19.1

Expression Proteomics Analysis in E2F1−/− T Lymphocytes

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E2F is a family of at least eight transcription factors (E2F1–8) that are important regulators of proliferation, differentiation and apoptosis. The E2F genes play an important role in the control of cell cycle progression in connection with the Retinoblastoma family proteins (Rb, p107 and 130) (Dyson 1998) which are critical elements of the G1/S check-point. Previous work showed interesting features in E2F1−/− and E2F2−/− genotype mice, especially in the development and regulation of T lymphocytes. E2F1 is a promoter of proliferation and apoptosis in T lymphocytes and E2F2 is a negative regulator of this type of cells (Murga *et al.*, 2001). Furthermore, recent work showed non-redundant functions for E2F1 and E2F2 in the immune system (Iglesias *et al.*, 2004). Our purpose is to identify differentially expressed proteins in E2F1 and E2F2 knockout mice T lymphocyte based on a 2DE gel approach. We worked on E2F2−/− mice, finding a set of differentially expressed proteins and identifying them. Some of these proteins are linked to cellular processes such as apoptosis or cell cycle progression, suggesting an alteration in these pathways and setting the starting point for future work. Now, using the same approach, we have focused our work in E2F1−/− mice to identify deregulated proteins and compare the expression patterns of both knockout mice in order to determine the mechanistic basis for the specificity of E2F function in T lymphocyte proliferation. In this work we compare 2-DE protein expression profiles of T lymphocytes obtained from E2F1−/− and wild-type controls. We show results for some proteins that are differentially regulated and related with the E2F transcription factors. Dyson, N. (1998) *Genes Dev.* 12, 2245–2262 Murga, M., *et al.* (2001) *Immunity* 15, 959–970 Iglesias, A., *et al.* (2004) *J. Clin. Invest.* 113(10),1398–407. This work has been supported by an ETORTEK grant from the Industry Department of the Basque Government.

19.2

Identification and Purification of Sperm Surface Proteins Related to Sperm Maturation

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Mammalian sperm differentiation and maturation occurs during the transit through the epididymis where spermatozoa acquire their motility and fertility. Epididymal maturation involved the progressive loss of most of the testicular sperm surface proteins and gain of new transient or permanent proteins on the surface membrane of mature and fertile spermatozoa. The aim of this work was to identify these sperm surface modifications in several domestic animals (boar, bull and ram).

Spermatozoa samples were collected all along the epididymis by microperfusion and washed on percoll gradient to remove any luminal epididymal protein contamination. Surface proteins of the gametes were labelled with sulfo-NHS-SS-biotin. Differential extractions of surface proteins were realised on intact sperm and on isolated membrane obtained by nitrogen cavitation. Peripheral proteins (low affinity membrane-bounded proteins) were released with high ionic salt buffer and integral proteins were extracted with a non-ionic detergent.

The biotinylated proteins from these different extracts were affinity purified on streptavidin column and separated by one and two dimensional gel electrophoresis (IEF/SDS-PAGE). Identification of the different proteins was done by mass spectrometry (MALDI-TOF and nano LC MS/MS).

Between mature and immature spermatozoa, several different surface proteins have been electrophoretically characterized and most of them have been identified by MS. Comparative analyses between species were carried out.

This systematic identification on several species shows that important modifications occurred both on the surface membrane and in the inner membrane of spermatozoa during epididymal maturation. Several of these proteins modifications may be associated with sperm fertility and potentially can be used as markers either in animal selection or human fertility pathology.

19.3

Human Epididymal Secretome and Proteome

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In all mammals the final stages of sperm differentiation occur outside the gonad and are not under the genomic control of germ cells. Testicular spermatozoa develop the ability to fertilize an ovum only when they transit the epididymis. In this organ, several successive differentiations occur to the gametes, particularly in their plasma membrane proteins. These sperm modifications occur always in the epididymal environment which regulated continuously, particularly in protein composition. Synchrony between changes in sperm and the epididymal milieu indicate that they are associated.

We present an analysis of the sequential changes in the protein composition of the epididymal luminal fluid and in the synthetic activity of the human epididymal epithelium. The secreted proteins were determined by incubating tubules in vitro and the luminal proteins were determined using luminal fluid collected by flushing lengths of tubules, and the preparations were analysed by two dimensional gel electrophoresis and nano LC MS/MS. In most species, epididymal activity is highly regionalized both in the secretory activity of the epithelium and the protein composition of the luminal fluid. However, in humans, this regionalization is present, but the number of sequential changes along the epididymis is reduced.

In conclusion, in all the species studied, the protein composition of the luminal fluid changed significantly according to the region of the epididymis. However in humans, there were only minor changes in the major proteins secreted. It is suggested that specificity of protein secretion could be related to the difference between species in the location in the epididymis where the sperm become fertile.

