrophoresis device (FFE) that separates mitochondria according to their surface properties, thereby able to significantly improve their degree of purity (Zischka et al., Proteomics 2003). Proteins were separated by 1- and 2-dimensional SDS-PAGE and identified by MALDI-TOF mass spectrometry. Mitochondrial fractions were further analysed using Western blot analysis and electron microscopy.

Results: We show that mitochondria are specifically involved in programmed cell death (PCD): cytochrome c release into the cytosol, mitochondrial production of ROS and distinct alterations at the mitochondrial proteome level are accompanied by respiratory deficiency and the emergence of caspase-like enzymatic activity in affected cells. ZE-FFE analysis revealed increased co-migration of microsomes and mitochondria upon apoptosis. Accumulation of polyubiquitinated proteins specifically in microsomal fractions indicates Cdc48p-S565G dysfunction in ER-associated protein degradation (ERAD) and ER stress.

Conclusions: The augmented attachment of ER-derived structures to mitochondria upon apoptosis suggests that impaired ER-mediated protein turnover confers susceptibility towards mitochondria-mediated PCD.
3.7 Signal through 4–1BB Ligand Inhibits Osteoclastogenesis by Increasing Interferon-Beta in Bone Marrow-derived Macrophages

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We have investigated whether intracellular signals are transmitted through 4–1BB ligand, using 4–1BBFc fusion protein and 4–1BB-deficient mice. Immobilized 4–1BB-Fc fusion protein strongly inhibited osteoclastogenesis induced by macrophage colony-stimulating factor (M-CSF) and receptor for activation of nuclear factor-κB ligand (RANKL) in bone marrow macrophages. Incubation of BMM with M-CSF increased the levels of 4–1BB ligand transcript and surface protein. Immobilized 4–1BB-Fc also dramatically reduced the number of TRAP-positive MNCs in BMM from 4–1BB deficient mice, suggesting that inhibitory effect of osteoclastogenesis is due to a signal through 4–1BB ligand. Reverse signaling by 4–1BB deficient mice, suggesting that inhibitory effect of osteoclastogenesis is due to a signal through 4–1BB ligand. Reverse signaling by 4–1BB-Fc caused increased level of INF-β in bone marrow-derived macrophages, resulting in inhibition of osteoclastogenesis. This work was supported by SRC fund to IRC, University of Ulsan from KOSEF and Ministry of Korea Sciences and Technology. This person (S. Kim) is supported by BK21 of UOU (2003–2005).

3.9 Proteomic Study of TGFβ Signal Transduction

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Tumor Growth Factor β (TGFβ) is a soluble ligand which binds to and activates the TGFβ receptors type I and type II. The TGFβ system plays a pivotal role in many aspects of cellular life, such as proliferation, differentiation, apoptosis, and control of important metabolic pathways. Through their serine/threonine kinase activity, TGFβ receptors activate Smad proteins, a family of signalling molecules which transduce signals from the activated receptors to the nucleus. Although up to now the majority of studies in TGFβ signalling have been focused on Smads, several lines of evidence suggest the involvement of other molecules for the accomplishment of the complex and differentiated TGFβ-dependent biological outcomes.

In order to obtain more insights on this point, we carried out a proteomic study of TGFβ receptor type I (TβR I)-interacting proteins. Using the cytoplasmic part of the constitutive active form of type I receptor as a bait, and a mass spectrometry analysis with a MALDI TOF/TOF instrument, we have been able to identify more than 10 proteins, which form complex(es) with TβR I. These proteins provide links of TGFβ signalling to small G proteins regulation, control of cell-cell adhesion, cytoskeletal morphogenesis. The present work contributes to a better comprehension of the TGFβ signalling, and discloses new perspectives for the treatment of TGFβ-related human diseases.

