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GLYCO 23
XXIII International Symposium on Glycoconjugates

Abstracts

September 15–20, 2015

Split, Croatia

Dear Friends,

Welcome to Glyco XXIII, the 23rd congress of the International Glycoconjugate Organization (IGO). Life on our planet is based on glycoconjugates and glycans are essential structural and functional elements of all living cells. However, due to their structural complexity and intricate biological roles, glycans are still ignored by the majority of "mainstream" science; therefore the knowledge about glycans is lagging significantly behind the knowledge about DNA and proteins.

The situation is now starting to change, since the progress in methods and technologies to synthesize, analyze and manipulate glycans enable glycoscience to reach out to other disciplines and provide them with tools to deepen our understanding of the world around us. As the largest glycoscience conference in 2015, Glyco XXIII will be an excellent opportunity for glycoscientists to exchange experience and ideas with colleagues from all around the world.

We glycoscientists know that glycans are important for nearly all biological processes, but we still have a long road ahead of us to make people studying these biological processes aware that studying glycans is important for their work. Therefore, in addition to "classical Glyco-sessions" and speakers who are experts in glycoscience, Glyco XXIII will host a number of sessions and speakers focused on different diseases and cellular processes in which glycans play an important role. Some of the speakers in these sections are not experts in glycoscience, but after Glyco XXIII, perhaps they decide to initiate some crossbreeding between glycoscience and their fields of science.

At Glyco XXIII we will continue the tradition of hosting a guest session of the Society for Glycobiology (SFG) and, for the first time, also host a guest session of the Asian Community for Glycoscience and Glycobiotechnology (ACGG). Another good tradition, the Young Glycoscientists' Symposium, will also be continued and will be intertwined with the main conference. As an experiment, we will also organize a pre-symposium training course "Introduction to Glycoscience" with introductory lectures by leading experts in the field. We hope that these lectures will help our young colleagues, but also experienced researchers from other disciplines, to better understand the dynamic and rapidly evolving field of glycoscience.

Our congress venue, the beautiful Le Meridien Lav hotel in Split got numerous awards, including the award for the best congress hotel in Europe, and I hope we are all looking forward to inspiring and enjoying week of glycoscience in Croatia.

Gordan Lauc
Chairman of Glyco XXIII
President Elect of IGO

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International Glycoconjugate Organisation Award 2015

Prof. Taroh KINOSHITA

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Date of birth: October 15, 1951
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Education

1970-1972	Department of Liberal Arts, University of Tokyo, Tokyo, Japan
1972-1974	Department of Biology, Faculty of Agriculture, University of Tokyo, B. Agr. 1974
1974-1977	Department of Biology, Faculty of Agriculture, University of Tokyo M. Agr. 1977
1977-1981	Department of Bacteriology, Osaka University Medical School, Osaka Japan Ph. D. 1981

Professional Experience

April, 1981-January, 1982	Postdoctoral Fellow, supported by a postdoctoral fellowship from the JapanSociety for the Promotion Science
February-August, 1982	Research Associate, Department of Bacteriology, Osaka University Medical School, Osaka, Japan
September, 1982-August, 1985	Postdoctoral Research Associate, Department of Pathology, New York University School of Medicine, New York NY
September, 1985-March, 1988	Research Associate, Department of Bacteriology, Osaka University Medical School, Osaka, Japan
April, 1988-October, 1990	Assistant Professor, Department of Bacteriology, Osaka University Medical School, Osaka, Japan
October, 1990-present	Professor, Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Osaka Japan
July, 1998-October, 2003	Director, Genome Information Research Center, Osaka University
October, 2003-October, 2007	Director, Research Institute for Microbial Diseases, Osaka University
October, 2007-present	Professor, Laboratory of Immunoglycobiology, WPI Immunology Frontier Research Center, Osaka University

Professional societies
The Japanese Society for Immunology
The Japanese Society of Bacteriology
The Japanese Biochemical Society
The International Complement Society
American Society for Biochemistry and Molecular Biology

Honors and Awards
19th Osaka Science Prize (2001)
The Commendation for Science and Technology by the Minister of Education, Culture, Sports, Science and Technology of Japan (2010)

Career highlights
During undergraduate course and Master course in the Faculty of Agriculture, The University of Tokyo, Tokyo, Japan, I studied Entomology, particularly Insect Pathology and self-defense system of insects.

For PhD course, I moved to Osaka University Medical School and started studies on mammalian immune system, especially complement system. Under the direction of late Professor Kozo Inoue, I was engaged in biochemical and functional studies on complement components C5 through C9 and their complexes. I purified and characterized C5 and C8 of guinea pig complement, and analyzed how membrane attack complex of complement, C5b-9, is formed and interacts with lipid bilayer.

After completion of PhD study in 1981 and further 1-year appointment as a post-doctoral research associate in Osaka University, I did 3-year post-doctoral research in the Department of Pathology, New York University Medical School. In the laboratory of Dr. Victor Nussenzweig, I took two projects. One was to identify and characterize complement receptors in mouse system. I was able to identify mouse complement receptor type 1 for the first time. The other was on a complement regulatory protein, decay accelerating factor (DAF). DAF was thought to be a critical protein involved in host defense from own complement. I succeeded to generate monoclonal antibodies to DAF and by using them demonstrated that blood cells from patients with paroxysmal nocturnal hemoglobinuria (PNH) consist of DAF-positive normal cells and DAF-deficient affected cells, and that the latter population is highly sensitive to complement. This showed the critical role of DAF in self-nonself discrimination in the complement system.

From 1985 to 1990, as a Research Associate and then an Assistant Professor in Osaka University Medical School, I continued research on mouse complement receptors. Major achievements in this area were 1) identification of mouse complement receptor type 2 and clarification of its relationship to type 1 receptor, and 2) demonstration that mouse complement receptor types 1 and 2 are important for antibody response to protein antigens. I also studied how C5 convertase of complement is generated. I found that within the trimolecular enzyme complex C4b2a3b, C3b is covalently-linked via an ester bond to the side chain of specific serine of C4b and that the C4b-C3b covalent adduct acts as a high affinity binding site of C5.