19.4

Comparative Proteome Analysis to Study the Blood Platelet Storage Lesion

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Platelet transfusion is a very common live-saving medical procedure for patients with platelet-deficient diseases such as leukemia. In contrast to other blood components, the availability of platelets is restricted since they have a limited shelf-life of 5 days for transfusion purposes. This is largely due to increased platelet clearance in the patient treated with long-term stored platelets. To overcome this problem and to extend the platelet shelf-life, it is important to understand the molecular mechanisms leading to blood platelet lesion during storage. We are using an approach that incorporates proteomics into functional biochemistry and cell biology to investigate time-dependent changes in the blood platelet proteome. The first step of our analysis consists of the separation of the platelet proteome using 2-dimensional (2D) gel electrophoresis at two different time points of storage, day 1 and day 8. After staining and comparative analysis, the identification of proteins changing in spot intensity, as determined by liquid chromatography and tandem mass spectrometry revealed, among others, that the amounts of integrins, and other proteins known to form receptor signaling complexes with these integrins, increased over the course of storage. This proves, for the first time, that there is an apparent link between blood platelet storage lesion and cell signaling. In order to shed light on the exact molecular mechanisms leading to platelet storage lesion, further analysis is now focusing on a more detailed time course of storage as well as on downstream proteins in the integrin signaling pathways. It is hoped that blocking these signaling pathways will help to reduce the effects of platelet storage lesion and to extend platelet shelf-life.

19.5

Analysis of Human Stored Platelet Proteome by 2D-DIGE

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Blood platelets play critical roles in normal human physiology by providing critical cellular components for hemostasis and wound repair, and in the pathophysiology of thrombotic disorders such as stroke and myocardial infarction, based in part on their ability to participate in the formation of vascular occluding clots. Since platelets are formed through a process of membrane budding from terminally differentiated megakaryocytes they are anucleate and contain no DNA and little RNA, thus providing little towards a meaningful correlation with physiology through a molecular analysis utilizing a genomics-centered approach. This, coupled with the fact that platelets are exquisitely suited to respond quickly to the microenvironment in which they find themselves, leads one naturally to a molecular analysis of platelet biology at the protein level. Analyses of the human platelet proteome under different physiological states may lead to enhanced understanding of both normal and abnormal platelet function. Recently, several laboratories worldwide have focused some attention on studying the human platelet proteome under differing sets of physiological conditions by use of various proteomic technologies. We have used 2D-DIGE analysis to characterize the changing platelet proteome with respect to time, when platelets are stored under conditions that mimic those used in the blood-banking industry for prolonged storage of blood platelets, in an effort to quantitatively characterize those changes in the platelet proteome that correlate with a loss of in vivo functionality of the stored platelets when re-administered to human patients. Data will be presented that represents our findings to date using this proteomic technology platform.

19.6

The Change of Tear Protein Pattern in Smokers and Contact Lens Wearers

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Human tear possess a complicated chemical structure containing many proteins, lipids and a number of inorganic substances. Tear film is composed of three distinct layers: the aqueous phase contains a variety of organic and inorganic substances such as proteins, glucose, urea and inorganic salts, the lipid layer reduces the evaporation from the aqueous phase and the mucous layer is in fact part of the aqueous layer and causes the adhesion of the tear to the epithelial cells. Tear proteins are the most important biochemicals present in aqueous layer. We have previously developed some reliable methods within our laboratory in order to study tear proteins qualitatively and quantitatively. The aim of this piece of research work was to compare tear proteins pattern by electrophoresis and HPLC in two groups (smokers and contact lens wearers) with healthy individuals. Tear samples from both healthy volunteers and the other group were collected with physical (rubbing the eye) and chemical (onion vapor). SDS PAGE electrophoresis was performed on each individual sample. The electrophoresis results showed that there was a similar pattern for tear proteins of healthy individuals. However it was slightly dependent on the method of tear collection, *i.e.* stimulated tears (physically or chemically) showed a slightly different electrophoretic pattern from non-stimulated tears. Electrophoretic protein pattern of tears from both affected groups showed a slight difference when compared to the healthy group. The important tear enzyme, lysozyme was detected by lysoplate and its biological activity measured spectrophotometrically. In this case, it was found that although the quantity of this important protein in tears change in both affected groups compared to healthy volunteers, its biological activity remained almost intact.