3.8 Deorphanization of Membrane Receptors Involved in Leukocyte Trafficking

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Our study focuses on the structural and functional study of peptides and proteins involved in signal transduction. G protein-coupled receptors represent the biggest protein family and are involved in all physiological aspects. A high number of these receptors remain orphan, their(s) specific ligand(s) remaining to be discovered. Some orphan receptors are closely related in terms of primary structure to the chemokine receptor family and so are potentially involved in the immune and inflammatory response. We optimised screening and high performance chromatography strategies to isolate specific activities for human orphan receptors, i.e. ChemR23 and FPRL2. Highly purified ligand preparations have been analysed by MALDI Q-TOF mass spectrometry and revealed that the product of human Tazarotene-Induced Gene 2 (named chemerin) and an acetylated 21 amino-acids-long amino-terminal peptide derived from Heme-Binding Protein (named F2L), are the specific natural ligands presenting a high affinity for human ChemR23 and FPRL2 receptors, respectively. Bioactive chemerin derives from the neutrophil proteases-mediated processing of inactive prochemerin by a specific deletion of the six and seven carboxy-terminal aminoacids. Ex vivo chemotaxis assays permitted to show that both ligands are novel potent chemoattractant molecules of antigen-presenting cells.

3.10 Analysis of Protein Kinases by Chemical Proteomics

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Small molecule inhibitors of protein kinases are widely used in signal transduction research and are emerging as a major class of drugs. Since the interpretation of biological results obtained with these reagents critically depends on their selectivity, efficient methods for the proteome-wide assessment of kinase inhibitor targets are required. To address this important issue, we have developed optimised affinity chromatography procedures that employ immobilized inhibitors for the selective enrichment of cellular protein kinases. Subsequent mass spectrometry analysis combined with various secondary assays consistently resulted in the characterization of previously unknown targets affected by widely used research compounds such as the p38 kinase inhibitor SB203580. Moreover, the combination of chemical proteomic methods with various biological assays permitted the identification of the angiogenesis inhibitor SU6668 as a cellular antagonist of Aurora kinase-mediated cell cycle progression and also revealed new kinase targets, which are potentially relevant for the in vivo mode of action of the marketed anticancer drug gefitinib. Our chemical proteomic procedures permit the characterization of protein kinase targets in the context of signal transduction analysis and drug development. Importantly, the established pre-fractionation techniques provide the experimental basis for proteomic studies focussing on the central regulatory functions of protein kinases in cellular signal transmission.
Cell Cycle Dynamics of Chromatin Proteome in *Xenopus* Egg Extract

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The *Xenopus* egg extract model system recapitulates most events of the early embryonic cell cycles on added demembranated sperm nuclei, including DNA replication and mitotic chromosome formation. We have employed this system for proteomic analysis of variations in chromatin composition during cell cycle progression, with the aim of searching for novel players in regulation of DNA replication. Reconstituted chromatin progressing through S-phase in the presence or absence of various replication inhibitors was separated from cytosol at different stages and proteins bound to DNA were eluted with a salt and detergent for the proteomic analysis. Using 2DE and LC/MS/MS approaches we have identified 50 candidate polypeptides that associate with chromatin in a cell cycle- and replication-dependent manner. Known components of replication machinery with expected behaviour, such as ORC and Mcm proteins, were among identified the polypeptides. To asses a functional role for candidate proteins we raised antibodies against 9 polypeptides, which were not previously associated with replication. An immunocytochemical analysis of replicating nuclei in *Xenopus* egg extract demonstrated that most antibodies (seven of nine) recognise epitopes only in interphase nuclei and show no staining on metaphase chromosomes. One antibody stains interphase chromatin and remains bound to mitotic chromosomes and one antibody failed to detect a target at all in nuclei and chromosomes. We are currently using these antibodies to verify the response of the proteins to cell cycle progression and to conduct functional studies.

Our preliminary data shows that two candidate proteins participate in a functional complex as they co-immunodeplete each other from *Xenopus* egg extract.

Temporal Analysis of Insulin-induced Changes in the Tyrosine-Phosphoproteome of Brown Adipocytes

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Type 2 diabetes is characterized by insulin resistance in muscle, liver, and fat and by defects in insulin secretion from pancreatic beta-cells. Insulin binding to its receptor initiates a complex network of events beginning with the activation of a tyrosine phosphorylation cascade that branches out to affect multiple endpoints.

As a model system to analyse the tyrosine-phosphoproteome we used brown adipocyte cell lines from wild-type and knock out mice models lacking key regulators of the insulin signaling pathway, such as the Insulin receptor substrate-1 (IRS-1).