From 1990 to date, as a Professor in the Research Institute for Microbial Diseases and WPI Immunology Frontier Research Center, Osaka University, I have been studying biosynthesis of glycosylphosphatidylinositol (GPI)-anchor and molecular basis of PNH, an acquired GPI-deficiency. I have also been studying GPI-biosynthesis genes in parasite, such as African trypanosomes. Followings are my major contributions.

1) I started a project to clone and characterize all the genes involved in GPI biosynthesis and post-translational attachment of GPI to proteins in mammalian cells. Our major approach has been expression cloning using GPI-deficient cell lines. In 1993, we first cloned PIG-A that is required for the initial step in GPI biosynthesis. Since then, we have cloned some 20 genes, such as PIG-B, PIG-C etc., that are involved in the pathway, nearly completing the project.

2) We demonstrated that mutation in PIG-A gene causes deficiency of GPI-anchored proteins, such as DAF, in PNH. We also showed that PIG-A mutation occurs somatically in hematopoietic stem cell, clarifying acquired nature of the disease and why only blood cells are affected. We further showed that PIG-A is X-linked and hence one hit in PIG-A (active allele of PIG-A in case of female stem cells) causes GPI-deficiency in both male and female.

3) We recently started a project that aims to identify genes involved in events that occur after attachment of GPI-anchor to proteins. GPI-anchored proteins generated in the endoplasmic reticulum are transported to the cell surface where they are concentrated in lipid microdomains. During the transport, GPI-anchored proteins are further modified structurally. We cloned and characterized four genes, PGAP1, PGAP2 and PGAP3, involved in lipid remodeling of GPI, and PGAP5 involved in glycan remodeling.

4) Since major cell surface proteins of protozoan parasites are GPI-anchored, GPI biosynthesis is essential for growth or critical for survival in the host. We cloned a GPI biosynthesis gene from Trypanosomabrucei for the first time and by gene knockout experiments showed that GPI is essential for growth of bloodstream form that causes sleeping sickness. We also showed that GPI is not essential for the insect stage parasite in vitro whereas GPI-anchored transsialidase, which is used to take up sialic acids from host sialoglycoconjugates, is essential for infection in tsetse fly.

The IGO Young Glycoscientist Award 2015

Prof. Xing CHEN

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Education

2007 Ph.D. in Chemistry, University of California, Berkeley
Advisors: Prof. Carolyn R. Bertozzi and Prof. Alex Zettl
2002 B.S. in Chemistry, Tsinghua University
Advisor: Prof. Yadong Li

Professional Experience

2011-present Principal Investigator, Synthetic and Functional Biomolecules Center, Peking University
2011-present Principal Investigator, Peking-Tsinghua Center for Life Sciences
2010-present Principal Investigator, Department of Chemical Biology, College of Chemistry and Molecular Engineering, Peking University
2007-2010 LSRF Postdoctoral Research Fellow, Immune Disease Institute, Harvard Medical School *Advisor: Prof. Timothy A. Springer*

Honors and Awards

2014 WuXi AppTech Life Science and Chemistry Award
2014 National Science Fund for Distinguished Young Scholars
2013 Chinese Chemical Society Prize for Young Scientists
2013 DuPont Young Professor Award
2012 SCOPUS Young Researcher Award
2009 Pfizer Fellow of the Life Science Research Foundation
2007 Chinese Government Award for Outstanding Self-financed Students Abroad
2006 Material Research Society Graduate Student Gold Medal Award

Publications

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TRAVEL AWARDS

Kathirvel Alagesan (Germany)
Fausto Almeida (USA)
Elisa Benedetti (Germany)
Joana Cabral (Ireland)
Samanta Cajic (Germany)
Elena Chiricozzi (Italy)
Ana Dias (Portugal)
Viktoria Dotz (The Nederlands)
Catarina Gomes (Portugal)
Imtiaj Hasan (Japan)
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Nari Seo (Korea)
Lan Wang (China)
Alexandra Wittmann (UK)
Linlin Yang (Germany)

Plenary Lectures

1. Glycan customization for optimizing biopharmaceutical properties

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Glycosylation is the predominant type of post-translational modification on biopharmaceutical proteins, with over 60 % of such molecules carrying either *N*-glycans, *O*-glycans, or both. Glycosylation is an inherent consequence of the need to express these proteins in eukaryotic cells. However, the attachment of glycans is not function-neutral, as these often large modifications can strongly modulate protein stability, pharmacokinetics and pharmacodynamics. Glycan macro- and microheterogeneity is an undesirable feature of the glycosylation process from the point of view of manufacturing downstream processing and product characterization. Over the past 10–15 years, biotechnologists have learned how to ply the glycosylation modifications of some of the most frequently used expression host cells (mammalian cells and yeasts in particular) to impart particularly desired properties onto biopharmaceuticals. I will provide an introduction to the achievements in this field of glycobiology, and will introduce our work on yeast glyco-engineering for the purpose of producing enzymes for lysosomal storage diseases. I will also present recent work that is geared towards effectively solving the issue of mammalian and yeast cell glycan heterogeneity and plant glycan immunogenicity. This 'GlycoDelete' technology allows for the production of glycoproteins that require *N*-glycosylation in the endoplasmic reticulum for proper folding, but in which heterogenous Golgi modifications are detrimental to the proteins' homogeneity or function.