19.7

A Preliminary Investigation of the Human Aortic Media Proteome

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Our research group has begun a multidisciplinary project that brings together proteomic and morphological technologies to study aortic media changes. This work represents the bottom layer for a large scale comparison of medial degeneration 2D-map to identify novel biomarkers. Aortic wall specimens were collected from patients undergoing surgery for aortic valve disease with associated ascending aortic dilatation. However, only samples without any strong microscopic changes were selected. At surgery, samples were harvested about 1 cm distal to the sino-tubular junction level. Aortic tissues were crushed in liquid nitrogen, treated with cold acetone to improve protein extraction and homogenised in lysis buffer. Urea soluble proteins were separated by two-dimensional gel electrophoresis. Selected proteins were excised and digested with trypsin and the resulting tryptic peptides analyzed by MALDI-TOF mass spectrometry. The obtained spectra were used to identify the proteins through searches in the SWISS-PROT protein sequence database. Aortic media proteins resolved into about 80 spots. Up to now, about 30 prominent proteins with various functional characteristics have been identified. The most abundant ones are those associated with contractile properties: actin, tropomyosin, calponin, myosin, profilin, dynamin and transgelin. The obtained data, considered as preliminary "aortic media proteome 2D-map," will be expanded and, in the meanwhile, used for studies of differential protein expression in samples with strong changes related to ascending aortic dilatation and other medial degenerative pathologies.

19.8

Comparative Proteomics Identified Nuclear Translocation of NFAT C1 in Ulcerative Colitis

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Ulcerative colitis (UC) is a chronic, relapsing and debilitating idiopathic inflammation of colon mucosa. It may cause toxic megacolon and perforation during acute exacerbation, while long-term inflammation increases the risk for colon cancer. We set out comparative proteomic approaches to identify pathogenic factors implicated in the development of UC. We used two-dimensional gel electrophoresis and mass spectrometry to compare the proteomes between the UC diseased and normal colonic mucosa tissues. A total of about a thousand of spots were analyzed, forty were differentially expressed and nineteen distinct proteins were finally identified. Of them, nuclear factor of activated T lymphocytes (NFAT C1) was up-regulated in the diseased colonic mucosa. Immunohistochemistry revealed that NFAT C1 was confined in the infiltrating lymphocytes and the increase was due to increased lymphocyte infiltration in the diseased mucosa. Confocal microscopy revealed the translocation of NFAT C1 from cytoplasm into nuclei in the infiltrating lymphocytes in the diseased colonic mucosa. We then examined the subcellular distribution of NFAT C1 within the infiltrating lymphocytes in the colonic mucosa in 89 cases of UC, 13 cases of Crohn's disease (CD) and 3 cases of infectious colitis, and found that nuclear translocation of NFAT C1 within the infiltrating lymphocytes was specific for UC, discrepant for CD, but not in infectious colitis. Since NFAT C1 is critical for induction of IL-4 and IL-5 expression, our results suggest that UC is primarily mediated by a Th2 immune perturbation. NFAT C1 may be utilized as a therapeutic target for UC.

19.9

Proteomic Analysis of Dysferlin-mediated Cell Membrane Repair Pathway

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Limb girdle muscular dystrophy type 2B (LGMD2B) and a distal myopathy (Miyoshi myopathy, MM) are caused by defects in the gene encoding dysferlin, a novel skeletal muscle protein. A recent study showed that dysferlin-deficient muscle fibers are defective in resealing disrupted membranes, highlighting a role for dysferlin in membrane repair. However, almost nothing is known about the mechanism or the molecular apparatus involved in dysferlin-mediated membrane repair. To elucidate the components of the membrane repair machinery and its signaling pathway, we used a proteomic approach to systematically analyzed alterations of protein expression in the skeletal muscles of dysferlin-deficient (*dysf*$^{-/-}$) mice compared to age-matched control littermates at different ages. Extracted muscle proteins were separated on large format 2D gels and visualized by silver staining. In our preliminary analysis of skeletal muscles from 12-month old *dysf*$^{-/-}$ mice, we identified at least 45 protein spots that were differentially expressed compared to controls. Of these, 36 protein spots showed increased expression while the other 9 spots were down-regulated. The identity of the protein spots are currently determined by MALDI-TOF mass spectrometry. By comparing the skeletal muscle proteomes of *dysf*$^{-/-}$ mice from different ages, we hope to reconstruct the membrane repair pathway mediated by dysferlin.

19.10

Proteomics of Tissue Samples Prepared by a Molecular-friendly Fixation and Processing System

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Background: Conventional tissue processing methods for clinical specimens have severe limitations for proteomic studies. This is mainly due to type of reagents used for fixation and processing. We have developed a formalin-free molecular-friendly standardized tissue fixation and processing system that includes reagents and automated instruments. We studied reliability of this system for proteomic studies.

Materials and Methods: Tissue samples were fixed in a novel fixative (UMFIX) and formalin for various periods of time. Samples were then processed using a microwave-assisted tissue processor or conventional overnight system. Total proteins were isolated both from fixed tissue and paraffin-embedded processed specimens. Two dimensional electrophoresis, SELDI-TOF (Ciphergen IMAC30-Cu and CM10chips) and western blot for various proteins and for their phosphorylated variants (n = "34") were performed. In addition, immunohistochemistry for 70 antigens was performed on tissue sections from the tissue blocks.