In order to precisely define the tyrosine-phosphoproteome of the insulin signaling pathway we employed stable isotope labeling by amino acids in cell culture (SILAC) to differentially label proteins in Insulin stimulated and unstimulated adipocytes. Combined cell lysates were enriched for phosphotyrosine-containing proteins by immuno-precipitation, digested with trypsin and analysed by LC-MS/MS using LTQ-FT mass spectrometer. The relative peak hights of the analyzed peptides are a direct measure of the relative amounts of the proteins.

In another attempt to characterize the temporal phosphotyrosine based changes after insulin stimulation we labeled three populations of adipocytes with different isoforms of arginine, respectively. Each population were stimulated for different length of time. The combination of two experiments (0, 1, 10 min, and 0, 5, 20 min) allowed us to generate a five time point kinetic of tyrosine-phosphorylated proteins upon insulin stimulation.

This approaches allowed us to identify and quantify many known proteins such as IRS-1/2, PI3K, and ERK1/2. Furthermore, we identified several novel effector proteins, which showed an activation/deactivation upon insulin stimulation.
Investigation of the Influence of Calpain on Membrane Ruffling

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Calpains have been implicated in a large number of physiological processes including cell spreading and migration, myoblast fusion, cell cycle control and apoptosis. However, the physiological functions of calpain and relevant substrates in vivo remain poorly understood. The calpains consist of a μ- or m-80-kDa large subunit (genes Capn1 and Capn2) and a common 28-kDa small subunit (Capn4). To gain further knowledge about the physiological functions of calpain we used fibroblasts obtained from Capn4−/− mouse embryos (Larsen et al., Protein Expr. Purif. 2004, 33(2): 246–55). These cells lack both μ- and m-calpain activities. The cells were investigated with a combination of proteome analysis and fluorescence microscopy. Capn4−/− and the wild type Capn4+/+ fibroblast expressing EGFP were compared by live cell fluorescence microscopy and revealed a clear phenotypic difference in plasma membrane ruffling. The Capn4−/− cells ruffled less actively than wild type cells. Proteome analysis of Capn4−/− and wild type Capn4+/+ fibroblast showed that 12 proteins where differentially expressed; the calpain small subunit, cofilin 1, retinoic acid binding protein, Doxin-dependent peroxide reductase, eIF-5A, isocitrat dehydrogenase 3β, binding protein, Alanyl-tRNA-syntase, Mannose-6-phosphate receptor and Rho-GDI-1, which is known to be a shortcoming of the shotgun SDS-PAGE/LC-MS/MS approach. However, no differential quantitative analyses could be performed. We therefore established chemical, enzymatic and metabolic isotope labelling procedures for relative quantification of peptides. The analysis of apoptotic and control cells using stable isotope labelling with amino acid in cell culture (SILAC) with leucine-2H3 and arginine-13C6, separation by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization mass spectrometry proofed the general feasibility of the established method. We currently combine the differential labelling technique with the SDS-PAGE/LC-MS/MS approach to finally achieve an automatable, differential and quantitative technology for the rapid identification of modified proteins. Since not all modified factors are involved in the regulation of apoptosis we set up a platform to functionally validate the identified proteins. Since not all modified factors are involved in the regulation of apoptosis we set up a platform to functionally validate the identified factors by loss of function screens (Machuy et al., Mol. Cell Proteomics, 2004). High throughput RNA interference with validated siRNAs turned out to be a powerful technology to identify new factors involved in the regulation of apoptotic signal transduction. The combination of state of the art proteome analysis with high throughput RNA interference has the potential for the rapid identification and functional characterisation of large numbers of proteins involved in different biological processes.

Phosphoproteomics of Angiopoietin Signalling in Lymphatic Endothelial Cells

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Lymphatic vessels play an important role in immune surveillance, fat absorption, and tumour metastasis. Proper function of the lymphatic system ensures drainage of excess tissue fluid, and thereby maintains homeostasis. The discovery of lymphatic defects in Angiopoietin 2 (Ang 2) knockout mice indicated that Ang 2 is an important regulator of lymphatic development or lymphangiogenesis. Gene knock-in replacement of Ang 2 with Angiopoietin 1 in mice revealed that these two ligands provided redundant function in lymphatic endothelial cells but not in blood endothelial cells. Whereas much is known about regulation of angiogenesis by the angiopoietins, little is known about the regulation of lymphangiogenesis by the angiopoietins. Phosphoproteomics is well suited for characterization of the signalling cascade in lymphatic endothelial cells induced by stimulation with the angiopoietins. We isolated lymphatic vessels from sheep mesentery and processed them to obtain primary lymphatic endothelial cell cultures. Using a phosphoproteomics approach, we are currently identifying phosphoproteins responsive to Ang 2 signaling in these cells.