2. Identification of NLS of *O*-GlcNAc transferase and its translocation into nucleus

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It has been reported that one of the downstream molecules generated from glucose via the hexosamine biosynthetic pathway (HBP) is uridine diphosphate-*N*-acetly glucosamine (UDP-GlcNAc). The dynamic cycle of addition and removal of *O*-linked-*N*-acetylglucosamine (*O*-GlcNAc) to Ser/Thr residues is involved in regulating nuclear and cytoplasmic proteins. Nucleocytoplasmic *O*-GlcNAc transferase (ncOGT) adds a single GlcNAc onto hydroxyl groups of serine and threonine residues. Interestingly, ncOGT dynamically modifies its target proteins in both cytoplasm and nucleus. For this reason, ncOGT has to be existed in these two compartments at the same time. The localization of ncOGT is important because it is related to the its substrate specificity, however, the mechanism of how this enzyme is sequestered in the cytoplasm and imported to the nucleus is not clear. Another interesting factor regarding ncOGT is that it is also modified by *O*-GlcNAc. Although it has been reported before, it is not known yet where the modification sites are and what their exact functions are. In this research, our aims are to answer these questions. First, we identified specific nuclear localization signal (NLS) in *O*-GlcNAc transferase that is required for nuclear transport. Also, we show that ncOGT binds importin α protein. Using ESI-Q-TOF mass spectrometry and site-direct mutagenesis we found two *O*-GlcNAc modification sites.

3. The structure and organisation of the glycan shield of HIV

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The envelope spike of HIV is highly glycosylated. These glycans help the virus evade neutralization by host antibodies. Paradoxically, however, they can also be targeted by broadly neutralizing antibodies that can develop over time in infected individuals. We wish to understand the structure of the glycan shield of HIV in order to help guide the design and production of vaccine candidates. Our focus has been to probe the glycosylation of HIV from viruses and determine which of the emerging recombinant mimics of the envelope spike most closely reproduce virion glycosylation. Using these recombinant models, we are also able to probe how the close packing of virion glycans limits the normal cellular glycan processing reactions and leads to a distinctive network of under-processed oligomannose-type glycans. Investigation into this so-called mannose patch indicates that it is notably unperturbed by changes in the configuration of the glycan shield and is prevalent across HIV strains. The conservation of the mannose patch renders it an attractive target in HIV vaccine design. Acknowledgements: My HIV research is supported by grants from the International AIDS Vaccine Initiative Neutralizing Antibody Center CAVD grant and the Scripps CHAVI-ID. The work described in this talk is very much a team effort and I am grateful to colleagues in

Oxford and collaborators worldwide for their significant contributions.

4. High sensitivity glycomics: windows to glycan function

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In partnership with bioscience collaborators worldwide, our laboratory specialises in the development and exploitation of high sensitivity mass spectrometry in the field of glycobiology with the aim of establishing the roles glycopolymers play in health and disease. Much of glycobiology research seeks to understand how glycans on glycoproteins and glycolipids engage with glycan-binding proteins to mediate adhesive and signalling events. Such recognition is central to cell-cell communication including interactions between mammalian eggs and sperm, between pathogens, parasites and their hosts, between cells of the immune system, and between the microbiome and the gastro-intestinal tract. With the help of examples from our collaborative research in glycoimmunology, reproductive glycobiology and infection, this talk will illustrate how high sensitivity glycomics can provide insights into some of these biological processes. Acknowledgements: This work is supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust. We are grateful to our students and postdoctoral scientists without whom this research would not be possible.

5. Novel functions and mechanisms of complex carbohydrates elucidated by glycosyltransferase gene knockout

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Carbohydrates on proteins and lipids have been considered to be involved in the modification of functions of complex carbohydrates. Genetic alteration of complex carbohydrates using experimental animals has been a powerful approach to clearly elucidate roles of glycosylations particularly in our bodies. In this lecture, novel functions and molecular mechanisms by which carbohydrates regulate cellular signals and tissue/organ functions will be introduced. First of all, we have analyzed roles of sialic acid-containing glycosphingolipids, gangliosides by establishing knockout (KO) mice of various glycosyltransferase genes, and have demonstrated that gangliosides are involved in the maintenance of the integrity of nervous tissues and in the regeneration of damaged nerves. These functions seem to be performed via the regulation of architecture of membrane microdomains, lipid rafts. Destructive changes in the lipid rafts induce inflammatory reaction such as complement activation and secretion of inflammatory cytokines in the central nervous tissues. These inflammatory reactions lead neurodegeneration as shown in Alzheimer's disease. Function of a neutral glycosphingolipid, globotetraosylceramide (Gb4) has been long not understood. Analysis of Gb3 synthase (A4galt) KO mice revealed that Gb4 is involved in the protection from cytotoxic effects of LPS by forming a complex with LPS receptor, TLR4/MD-2 complex. The binding of Gb4 to TLR4/MD-2 was specific for the carbohydrate structure. Furthermore, only ceramides consisiting of saturated forms of fatty acids could enable Gb4 to form a complex with TLR4/MD-2, supporting that this interaction takes place in lipid rafts. Generally, all these results suggest that glycosphingolipids play roles in the regulation of inflammation. The implication of sialylation in the *N*-glycan on IgG in the regulation of inflammation will be also introduced in the same line.

6. A little sugar goes a long way: *O*-GlcNAc and epigenetics

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O-GlcNAcylation is a nutrient-driven modification linked to cellular signalling and epigenetic regulation of gene expression. Utilizing precursors derived from metabolic flux, *O*-GlcNAc is uniquely poised as a homeostatic signalling cassette. The enzymes of *O*-GlcNAc cycling, OGT and *O*-GlcNAcase act in consort with a host epigenetic 'writers' and 'erasers' including kinases, methyltransferases, acetyltransferases, and the DNA-acting TET proteins. OGT acts preferentially on intrinsically disordered protein domains that are abundant in the nucleus. One of the key epigenetic effectors upon which *O*-GlcNAc acts is the carboxyl-terminal domain (CTD) of RNA Pol II. Thus, *O*-GlcNAc is part of the complex 'CTD-code'. By integrating nutrient information and communicating with the Histone and CTD codes, *O*-GlcNAc cycling provides a direct link between cellular metabolic status and the transcriptional machinery. *O*-GlcNAc cycling is also important for coordinating the interaction between the