Results: High-resolution protein profiles were obtained from tissues processed by the new method using 2D gel and SELDI-TOF. In contrast, no such pattern was observed in conventionally fixed and processed samples. Similar profile was obtained after long-term preservation (6 months) at room temperature from paraffin-embedded tissue blocks using our new method. Antigen preservation in immunohistochemical studies was excellent, as all antibodies were reactive in UMFIX samples; some not requiring antigen retrieval.

Conclusion: We have developed a new tissue fixation and processing system that can be used as a standard platform for clinical proteomic studies.

19.11

The Influence of Hypothermia on Renin-Angiotensin-Aldosterone Axis in Rats

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Hypothermia in nature occurs in animals which they are in winter sleeping. Hypothermia researches have applications in open heart surgery, heights medicine, adults' medicine. Despite the vastness of studies on hypothermia many of its biologic and physiologic effects on endocrine system changes aren't still recognized perfectly. The study was designed to investigate the influence of hypothermia on Renin-Angiotensin-Aldosterone axis function. In this study after general anaesthetize by injection of chloral hydrate (a 100cc /0.5gr body weigh), into ten male rats (albino, wistar race) were placed in hypothermia system and their body temperature were reduced to 25 °C. Angiotensin and aldosteron levels of serum were measured before and just after hypothermia, repeated every 24 hours for 3 days by radio-immunoassay. Plasma renin activity were determined by using the standard way of angiotensin I at two temperature of 4 °C and 37 °C. Based on results plasma renin activity, angiotensin and aldosterone increased immediately after hypothermia. This increment in angiotensin I and aldosterone rather than control group has significant difference. ($p < 0.03$) later all of them decreased to basal level except in aldosterone which maintained its increased level significantly for 24 hours ($p < 0.05$). Appears that medium hypothermia have stimulatory effect on renin-angiotensin-aldosterone axis activity.

19.12

Proteomic Analysis of Apical Microvillous Membranes of Syncitiothrophoblast Cells Reveals a High Degree of Similarity with Lipid Rafts

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Microvilli are highly enriched in cholesterol- and glycosphingolipid-containing membrane microdomains, also known as lipid "rafts." In addition to its lipid composition, interest has turned on the protein composition in order to define properly the role of this characteristic structure. Intrinsic variability is now becoming apparent although the data obtained to date remain controversial as the issue of distinguishing lipid-raft specific proteins from possible contaminants remain largely unresolved. However, despite the use of different experimental approaches, there is a remarkable degree of coincidence in the protein composition of lipid rafts. This coincidence is particularly interesting after attending to the heterogeneity of the sample sources. From these and other data, lipid rafts are believed to play a role in the major routes of membrane trafficking, transport of GPI-anchored proteins and glycosphingolipids to the cell surface, regulated secretion, and transport for the endosomes to the Golgi apparatus and internalization via both caveolae and chlatrin-coated pits. A plethora of specific modifications help proteins for their targeting to lipid rafts. Here we describe up to 58 proteins identified by Multidimensional nano-LC ESI-MS/MS from microvillous apical membranes purified from placental syncitiotrophoblast cells. To obtain the samples we used a non-detergent based method which allows simultaneous isolation of apical and basal membranes free of mitochondrial membranes. Among the proteins identified include membrane-bound receptors, GPI-anchored proteins, several ion-channels, proteins involved in vesicular trafficking, membrane scaffold proteins and regulatory and signal proteins. Despite the use of a non-detergent bases method to obtain the sample, most of the proteins identified here have been described as belonging to lipid rafts and some of them are used as lipid-raft markers. This job demonstrates that microvillous membranes obtained from apical membranes of placental syncitiotrophoblast cells are highly enriched in lipid rafts as the protein composition is very similar to that described for lipid-rafts.

19.13

Proteome Analysis of the Red Blood Cell; What More Can Be Learned?

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The composition of the human red blood cell (RBC), the major cellular constituent of blood, has been extensively studied over the last 40 years One main role of the RBC is to transport oxygen from the lungs to the tissues and carbon dioxide from the tissues back to the lungs, but it may be a key player in several other processes. For example, the RBCs transfer and acquire GPI-linked proteins from other cells. As the only oxygen transporter diseases compromising RBC performance, may be particularly serious, and warrant in depth studies. Such diseases are frequently linked to altered protein expression, whether by absence or the expression of abnormal protein forms. To understand such diseases accurate and sensitive techniques are required to determine the normal RBC protein profile. Recent advances in technology have provided sophisticated tools such as proteomics and microarrays that allow global approaches to the identification of proteins and the study of their expression. As mature mammalian RBCs do not posses a nucleus and are therefore not actively involved in the synthesis of new proteins, microarray methods screening for m-RNA cannot be used and proteomics becomes the method of choice to study the RBC proteins. The aim of this study was to obtain the most complete and informative human RBC proteome possible combining the use of state of the art protein identification technology (Q-star and LTQ-FT) with selected biochemical procedures for sample preparation. In the study a total of 341 membrane proteins and around 200 soluble proteins were identified and validated. A critical screening of the splice isoforms was also undertaken. An initial categorization in terms of sub-cellular localization, family and function has also been made through interrogation of available annotation databases.