Identification of New Apoptosis Regulators by Differential, Multidimensional Proteome Analysis Combined with High Throughput RNA Interference

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Post-translational modification is the major mechanism underlying signal transduction during apoptosis. Particular important and frequent modifications are cleavage by caspases and translocation between different cellular compartments. We established a shotgun SDS-PAGE/LC-MS/MS approach for the rapid identification of caspase substrates. To discover degraded proteins, highly resolving, large (30 cm) SDS-PAGE gels were used to separate lysates and cellular compartments of apoptotic cells. 100 gel slices were analysed and identified by LC-MS/MS. The SDS-PAGE/LC-MS/MS approach was technically simpler than the 2-DE/MS approach because fewer gels had to be generated and the elaborate gel comparisons to detect altered proteins spots in 2D gels are not required. However, a shortcoming of the shotgun SDS-PAGE/LC-MS/MS approach was that no differential quantitative analyses could be performed. We therefore established chemical, enzymatic and metabolic isotope labelling procedures for relative quantification of peptides. The analysis of apoptotic and control cells using stable isotope labelling with amino acid in cell culture (SILAC) with leucine-2H3 and arginine-13C6, separation by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization mass spectrometry proofed the general feasibility of the established method. We currently combine the differential labelling technique with the SDS-PAGE/LC-MS/MS approach to finally achieve an automatable, differential and quantitative technology for the rapid identification of modified proteins. Since not all modified factors are involved in the regulation of apoptosis we set up a platform to functionally validate the identified factors by loss of function screens (Machuy et al., Mol. Cell Proteomics, 2004). High throughput RNA interference with validated siRNAs turned out to be a powerful technology to identify new factors involved in the regulation of apoptotic signal transduction. The combination of state of the art proteome analysis with high throughput RNA interference has the potential for the rapid identification and functional characterisation of large numbers of proteins involved in different biological processes.
A Proteomic Screen for Host Cell Proteins Interacting with Tyrosine-phosphorylated Bacterial Effectors

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Many bacterial pathogens use type III or type IV secretion systems to inject effector proteins into the cytoplasm of mammalian cells. Some of these proteins are phosphorylated on tyrosine residues by kinases of the host cell. The growing list of tyrosine phosphorylated bacterial effector proteins includes Tir from enteropathogenic Escherichia coli, CagA from the gastric pathogen Helicobacter pylori, Tarp from the obligate intracellular parasite Chlamydia trachomatis and the recently described Bep proteins from the zoonotic pathogen Bartonella henselae. The analysis of how these effector proteins subvert various host cell functions has shed new light on both microbial pathogenesis and basic cell biology. For example, the adaptor protein Nck has been identified as a target protein of phosphorylated Tir and CagA was reported to bind to the tyrosine phosphatase Shp-2. Despite these insights, however, much is yet to be understood about the interaction of microbial effectors. There has not been a systematic screen for target proteins specifically interacting with tyrosine phosphorylated effectors and no interaction partners for Tarp and Beps were reported yet. Recent developments in liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) have made it possible to study protein-protein interactions via affinity-based isolations on a proteome-wide scale. A method called “stable-isotope labelling with amino acids in cell culture,” or SILAC has been described that makes it possible to compare two samples quantitatively. Here, one cell state is metabolically labelled by, for example, 13C-labelled lysine. Pairs of chemically identical analytes of different stable-isotope composition can be differentiated in a mass spectrometer owing to their mass difference. The ratio of signal intensities for such analyte pairs accurately indicates the abundance ratio for the two analytes. We are using short peptides derived from the tyrosine phosphorylation sites of Tir, CagA, Tarp and Beps in order to pull down proteins from differentially SILAC labelled host cells. Host cell proteins specifically binding to the tyrosine phosphorylated peptide are identified by LC-MS/MS. First results indicate that there are remarkable differences in the host cell proteins binding to different bacterial effectors.