nucleus and mitochondria. We used genetic and biochemical approaches in model organisms to model how perturbations in *O*-GlcNAc cycling may influence human physiology and disease. In *Drosophila*, we showed that *O*-GlcNAc cycling plays a critical role in glucose-insulin homeostasis and nutrient-dependent oogenesis. Null alleles of OGT and OGA are viable in *C. elegans* providing a unique genetic model for studying the physiological roles of *O*-GlcNAc. Cre recombinase-based targeted conditional disruption of mouse *O*-GlcNAcase results in defective metabolic homeostasis linked to obesity and insulin resistance. The mice show deficits in innate and cellular immunity as well as defects in neural development and clasping phenotypes indicative of neurodegeneration. These findings solidify a role for the *O*-GlcNAcase in metabolic homeostasis, immunity, and neurodegeneration providing a new model for studying the role of *O*-GlcNAc in disease.

7. Overlapping mechanisms of sepsis, diabetes and inflammatory bowel disease

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N-glycosylation is the major form of modification detected among circulating proteins in the blood. Previous and recent findings have increasingly linked *N*-glycan structures to the control of protein function by altering glycoprotein homeostasis in multiple cell and organ systems of mammals. Protein half–lives and turnover contribute substantially to the composition and functions of the blood proteome. An intrinsic mechanism that controls the homeostasis of hundreds of blood proteins each with different half-lives has not been identified previously. The repertoire of blood proteins is structurally diverse however most are post-translationally modified with identical saccharide sequences at the termini of multi-antennary *N*-glycans. We have identified multiple endogenous circulating glycosidases that progressively remodel the *N*-glycan linkages of aging blood proteins. The basal rate of *N*-glycan remodeling was indicative of the different half-lives of distinct proteins, while glycosidase inhibition blocked *N*-glycan remodeling and elevated blood protein half-lives and abundance. These findings reflected the participation of endocytic lectin receptors that distinguished multivalent *N*-glycan ligands sequentially unmasked during protein aging by multiple glycosidase activities. We investigated various lectin receptor deficiencies and observed the accumulation of aged blood proteins bearing remodeled *N*-glycans of the corresponding lectin binding specificities. This intrinsic multi-factorial mechanism of secreted protein senescence and turnover operates among hundreds of plasma glycoproteins that contribute to health and disease. These and other studies have revealed that the control of *N*-glycan structures by various means including diet, genetic background, and metabolism contributes to homeostatic disruptions of glycoprotein abundance and function that can trigger diseases including diabetes, the lethal complications of sepsis, and inflammatory bowel disease.

8. Glycoscience and the systems biology of cancer

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Rapid advances in all –omics analytical technologies have enabled a more detailed understanding of the biological systems that are disrupted in cancer. This provides a better overview based on Systems Biology in which the aims to link the consequences of many molecular changes on disease pathogenesis. In addition to changes in genes, proteins, metabolites and so on, glycosylation pathways are frequently altered in tumour cells as a result of mutations and changes in the organization of cellular organelles. Tumor cells can also secrete cytokines that alter the transcription levels of glycosylating enzymes in remote tissues. Particular glycosylation changes, such as the presentation of SLex, support the metastasis of tumour cells that can alter their cell surface glycosylation to present these structures. Monitoring glycosylation changes in patients provides potential markers for patient stratification that often outperform existing markers and, in particular, give insights into the pathways involved in disease, linking the genome and transcriptome to the proteome, glycome and epigenetic modifications. Automated detailed quantitative *N*-glycan analysis using a robotic HPLC based platform coupled with computer assisted data interpretation now enables high throughput analysis of samples in 96/384-well plates. In addition to suggesting potential clinical markers, analysis of the serum glycome and the glycosylation of specific glycoproteins provides valuable insights into disease processes.

9. Glycoengineering cell migration: achieving the promise of cellular therapeutics

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The success of all cell-based therapeutics depends critically on the ability to deliver the pertinent cells to sites where they are

needed. Direct injection of cells into tissues has limited applicability and can itself cause tissue injury, prompting strategies to optimize vascular delivery of cells into target tissues. E-selectin, a molecule only found on mammalian endothelial cells, binds sialofucosylated lactosaminyl glycans such as the tetrasaccharide called "sLex". E-selectin is expressed constitutively on microvascular endothelial cells of bone marrow and skin, however, inflammatory cytokines (such as TNF and IL-1) markedly induce E-selectin expression on microvascular endothelial cells at all sites of tissue injury. Binding of E-selectin to its sialofucosylated ligands displayed on circulating cells initiates shear-resistant adhesive interactions between cells in blood flow and endothelium, the key first step in recruitment of circulating cells to any target tissue. The most potent E-selectin ligand expressed on mammalian cells is a molecule known as "Hematopoietic Cell E-/L-selectin Ligand" (HCELL). HCELL is a specialized sialofucosylated glycoform of a cell surface molecule called "CD44". CD44 is a transmembrane glycoprotein that is expressed at high levels on essentially all mammalian cells, but the HCELL glycoform is natively found only on hematopoietic stem cells. We have developed a glycoengineering platform technology called "Glycosyltransferase-Programmed Stereosubstitution" (GPS) for custom-modifying CD44 glycans to create HCELL on the surface of living cells. Data from preclinical studies have shown that systemic infusion of $HCELL^{+}$ cells into mice with inflammatory conditions augments cell infiltrates into affected tissues, with profound tissue-restoring effects. These studies highlight the utility of GPS-based cell surface glycan engineering to, literally, navigate cell migration toward achieving the promise of cellular therapeutics.