19.14

Large Scale Proteomic Analysis of Human Seminal Plasma

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Large-scale proteomics has been successfully used to study complex samples, such as blood or whole subcellular structures. Here we present the most thorough analysis of human seminal plasma, based on GeLCMS (Gel enhanced Liquid Chromatography-Mass Spectrometry) experiment. To improve peptide identification, the combination of linear ion trap (LTQ) and FTICR instruments was used. It enabled the high accuracy parent ion scan and MS3 spectra analysis to be performed simultaneously, which provided incomparable data accuracy. Together with a very rigorous data analysis, it gave rise to extremely high data quality.

1 mg of material was separated on 1D SDS-PAGE, 14 gel slices were excised and subjected to in-gel trypsin digestion protocol. The LC-MS analysis was performed on LTQ-FT mass spectrometer connected to an Agilent 1100 nanoflow LC system. The mass spectrometer was operated in the data-dependent mode to enable automatic switch between MS, MS2 and MS3 acquisition.

Human seminal plasma is one of the easily available body fluids, which constituent proteins remained mostly unknown. The elucidation of its content may shed light onto processes involved in fertilization or infertility. Seminal plasma might be as well a potential source of valuable biomarkers. So far, over 500 proteins have been confidently identified, from which 73% of proteins have been assigned a subcellular localization: 33% of those have been allocated as extracellular or secreted, the rest belonging to various cellular locations. By extensive Swiss-Prot database analysis, we determined that only 11% of all proteins were previously known to be expressed in various parts of the male genital tract. This number is likely to rise during literature search. We expect that many proteins formerly known only in other compartments will be assigned a biological role as part of seminal plasma proteome. Our data proves the utility of the described approach, combining high quality identification with a relatively short time of analysis and opens new horizons for subsequent quantitative studies, the quest for biomarkers as well as characterization of many different tissue proteomes.

19.15

Seminal Plasma Levels of 15-F_{2t}-Isoprostane, Malondialdehyde, and Total Homocysteine in Normozoospermic and Asthenozoospermic Males

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It has been proposed that oxidative stress plays an important role in male infertility. The aims of this study were to compare seminal plasma levels of 15-F_{2t}-isoprostane (8-iso-$PGF_{2\alpha}$), malondialdehyde, and total (sum of free and bound) homocysteine (tHcy) from normozoospermic vs. asthenozoospermic men, and to examine the relationships between tHcy and lipid peroxidation products. The study was a case-control study with a simple random sampling. The case group was consisted of 15 asthenozoospermic males. This group was compared with 15 normozoospermic men. Seminal plasma levels of 15-F_{2t}-isoprostane and tHcy were measured using commercially available enzyme immunoassay (EIA) kits. MDA levels were determined by the thiobarbituric acid (TBA) assay. The Mann-Whitney U test was used to compare two groups. Coefficients of correlation were calculated using Spearman's correlation analysis. All hypothesis tests were two-tailed with statistical significance assessed at the p value <0.05 level. MDA levels were higher in asthenozoospermic subjects than in control subjects (0.72 ± 0.06 μM *vs.* 0.40 ± 0.06 μM; $p < 0.05$). No differences were seen in 15-F_{2t}-isoprostane levels in asthenozoospermic subjects and controls (65.00 ± 3.20 pg/ml *vs.* 58.17 ± 4.12 pg/ml; $p > 0.05$). Interestingly, tHcy levels were to be slightly higher in asthenozoospermic subjects than in controls (6.18 ± 1.17 μM *vs.* 4.8 ± 0.52 μM). Sperm motility was inversely correlated with seminal plasma 15-F_{2t}-isoprostane and MDA levels, respectively ($p < 0.05$). In summary, seminal plasma levels of 15-F_{2t}-isoprostane and tHcy showed no significant difference between normozoospermic and asthenozoospermic men. Sperm motility was not correlated with seminal plasma levels oftHcy. No relationship was found between tHcy and lipid peroxidation.