Growth-related Oncogene (GRO) Is Produced in Human Breast Cancer Cells and Regulated by Syk Protein-Tyrosine Kinase

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Syk, a non-receptor type of protein-tyrosine kinase widely expressed in hematopoietic cells, was recently shown to be a candidate suppressor gene in human breast cancer. Reduced expression of Syk protein is correlated with poor prognosis while its overexpression can reduce the malignant phenotype of breast cancer cells. The mechanism of action of Syk remains unclear. In this study, we have utilized low-Syk-expressing, highly invasive MDA-MB-231 and high-Syk-expressing, less invasive MCF-7 breast cancer cells to investigate the possibility that part of the functional effects of Syk are mediated by cytokines known to play roles in cell migration, invasion, or metastasis. Using protein array technology, we have determined that MDA-MB-231 cells secrete a number of cytokines known to regulate cellular growth and motility. One such cytokine, growth-related oncogene (GRO), has previously not been described in breast cancer. Of the compounds detected in the culture supernatant of MDA-MB-231, GRO was the only one that was significantly altered by modulation of Syk expression; overexpression of Syk caused a marked reduction in secreted levels of GRO. Conversely, downregulation of the relatively high levels of Syk produced in MCF-7 cells was seen to upregulate GRO secretion. At the mRNA level, overexpression of Syk in MDA-MB-231 differentially regulated the three GRO isotypes such that message levels of GRO-alpha and gamma were downregulated while that of GRO-beta was not affected. Matrigel invasion assays demonstrated a link between Syk expression and resulting GRO activity in mediating the invasive potential of MDA-MB-231 cells. In summary, our findings provide the first evidence that human breast cancer cells express and secrete GRO and have implicated this cytokine as an essential mediator of the anti-invasive properties of Syk tyrosine kinase.
3.18
Proteomics of Human Breast Cancer
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Proteomics has proven to be a useful tool for an exploration of mechanisms of human tumorigenesis and in a search for prognostic markers. Using 2D-GE and MS, we have identified a number of proteins which are differently expressed in human breast epithelial cells of various degree of transformation, e.g. non-transformed cells, immortalized but not tumorigenic, and tumorigenic cells. Their role in cellular transformation has been under investigation with use of large scale modeling (systems biology tools), molecular and cellular biology. Functional proteomics of transforming growth factor-β (TGFβ) signalling in breast epithelial cells has delivered more than 300 proteins regulated by TGFβ. Some of these proteins, e.g. Rad51, BRCA1, CIDE, TFII-I, RPA and RAP, are involved in DNA damage repair and regulation of cell cycle checkpoints important for tumorigenesis; roles of these proteins in TGFβ-dependent regulation of cell proliferation, apoptosis and DNA damage repair have been explored. Development of specific inhibitors of TGFβ signalling allows us to design therapeutic applications, based on addressing unveiled changes in proteomes. Using 2D-GE of plasma of patients suffering from breast cancer, we identified three proteins, which may be used for multiparameter diagnostics of cancer. Our proteomics-based studies of cellular transformation, signaling of TGFβ and search for plasma markers of breast cancer will be discussed.