10. Integrated omic studies for common complex traits & personalized medicine

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We are using a large-scale multi-omic study of 5,000 adult twins to explore the potential of omics in personalized medicine. The TwinsUK resource contains the most studied individuals on the planet with all subjects having over 3000 phenotypes and GWAS SNP chips. Over 3000 now have whole genome sequencing at 6–30 X depth. The omics being explored includes sub-studies used to explore the basic heritability, QTLs and age relationships as well as disease associations. This includes (EpiTWIN) www.epitwin.eu using whole genome methylation sequencing and 450 k for epigenetics, which also uses the discordant twin design – using for replication 30,000 twins from the Discotwin European consortium. The same individuals are tested using an integrated omics approach including—the Multiple Human Tissue Expression Resource (MuTHER) and with RNA array and sequencing in three tissues. In addition we have analysed 1000 blood metabolites using metabolomics (Mass Spec and NMR), >100 glycomics markers (IgG and total plasma), immunophenotyping (with 78,000 White cell sub-types) and gut microbiomes (16 s and metagenomics) on the same twins. Most subjects are being followed longitudinally to assess prediction of incident disease. Examples of recent integrated Omic analyses include prediction of age, pain sensitivity, renal function, hypertension, cancer, depression, obesity and diabetes. Longitudinal twin studies with global collaborations are the ideal resource to study the potential of personalized medicine.

11. Glycosylation: chemical approach to disease biology

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Biological glycosylation is one of the most complex biological processes and is known to modulate the structure and activity of proteins, lipids and natural products. Development of new tools and methods for use to identify unique carbohydrate markers exclusively expressed on disease cells and to understand the roles of carbohydrates in disease progression may provide new solutions to the unsolved disease problem. This lecture will describe our efforts in this regard with focus on the development of carbohydrate-based vaccines in combination with the use of designed glycolipid adjuvants to induce a class switch, thereby overcoming the common problem of carbohydrate-based vaccines.

IGO Award Lectures

12. Chemical tools for probing glycosylation dynamics *in vivo*

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Glycosylation plays a key role in mediating molecular recognition, development, and cell signaling. Intriguingly, cellular glycans are highly dynamic in various physiological and pathological processes. Our research group works on developing

chemical tools to label, visualize, and modulate glycosylation in living systems. Here, we present the development of a liposome-based strategy for cell-selective metabolic labeling of glycans and a FRET-based method for protein-specific imaging of cell-surface glycans. Furthermore, we have applied those chemical tools to dissect the glycosylation changes during cardiac hypertrophy and tumor progression. Finally, we are interested in developing new imaging modalities other than the more conventional fluorescence microscopy for glycan visualization. A recently developed bioorthogoal Raman imaging technique will be discussed.

13. Glycosylphosphatidylinositol-anchored proteins: biosynthesis, transport, shedding and deficiencies

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Many cell surface proteins are anchored to the outer leaflet of plasma membrane via glycosylphosphatidylinositol (GPI). GPI is synthesized in the endoplasmic reticulum (ER) from diacylphosphatidylinositol (PI) through sequential reactions. In mammalian cells, GPI biosynthetic pathway consists of 11 steps including four mediated by glycosyltransferases. In step 5, diacylglycerol moiety is remodeled to diradylglycerol, of which 1-alkyl-2-acyl glycerol is the major form. The complete GPI precursor is attached to proteins bearing a carboxyl-terminal GPI attachment signal peptide by GPI transamidase, an ER-resident enzyme complex, which replaces the GPI attachment signal peptide with GPI. Both lipid and glycan moieties of the nascent GPI-anchored proteins (GPI-APs) are then remodeled, generating GPI-APs competent for cargo receptor binding and subsequent packaging into COPII coated vesicles for transport to the Golgi apparatus. In the Golgi, GPI-APs are subjected to fatty acid remodeling in that sn2-linked unsaturated fatty acid is exchanged to a saturated fatty acid, mainly stearic acid. The fatty acid remodeled GPI-APs are preferentially associated with membrane microdomains rich in sphingolipids and cholesterol, and are expressed on the cell surface. At least 150 different human proteins are GPI-anchored. Some of them are shed from the cell surface by GPI-anchor cleaving enzymes. My laboratory has been working on identification and characterization of genes/proteins involved in biosynthesis, transport and shedding of mammalian GPI-APs. Molecular bases of GPI deficiencies, namely, paroxysmal nocturnal hemoglobinuria and inherited GPI deficiencies are also among our main targets. I will summarize our achievements in this area and discuss future direction.

Animal Lectins

Keynote Lecture

14. Galectins in innate immunity

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Galectins are a family of β-galactoside-binding proteins. They can engage cell-surface glycans, thereby affecting a variety of cellular processes, but can also function intracellularly in a glycan-independent fashion. We have investigated the role of galectin-3 in induction of Th17 immunity through dendritic cells (DCs) and concluded that intracellular galectin-3 negatively regulates Th17 polarization in response to the dectin-1 agonist curdlan (a fungal antigen). This function is associated with galectin-3's suppression of the Th17-axis cytokine IL-23. We have confirmed this modulatory effect of galectin-3 in DCs *in vivo*. We have also studied the role of galectin-3 in HIV infection in T cells and our results suggest that the efficiency of viral budding is correlated with the amount of galectin-3 in the cell. As evidenced by immunofluorescence microscopy, galectin-3 is colocalized with HIV Gag and Alix in HIV-1-infected cells, the latter being a component of endosomal sorting complex required for transport (ESCRT) that we previously identified as a galectin-3-binding partner. Additional results suggest that endogenous galectin-3 facilitates HIV-1 budding by promoting the Alix-Gag p6 association. A picture that has emerged recently is that galectins can bind to cytosolic glycans presented as a danger signal when cells are infected by intracellular microbes. Galectin-8 has been shown to target autophagy to *Salmonella*-damaged vesicles and inhibit proliferation of the bacteria. We found galectin-3 accumulated around LM that had escaped from phagosomes through binding to host glycans on the membrane of ruptured phagosomes that initially contained the bacteria. Moreover, our results suggest that through this mechanism, galectin-3 suppresses autophagy induced by *Listeria* infection and has a negative role in macrophage Listericidal activity. Thus, both galectin-3 and galectin-8 bind to cytosolic host glycans exposed to the cytosol but have disparate functions.