19.16

Purification and Characterization of Human Articular Chondrocyte Mitochondria for Proteomic Analysis

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Background: The chondrocyte is the only cell type present in mature cartilage, and its death contributes to the progression of osteoarthritis. Chondrocyte mitochondria play a key role in the development of this pathology, as their respiratory activity is significantly altered in osteoarthritic chondrocytes. This feature turns chondrocytic mitochondria into perfect targets to study such a disease.

Objective: We set up a method to isolate pure mitochondria from human articular chondrocytes and obtained a two-dimensional reference map of their proteins.

Methods: Chondrocytes were isolated from cartilage obtained from normal autopsy cases. Cultured cells were used to obtain total cell lysates and to purify chondrocyte mitochondria by differential centrifugation and subsequent density gradient ultracentrifugation. Western blot analyses were performed to test the purity of the samples. Mitochondrial proteins were resolved by means of two-dimensional electrophoresis (2-DE), and visualized by silver nitrate staining. For protein identification, proteins were analyzed using MALDI-TOF or MALDI-TOF/TOF mass spectrometry.

Results: The isolation method we present lead to the obtaining of around 4 μg of pure mitochondria per million chondrocytes. 2-DE separation of mitochondrial proteins allowed detecting more than 900 proteins. A hundred of them were excised and identified in order to obtain a chondrocyte mitochondrial reference map. The study lead to the identification of many mitochondrial proteins, but also of proteins either not previously characterized as mitochondrial (like Annexins 2 and 5), or classically localized in other organelles (Grp78), but to whom a role associated to mitochondrial function has been suggested.

Conclusion: The purification method carried out in this work is valuable for mitochondrial proteomic analysis in chondrocytes. Moreover, the reference map obtained will serve as a basis for future studies of mitochondrial alterations in osteoarthritis.

19.17

Proteomics of Murine Prostate Secretion

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The role of androgens in the growth and differentiation of the rodent ventral prostate gland is well known. More recently we have found that estrogen receptor beta (ERβ) is also involved in terminal differentiation of the prostate gland and in the regulation of androgen receptor (AR) levels. In ERβ−/− mice the prostate epithelium is not fully differentiated, AR levels are high and the proliferation index is higher than in wild type (WT) mice. Poor differentiation of the prostatic epithelium is expected to affect prostatic secretion. In the present study we are characterizing the proteins in prostatic secretion in wild type and ERβ−/− mice by 2D-gelelectrophoresis in order to clarify the role of ERβ in prostate secretion. One of the proteins whose level was found to be reduced in the secretion from ERβ−/− mice is calreticulin, also known as a complement receptor. Calreticulin is an androgen regulated gene. So its reduced level in prostate secretion when androgen levels are high was unexpected and seemed to indicate that the lack of ERβ was influencing its expression. We have, therefore, used immunohistochemical techniques to study the role of ERβ in calreticulin expression. We found that calreticulin is indeed regulated by ERβ in the prostate but also in the cardiovascular system, the central nervous system, and the immune system and can be used as a marker for ERβ signaling during development of these tissues.

19.18

Identification of Crystallin Family Proteins in Endotoxin-induced Uveitis (EIU) Rat Vitreous Body; Implication of Crystallin Truncation in EIU Pathogenesis

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Crystallin is a major protein of the mammalian lens in most species. Crystallin sequence has a homology with the small heat shock protein family. Like many other heat shock proteins, crystallin exhibits chaperone-like activity, including the ability to prevent the precipitation of denatured proteins and to increase cellular tolerance to stress. Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation. To characterize the mechanism of the EIU, we have analyzed the expression level of proteins in vitreous body from EIU-induced rat and normal rat by using two dimensional electrophoresis (2-DE) and micro LC/LC-MS/MS. Thirty-three spots were observed as the increased molecules over 3-fold in uveitic vitreous compared to normal group. Fourteen spots of them were turned out to be the crystallin family. While others, the chain B-crystallin was decreased in the uveitic vitreous. While the truncated form of crystallin appeared predominantly in normal vitreous, there was no truncated form of crystallin in LPS-injected EIU. These results suggest that the crystallin family proteins are major group in uveitic inflammation and further C-terminal truncation of crystallin may play a role in EIU-related disease progression.