3.19
Identification of NPM-ALK-interacting Proteins Involved in the Transformation of Anaplastic Lymphomas
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Anaplastic Large Cell Lymphomas (ALCL) are a subgroup of Non Hodgkin’s lymphomas characterized by a reciprocal translocation t(2; 5)(p23;q35). As a consequence, the Anaplastic Lymphoma Kinase (ALK) fuses to Nucleophosmin (NPM) and becomes activated via the constitutive dimerization of the NPM domain. Although several signaling molecules such as Ras/Erk, PLC-g, PI3K/Akt and Jak3/Stat3 have already been described to interact with NPM-ALK, many of the pathways triggered by NPM-ALK tyrosine kinase are still unknown. The identification of new NPM-ALK interacting molecules could elucidate the molecular mechanisms leading to NPM-ALK transformation. We developed a proteomic approach based on the immunoprecipitation with anti-ALK and anti-phosphotyrosine antibodies. Immunoprecipitated proteins from human HEK293 cells with tetracycline-inducible NMP-ALK expression were separated by monodimensional SDS-PAGE and silver stained. Specific bands were excised from gels, digested with trypsin and analyzed by nanoflow reverse-phase LC-MS/MS. We identified previously reported proteins in ALK signaling pathways, as well as new interactors such as IRS-4, Grb7 and Shp2. As Shp2 has been described to be involved in the transformation of hematologic malignancies, we further demonstrated by reciprocal immunoprecipitation that Shp2 was phosphorylated by NPM-ALK in HEK293 cells and in human ALCL cell lines. This phosphorylation was dependent on ALK kinase activity as it was absent either in presence of the kinase-dead mutant NPM-ALK(K210R) or in human ALK negative lymphoblastoid cell lines. Since mutations responsible of Shp2 activation are oncogenic, NPM-ALK mediated Shp2 continuous activation could have a role in lymphoma transformation as well.
3.20  
**Optimized Sample Preparation Tools for the Analysis of Oncological-relevant Signal Transduction Pathways**  
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Besides classical protein expression profiling, the functional characterization of proteins, including the study of subcellular redistribution and post-translational modification events (both of which are key in signal transduction) is extremely important. Previously described subcellular fractionation techniques have the drawback that they usually require relatively large amounts of starting material, are time and cost intensive and generally yield only a subset of the total proteome. This is virtually impossible to study protein redistribution events. Furthermore, current phosphopeptide-enrichment procedures do not generally result in sufficient selectivity and yield. Here, we present the recently developed ProteoExtract tools including optimized Subcellular Proteome Extraction and Phosphopeptide Capture procedures that work in concert with one another, and demonstrate their utility for the analysis of signal transduction pathways. Human cancer cell lines, some of which had been stimulated or treated with EGFR kinase or MAPK pathway inhibitors, were fractionated using the recently developed native subcellular and membrane extraction procedures. Extracted subcellular protein fractions were digested by Trypsin and precipitated in order to remove unwanted contaminants. Half of the samples were split and used to specifically enrich phosphopeptides with Zirconium-activated magnetic particles. Subsequently, polypeptide profiles of the obtained subcellular fractions were generated using Ulti-mate HPLC equipped with a 100 μm CapRod RP18e monolithic column coupled to an Esquire 3000 plus ion trap. The mass spectrometer was operated in the positive ion mode and peptides were fragmented using auto-MS/MS. Protein identifications were made using the Matrix Science Mascot algorithm. It is shown that the procedure can be used for kinase-inhibitor profiling studies to unravel mode-of-action mechanisms in colon cancer or epidermoid carcinoma cells. In conclusion, the presented approach is extremely useful to facilitate convenient gel-free analysis of signal transduction events.

3.21  
**Analysis of Human Proteins Regulated by Stimulation with Muramyl Dipeptide**  
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The changes in protein profiles reflecting inflammatory signaling were investigated in collaboration with the Mucosa Immunology group at Kiel University (Philip Rosenstiel, Dirk Seegert and Stefan Schreiber). To this end, total protein extracts of human cultured cells were analysed by two-dimensional polyacrylamide gel electrophoresis (2D-GE) and mass spectrometry. Protein components regulated by inflammatory signaling were detected by statistical analysis of protein spot intensities using the Proteoweafer software. The response of protein profiles to muramyl dipeptide (MDP) was monitored in three different human cell lines. When comparing HEK293 cell cultures with isogenic cell cultures carrying a construct overexpressing the NOD2 regulator gene many NOD2-dependent alterations in the proteome profiles were observed. When a mutant allele of NOD2 (SNP13) that renders individuals susceptible for Crohns disease was overexpressed, several protein species failed to show the response observed with wild type NOD2 overexpression. Clearly, the cellular response to MDP depended on the NOD2 allele overexpressed. Of the protein spots monitored in this study, 198 were identified by MALDI-TOF-MS peptide mass fingerprinting and MALDI-MS/MS. Among the proteins found regulated by MDP addition or by overexpression of NOD2, a wide spectrum of functional classes was observed. In addition to metabolic enzymes, structural proteins and components involved in protein biosynthesis, a number of proteins involved in folding, assembly or degradation of proteins, as well as in the regulation of cell division or apoptosis were identified. Evaluating the comparison of wildtype NOD2 and the mutated NOD2 form, we have identified a proteomic signature pattern that may lead to an understanding of the influence of the NOD2 genotype on the etiopathogenesis of chronic inflammatory bowel disease.