Lectures

15. Role of Galectin-7 in lung cancer progression

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Galectin-7 belongs to prototype subfamily of galectins, which are a family of evolutionary-conserved carbohydrate-binding proteins. Several members of the galectin family have been shown to be involved in cancer progression and metastasis. In the case of galectin-7, its expression can range from being completely down-regulated to highly up-regulated in a variety of tumors. Several studies have reported alterations in its expression pattern during cancer progression. Galectin-7 has generally been considered as a pro-apoptotic protein under the control of p53; it is also reported to promote tumor progression in breast cancer and lymphoma. Here, we investigated the role of galectin-7 in lung cancer progression. Non small cell lung cancer (NSCLC) is one of the most common cancers and the leading cause of cancer-related death in many countries around the world. In this study, we found that galectin-7 was highly expressed in several lung cancer cell lines and associated with lung cancer progression. Knocking down of galectin-7 promoted lung cancer cell migration while overexpressing galectin-7 decreased lung cancer cell migration. We also found that knockdown of galectin-7 increased colony formation of lung cancer cell line as well as enhanced sphere formation ability and enlarged the sphere size of lung cancer stem cells. These data suggest that galectin-7 may play a role in tumor metastasis and recurrence. Our immunohistochemistry staining of samples obtained from stage 1 lung adenocarcinoma patients also showed that patients with galectin-7 expression in tumor sites exhibited lower recurrence rate and higher overall survival rate. Taken together, galectin-7 may serve as a tumor suppressor in lung cancer.

16. Galectin ligand-binding, specificity, mechanism and function

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Galectins bind galactose containing glycans, but the structural context of the galactose may enhance, decrease or block the binding resulting in a detailed fine-specificity for each galectin. We ask what role does this fine specificity play in decoding the function of glycan profiles? What is the galectin affinity for a natural glycoprotein with different glycan profiles? How different do the profiles need to be for a functional consequence? Is affinity determined solely by glycan galectin interaction? Do galectins have other ligand binding-sites besides the one for galactosides? Can galectin-glycoprotein interaction be targeted as drug therapy? Galectin-ligand interactions have been analyzed by fluorescence anisotropy, affinity chromatography, microscale thermophoresis and other binding-assays, and interactions have been mapped using galectin mutants, galectin orthologues from different species, and an array of different natural and synthetic ligands. Serum glycoproteins (*e.g.* transferrin and haptoglobin) have been analysed as a relatively abundant source of natural glycoproteins, but the assays have also been miniaturized to permit analysis of more scarce natural glycoproteins (*e.g.* TLR4, HIV gp120, IL-6). Small molecules with high galectin-inhibitory potency have been developed. Two aspects of cellular function of galectin-glycoprotein interaction were studied: intracellular traffic after endocytosis, and accumulation of galectins around disrupted intracellular vesicles. While galectins bind galactose with K_d in the 10 mM range, they may show monovalent binding to glycoproteins with K_d<1 μM, and to some artificial disaccharide inhibitors down to low nM. Galectin-glycoprotein binding can lead to different intracellular sorting after endocytosis (transferrin and haptoglobin), signaling (TLR4), or effects on infectivity (HIV-gp120). Small molecules can inhibit the intracellular accumulation of galectins around disrupted vesicles.

17. The mammalian lectin Galectin-8 induces rankl expression, osteoclastogenesis and bone mass reduction in mice

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Skeletal integrity is maintained by the coordinated activity of osteoblasts, the bone forming cells and osteoclasts, the bone resorbing cells. Here we show that mice overexpressing galectin-8, a secreted mammalian lectin of the galectins family, exhibit accelerated osteoclasts activity and bone turnover, which culminates in reduced bone mass, similar to cases of post-menopausal

osteoporosis and cancerous osteolysis. This phenotype can be attributed to a direct action of galectin-8 on primary cultures of osteoblasts that secrete the osteoclastogenic factor RANKL upon binding of galectin-8. This results in enhanced differentiation into osteoclasts of bone-marrow cells co-cultured with galectin-8-treated osteoblasts. Secretion of RANKL by galectin-8-treated osteoblasts can be attributed to binding of galectin-8 to receptor complexes that positively (uPAR and MRC2) and negatively (LRP1) regulate galectin-8 function. Our findings identify galectins as new players in osteoclastogenesis and bone remodeling, and highlight a potential regulation of bone mass by animal lectins.

Posters

18. Effect of low temperature plasma on the expression of galectins in wound healing skin

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The purpose of this study is to clarify the effect of low temperature plasma on the expression of galectins in the wound healing skin. The low temperature plasma is effective for hemostasis, promotes the wound healing of skin, and promotes the regeneration of epithelial tissue. Galectins play many important roles such as cell adhesion, differentiation, transcription, and wound healing. Recently, it was reported that galectin-1 accelerates wound healing by regulating the neuropilin-1/Smad3/NOX4 pathway and ROS production in myofibroblasts. However, the effect of low temperature plasma on the expression of galectins in the wound healing skin has not been clarified yet. In this study we examined the effect of low temperature plasma on the expressions of galectin-1, −2 and −3 in the wound healing skin to elucidate the mechanism of promotion of the wound healing by low temperature plasma. Two wounds per mouse were created in the dorsal skin. Generated wounds were treated by either plasma technology or high-frequency electrical coagulator. We used our low temperature glow-like plasma which has been developed by us to achieve blood coagulation. The generated plasma prompted blood coagulation keeping thermal elevation less than 40 °C without arc-like plasma formation. We compared the effect of low temperature plasma on the expression of galectin-1, −2, and −3 in the early stage of wound healing with that of high-frequency electrical coagulator. The morphological changes of cell and extracellular matrix were scarcely observed in the low temperature plasma irradiated skin, while those were observed in the electrical coagulator treated skin. The expressions of galectin-1, −2 and −3 were increased in the skin treated with low temperature plasma, while those were decreased in the skin treated with electrical coagulator. These results suggest that the increase of galectin expression may cause the promotion of the wound healing by low temperature plasma.