19.19

Proteomic Analysis of *in Vitro* Differentiating Cytotrophoblasts

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Human placenta is a transient organ, which forms the feto-maternal interface during pregnancy. The outer layer of the placental villi which are free floating in the maternal blood is formed by a multinuclear syncytium, the syncytiotrophoblast. It is assumed that this cell layer plays a pivotal role in the control of diaplacental transport, placental hormone production and metabolism. Syncytiotrophoblast are formed *in vivo* by fusion and differentiation of cytotrophoblasts. These progenitor cells can be isolated and cultured showing the differentiation *in vitro.*

As several trophoblast related pregnancy diseases are suggested to be related to insufficiency of cytotrophoblast differentiation we addressed the changes of intracellular protein pattern during this physiological process. We isolated cytotrophoblasts from human term placentas and cultured them. Purity of the isolate was controlled by immunofluorescence staining. *In vitro* differentiation of cytotrophoblasts was controlled by light microscopy showing the formation of a multinuclear syncytium in culture. Quantification of the pregnancy hormone, β-human choriogonadotropine (β-hCG), in the culture supernatant was used as a biochemical marker of differentiation and showed the expected increase. Initially, interindividual variability of the intracellular protein pattern of freshly isolated cytotrophoblasts was determined by comparative profiling of silver stained 2-DE gels. Analysis of different placentas revealed a high similarity. Thus, subsequently differential in gel electrophoresis applying Cy-dyes (DIGE) was carried out to examine the differentiation associated changes in the protein profile. Differentially expressed proteins that were identified by mass spectrometry include cytoskeleton proteins and proteins involved in transcytotic pathways, gylcolysis and purine metabolism. The implication of the changes the in the protein profile will be discussed.

19.20

Proteomic Study of Phenotypic Variability in a Murine Model of Osteogenesis Imperfecta

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Osteogenesis Imperfecta (OI), also known as "brittle bone disease," is a dominant negative disorder of connective tissue caused by mutations in the alpha chains of type I collagen, the major structural protein of the extracellular matrix of bone, skin and tendon. The typical clinical features of this pathology are bone fragility and skeletal deformities, within a broad phenotypic range. A more detailed relationship between genotype and phenotype in OI is still incompletely understood. The development of transgenic technology in recent decades made possible to create murine models for many human disorders. Brittle IV mouse is a knock-in murine model for Osteogenesis Imperfecta reproducing molecular defect, type of transmission and phenotypic variability reported for OI human patients. In presence of the same Gly349Cys substitution in one col1a1 allele, BrtIIV mice show both the lethal (70%) and the moderately severe (30%) phenotype. In order to obtain a better knowledge of this variability and OI pathophysiology, we performed proteomic analysis. Proteins from skin biopsies of wild-type, mutant lethal and mutant alive mice were extracted and separated by high-resolution 2D electrophoresis and three reference electropherograms were acquired. Computer analysis of the 2DE protein patterns revealed the presence of several reproducible qualitative and quantitative differences. The identification of protein spots differentially expressed in skin maps was carried out by MALDI-ToF mass spectrometry. These data may be considered a further step toward the understanding of the relationship between collagen mutations and clinical outcome of OI.

19.21

Subproteomic Identification of New Metal-Protein Interactions in Human Antigen Presenting Cells (APC) Reveals a Potential Link to Metal-specific Immune Responses in Human Nickel Allergy

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Nickel (Ni) represents the most common occupational as well as public contact allergen, causing allergic reactions in about 10–15% of the human population. However, the molecular/proteomic events underlying this disease still have to be elucidated. Moreover Ni has been shown to be toxic and cancerogeneic. Low molecular weight agents, such as Ni, cobalt and copper, are non-antigenic in the free state (hapten), but have the potential to interact with proteins (metal-protein-interactions) in a way that renders them antigenic. We have previously demonstrated that such Ni-protein-interactions involving human serum albumin (HSA) lead to functional, Ni-specific human T cell clone activation. T cell receptor (TCR) - transfected cell lines were also activated in a Ni-dependent and HLA-restricted manner by such HSA-Ni metalloprotein-complexes (Thierse, H. J., *et al.*, 2004, *J. Immunol.* 172.1926). With the aim of identifying unknown cellular Ni-protein interactions in human blood-derived professional antigen presenting cells (APC), we used in vitro generated human dendritic cells (DC), as a model system for a metal-specific subproteomic approach (metalloproteome). Results were compared to Ni-interacting proteins in human B cells, which have just recently been described by our laboratory (Heiss, K., *et al.*, *Proteomics*, in press).

Methods: Ni-protein interactions were detected via Ni-NTA-enrichment, 2-D electrophoresis, mass spectrometry and database analysis. If possible, data were confirmed by Western blotting, graphite furnace atomic absorption spectrometry and/or Biacore analysis.

Conclusions: In DCs 32 out of 57 isolated Ni-interacting proteins were identified. Comparative subproteomic analysis of both cell types revealed differential Ni-interacting molecules in B cells and *in vitro* generated human DCs. Among others, several chaperones/heat shock proteins were detected, which may be involved in Ni-epitope presentation and/or cellular stress responses towards heavy metal Ni. Functional understanding of these metal-protein interactions potentially helps to elucidate the development and pathophysiology of T cell-mediated human nickel allergy and/or processes like Ni-induced cell toxicity.