3.22  
**Proteomics of TGF Beta Signaling in “Normal” Human Breast Epithelial Cells**  
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TGFβ is a potent regulator of cell growth, differentiation, migration and apoptosis. TGFβ signaling plays an important role in appearance and spreading of tumor cells. To identify novel signaling targets for TGFβ, the human breast epithelial cell line 184A1 was used. This cell line is immortalized but does not form tumors in mice and undergo senescence at a high rate. Thus, 184A1 cells may reflect the first step in transformation of human breast epithelial cells. Subsequent to TGFβ treatment (5 ng/ml) for 0, 1, 2, 8 and 24 hours proteins from total cell extract were separated, using 2D gel electrophoresis. Proteins were detected by staining with silver. Incorporation of [35S]methionine in proteins was also measured. Proteins observed to be up or down regulated by TGFβ were then subjected to identification by peptide mass finger printing by MALDI TOF MS. In total 142 and 97 proteins were identified to be regulated by TGFβ in the silver stained images and the 3SS images, respectively. Subsequent validation of changes in protein expression upon TGFβ treatment observed in 2D gels was performed using immunoprecipitation and Western blotting. Validation study of four proteins confirmed changes in expression of these proteins observed in 2D gels. For further analysis Gene Ontology via the GoMiner program was used for comprehensive functional clustering. Such computer assisted analysis suggested involvement of identified proteins in regulation of cell proliferation, metabolism, apoptosis, organogenesis and developmental processes. Thus, our technology allows to detect changing proteins in numbers which would be predicted from theoretical analysis of proteome.
3.23

Comparative Proteomics Analysis of Rat Mesangial Cell Apoptosis Induced by Lovastatin

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Lovastatin, as an inhibitor of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, has shown a diversity of cellular effects independent of its lipid-lowering capacity such as apoptosis induction, differentiation induction, G1/S arrest initiation, and anti proliferative effects. We evaluated the time and concentration dependence of lovastatin-induced apoptosis in primitively cultured rat mesangial cells by MTT cell proliferation assay and PI-FACS analysis. Then the early stage or low-grade mesangial cell apoptosis induced by 20 μM lovastatin for 48h, and the late stage or heavy-grade mesangial cell apoptosis induced by 20 μM lovastatin for 72h were compared to control mesangial cells through global comparative proteomics analysis based on two-dimensional gel electrophoresis (2D-PAGE) and isotope-coded affinity tag (ICAT) technique. More than 200 differently expressed proteins or protein spots were characterized. While many heat shock proteins were up-regulated, most structure proteins and ribosome proteins decreased in lovastatin-treated mesangial cells. Moreover, many signaling proteins such as RhoA and Cofinin, and metabolic enzymes such as Adenylate kinase and nitrilase, significantly changed at protein lever. Such a global view of the differentially expressed proteins in lovastatin-induced mesangial cell apoptosis would be in favor of elucidating the pharmacology mechanism of lovastatin and the molecular mechanism of apoptosis.

3.24

Quantitative Proteomic Analysis of Proteins in Membrane Lipid Microdomains Isolated from 3T3-L1 Preadipocytes with or without Caveolae


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The proteins associated with plasma membrane lipid microdomains have been indicated to be important for most of the cellular functions played by the membrane lipid microdomains. Caveolae and non-caveolar lipid rafts are two distinct forms of lipid microdomains and share the similar lipid composition of enriched cholesterol and sphingolipids. Using RNA interference (RNAi) to suppress the expression of caveolin-1 in 3T3-L1 cell, caveolae on the plasma membrane were ablated completely. Thus, the membrane lipid microdomains isolated from the caveolin-1 RNAi 3T3-L1 cells contained lipid rafts but not caveolae. Using SILAC (stable isotope labeling by amino acid in cell culture) method, proteins in wild type 3T3-L1 cells were labeled with 13C-Leucine. Mass spectrometry analysis of Leucine-containing peptides in membrane lipid microdomain isolated from 1:1 ratio mixed labeled 3T3-L1 cells and unlabeled caveolin-1 RNAi cells indicated that proteins associated with membrane lipid microdomains were not diminished by the ablation of caveolae and caveolin. The association of proteins with membrane lipid microdomains was independent from caveolin. Lipid rafts associated proteins were identified by mass spectrometry analysis, which contained receptors, membrane channels, cell surface antigens, glycosylphosphatidylinositol (GPI)-anchored proteins, G proteins and cytoskeleton associated proteins.