19. A novel *N*-acetylhexosamine-binding lectin from slipper lobster caused apoptosis and endocytosis against cancer cells

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A novel anticancer lectin termed “iNL” was purified from blood of slipper lobster, *Ibacus novemdentatus* by using GlcNAc immobilized affinity column. Administration of iNL against cancer cells such as breast (MCF7, T47D) and ovarian cancer (HeLa) cells induced the cell death dose-dependently. iNL treated HeLa cells showed the DNA fragmentation and activation of caspase-3 which were enforced the apoptosis. iNL showed the strong hemagglutination activity against sheep erythrocytes which bear the Forssmann antigen (GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc) in cell surface. And its activity was canceled by the co-presence of *N*-acetyl group containing monosaccharides such as *N*-acetyl-D-mannosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and *N*-acetyl-D-neuramic acid. By consideration of sugar specificity, induction of cell death was caused by the interaction of *N*-acetylhexosamine glycan of cancer cell surface. iNL was existed as 500 kDa in the solution which were consisted of 70, 40 and 30 kDa polypeptides via disulfide and hydrophobic bond. Even in the large molecule, endocytosis of lectin was able to observe in endosome and transported to lysosome according to

the incubation time by fluorescent microscopic analysis. Cancellation of endocytosis was confirmed by the co-presence of *N*-acetyl-D-mannosamine. These results suggest that iNL cause the cytotoxicity and endocytosis against cancer cells through the interaction of glycan on the cell surface.

20. Murine and human Langerin do not share specificity for bacterial glycans

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Many members of the C-type lectin receptor family are conserved among mammalian species and important representatives serve as pattern recognition receptors on myeloid cells. The assumption prevails that genetic homologs share similar function among species. Here, we focus on Langerin, a C-type lectin receptor expressed on Langerhans cells and specific dendritic cell subsets. It is conserved in many mammalian species and serves as an uptake receptor for pathogens, which are subsequently processed for antigen presentation to T cells. Its role in pathogen recognition and immune cell activation renders Langerin an attractive receptor in both human and murine model systems. Thus far, results from both systems have been considered interchangeable, however functional proof is lacking. We therefore investigated human and murine Langerin side-by-side with respect to their capability to recognize microbial glycans. To this end, Langerin homologs were expressed recombinantly and tested on a glycan microarray comprising over 300 naturally occurring microbial polysaccharides. The hits were further investigated by ELISA-based as well as cell-based assays and are currently followed up by direct binding studies to live bacteria. We show that human and murine Langerin differentially recognize complex bacterial glycans while exhibiting similar binding properties towards yeast and simple glycans. Our findings have important implications for the use of murine infection models to study human disease.

21. Expression and characterization of recombinant rhamnose-binding lectin with novel domain structure

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Rhamnose-binding lectins (RBLs) have been found mainly in fish eggs and known to have a characteristic tandem repeat structure of carbohydrate recognition domains (RCLDs). Recently, some of their physiological functions are deduced, *e.g.*, involving in innate immunity as a pattern recognition receptor or as a regulator of cytokines. In our previous study, we have isolated four RBLs from rainbow smelt (*Osmerus mordax dentex*) eggs and termed OML1~4. To elucidate their primary structure, we tried to get a full length cDNA of OML. We could obtain only one cDNA clone from mRNA of female fish liver. Interestingly, currently established OML cDNA encoded 245 amino acid residues with two RCLD domains separated by 43 residues insertion. Calculated molecular mass and pI of the deduced sequence were 26046 and 4.81, respectively. RBLs are commonly composed of two or three domains without insertion sequence. Therefore, obtained OML structure was rare case in this family. Since inserting peptide was partially homologous to the RCLD sequence, cloned OML might be a variant from alternative splicing of OML gene. Recombinant OML (rOML) was expressed as an inclusion body in the bacterial KRX strain and refolded with dilution and dialysis using arginine-containing buffer. rOML kept an affinity for galactose-sepharose resin and hemagglutinating activity for rabbit erythrocytes as well as naturally obtained lectin. The hemagglutinating activity was inhibited by L-rhamnose and melibiose, but not by sucrose. To evaluate the necessity of the insertion sequence, the deletion mutant of rOML (rOML-del) was constructed by inverse-PCR method. Against our expectations, rOML-del lost its lectin activity, even though its primary sequence and domain structure was closely similar to those of *O. lanceolatus* lectin. These results indicate that the insertion peptide might affect refolding process of denatured rOML in a disulfide-bond formation or a conformational structure.

22. A food polysaccharide inulin is a novel carbohydrate ligand of Dectin-2

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Dectin-2 is a C-type lectin receptor expressed on the surface of myeloid cells such as dendritic cells and macrophages. Upon carbohydrate-ligand binding, Dectin-2 elicits cellular activation. Furthermore Dectin-2 is documented as a mannose-specific lectin responsible for immunity against pathogenic fungi and mycobacterium. Fungal α-linked mannan has been identified as a preferable ligand for Dectin-2. We explored other food-related polysaccharides for their binding to Dectin-2, as some of food polysaccharides seem to have immunomodulatory properties perhaps through interacting with C-type lectins. Here we identify inulin, a fructofuranose polymer, as a new carbohydrate ligand for Dectin-2. Inulin is a common food polysaccharide found in several fruit and vegetables such as banana, chicory and onion, and has been suggested to modulate immune function. Using a Dectin-2 expressing reporter cell assay, we screened various food polysaccharides for their binding to mouse Dectin-2. This assay enables us to monitor the Dectin-2 binding to polysaccharides by measuring beta-galactosidase expression. We found Dectin-2 bound to inulin in a dose-dependent manner. Compared to the known Dectin-2 ligand (yeast α-mannan), inulin exhibited lower affinity to Dectin-2. Furthermore, Dectin-2 mutant, which possess mutations in the carbohydrate recognition domain, failed to bind to inulin. These data suggest inulin binds to Dectin-2 through the carbohydrate-binding site. Our results demonstrate for the first time that Dectin-2 is capable of recognizing a fructofuranose polymer, which is a common food polysaccharide. This finding may encourage investigating the potential of inulin and other fructose-based polymers in modulating immune function through Dectin-2.