19.22

Proteomics of Human Sputum

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Several techniques are currently available to explore the distal airways. Induction of sputum with hypertonic saline and bronchoalveolar lavage (BAL) with isotonic saline performed during fiberoptic bronchoscopy open the way to evaluate the lung proteome. While the BAL fluid investigates the alveolar compartment, the sputum is more related to the the tracheobronchial tree. Spontaneous sputum is a sign of disease and indicates excessive production and retention, which occurs in patients with respiratory infection, bronchitis, asthma and cystic fibrosis. BAL fluid (BALF) and sputum contain cells (lymphocytes, neutrophils, eosinophils, . . .) and a wide variety of soluble compounds (proteins, lipids, . . .) from the respiratory tract secretions. These last 10 years, in contrast with the sputum, BALF proteome has been extensively investigated. In this work, we studied the proteome in supernatants of induced sputum, obtained in normal subjects. Inter-day, intra-subject and inter-subject reproducibility of the method are evaluated. BALF and sputum proteomes show a high degree of similarity except for some proteins that are currently under investigation. Moreover, the analysis of the sputum proteome was used to evaluate the inflammatory response to inhaled lipopolyssacharide (LPS) in healthy volunteers. Firstly, an increase of the total protein level similar to the modification of cell contents was observed after 7h and 24h following LPS inhalation. Secondly, preliminary results show an increase in the content of small proteins such as calgranulin B, albumin fragments . . .

19.23

Human Salivary Proteome

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Human saliva contains proteins that potentially can be used as disease biomarkers. Several human salivary proteomic analyses have been published using mass spectrometry. In this study, we compiled salivary proteins from the literature and constructed the first non-redundant human salivary proteome (HSP). The initial HSP contained a total of 680 proteins which were referenced by accession numbers (AC) obtained from several protein databases. To standardize the protein identifier, the HSP proteins were queried against the International Protein Index (IPI) database. AC of the IPI was selected and used as the unique identifier if the protein from IPI was cross referenced to the AC of the query or its sequence has >95% identity over 75% of the query sequence length based on the BLAST result. Redundancy was eliminated first by finding the unique ACs and then by clustering those sharing >95% sequence identity over 95% of the sequence length. The remaining 339 proteins formed the nonredundant list. The nonredundant HSP was then characterized based on Gene Ontology (GO) annotations and compared with the human plasma proteome (HPP; www.bioinformatics.med.umich.edu/hupo/ppp/; www.ebi.ac.uk/pride). In GO molecular functional categories, HSP and HPP possess similar proportions of transporter, catalytic, protein binding and structural molecule activities (< 50% difference), but HSP demonstrates enhanced activities in calcium ion binding and antioxidant activity (>1.5 fold). In biological process categories, proportions in cellular metabolism, signal transduction, transport and development are similar between HSP and HPP. Interestingly, proportions in regulation of enzyme activity, response to stimulus and homeostasis are enhanced in HSP. These results may reflect the known functions of saliva and provide a foundation for future salivary proteomics studies.

19.24

Proteome of Normal Human Kidney Glomerulus; Database, Differential Expression, and 2-DE Profiling of Glomeruli Isolated from Biopsy Tissues

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We have analyzed the glomerulus of normal human kidney by 2-DE and identification through MALDI-TOF MS and/or LC-MS/MS. Glomeruli were highly purified from kidney cortices with normal appearance obtained from patients under surgical nephrectomy due to renal tumor. Glomerular proteins were separated by a large format 2-DE, silver-stained, and valid spots were processed for identification by MS. In a synthetic gel image constructed from 2-DE gels of 4 subjects with no apparent pathological manifestation, 1,713 valid spots were detected, of which 1,559 spots were commonly observed in all the 2-DE gels. Among the 1,559 protein spots, 327 protein spots, representing 212 proteins, were so far identified. Although most of proteins identified include cell structural proteins (50), metabolic enzymes for energy (42), and protein metabolism (30), significant number of proteins implicated in signal transduction (25), cell cycle and proliferation (10), stress response (13), and cell adhesion (7) were also identified. As a separate study intended to complement the proteomic analysis of the glomerulus, we have also analyzed differential protein expression in the glomerulus, cortex and medulla in the normal human kidney by using 2-DE and MS analysis. All the data obtained in this study and more knowledgeable information relevant to identified proteins obtained through further search of databases in public domains are converged to construct an XML-based database, which is deposited on an Web site accessible to researchers. In addition, we have attempted to separate a minimal amount of proteins of glomeluli isolated from biopsy tissues on large format 2-DE gels by exploiting extremely sensitive fluorescent dyes (Cy3- and Cy-5 saturation dye). Only 5 to 10 microgram of glomerular proteins gave highly resolved 2-DE profiles suggesting the applicability of 2-DE to analyze glomerular proteome in biopsy specimens.