Biosynthesis and Metabolism of Glycoconjugates

Keynote Lectures

23. Human genetic disease offers novel insights in protein glycosylation

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Protein glycosylation is increasingly recognized as crucial modulator of protein function, offering a third layer of biological information over genomics and proteomics. Numerous human disorders, both genetic and acquired, have been associated with deficient glycosylation. However, the biochemical mechanisms underlying abnormal protein glycosylation in human disease are poorly understood. To elucidate disease mechanisms, we aim to unravel the genetic and metabolic factors that influence protein *N*-glycosylation in human genetic disease. *N*-glycosylation is a ubiquitous process, and consequently, genetic defects in this pathway result in a multisystem disease affecting all organs. Nevertheless, many patients with unsolved defect present with tissue-restricted clinical symptoms, hinting to the presence of cell- and tissue specific biochemical mechanisms. To identify causative disease genes, we typically employ a combination of whole-exome sequencing and novel protein-specific glycomics methodologies. Via this complementary approach, disease gene identification was successful in >70 % of patients, revealing diverse mechanisms such as disturbed intracellular pH and Golgi trafficking, and a significant group of defects in cytosolic sugar metabolism. As an example, defects in dolichol kinase and polyprenol reductase, subsequent steps in dolichol-P-mannose synthesis, resulted in contrasting disease symptoms such as cardiomyopathy versus neurological symptoms. Biochemical studies indicated the presence of tissue-specific alternative pathways. In a more recent example, we identified mutations in the cytosolic phosphoglucomutase PGM1 as cause of protein-specific glycosylation abnormalities and tissue-specific clinical symptoms. Our current aim is to elucidate the biochemical mechanisms in sugar metabolism that influence protein *N*-glycosylation in a tissue-specific way, via genome-editing in tissue-specific cell models and quantitative analysis of sugar metabolites.

24. Glycosyltransferase inhibitors: chemical tools for glycobiology

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Glycosyltransferases (GTs) are Nature's glycosylation reagents: enzymes that catalyse the transfer of a mono- or oligosaccharide from a glycosyl donor to an individual acceptor molecule, *e.g.* another saccharide, peptide, protein, lipid or secondary metabolite. In both eukaryotic and prokaryotic organisms, the biosynthesis of complex glycans and glycoconjugates requires a multitude of GTs with different acceptor, regio- and stereo-specificities. Potent and selective GT inhibitors are therefore of considerable value as chemical tools to dissect these complex biosynthetic pathways. In order to be practically useful, such inhibitors must also possess the requisite physicochemical properties for applications in cell assays and whole animals (*e.g.* cell penetration, chemical and enzymatic stability). The development of GT inhibitors

that meet all, or most, of these requirements remains a formidable challenge. In this presentation, selected recent examples for the development and application of GT inhibitors will be discussed. We have recently developed a new class of donor-based galactosyltransferase (GalT) inhibitors, which interfere with the conformational changes during GalT catalysis. Herein, we show how these inhibitors can be used to study the expression of the cell-surface glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1, CD162) in human monocytes. As the main physiological selectin ligand for leukocyte rolling on activated endothelial cells, PSGL-1 is a key mediator of inflammatory cell recruitment and transmigration into tissue. Inhibitors of PSGL-1 biosynthesis are therefore of considerable interest not only for chemical biology, but also for anti-inflammatory drug discovery. We will also present the latest results from our ongoing efforts to develop non-substrate-based inhibitors for bacterial GTs involved in bacterial virulence.

Lectures

25. Biosynthesis of the O antigen repeating unit of the intestinal pathogen *Escherichia coli* O104

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Lipopolysaccharides (LPS) are important virulence factors in Gram-negative bacteria and our goal is to understand the enzymes and mechanisms involved in the assembly of the outer O antigenic polysaccharides of LPS. O Antigens are assembled as repeating units of oligosaccharides which are further processed to form LPS. Biochemical identification of the biosynthetic enzymes is essential to prove their functions. *Escherichia coli* (*E.coli*) serotype O104:H4 (ECO104) is a powerful intestinal pathogen that can cause severe diarrhea, hemolytic-uremic syndrome and death. The ECO104 repeating unit has the structure [4-D-Galα1-4Neu5,7,9Ac$_3$α2-3-D-Galβ1-3-D-GalNAcβ1-]$_n$ which contains a mimic of the human sialyl-T antigen. The O antigen synthesis gene cluster has been cloned. We have now characterized the gene products, the glycosyltransferases that assemble the ECO104 repeating unit based on a synthetic substrate, GalNAcα-PO$_3$-PO$_3$-(CH$_2$)$_{11}$-O-phenyl. The second enzyme in the pathway, β1,3-Gal-transferase WbwC has an absolute requirement for the diphosphate in the acceptor. Surprisingly, the third enzyme, α2,3-sialyltransferase WbwA, also has this requirement. Although these enzymes synthesize a structure found in human glycoproteins, their amino acid sequences bear little resemblance to those of the human enzymes. The fourth enzyme in the ECO104 pathway is α1,4-Gal-transferase WbwB that acts on acceptors terminating in sialic acid but has a distinct preference for the substrate with the intermediate repeating unit structure containing diphosphate. Interestingly, a mixture of the enzymes greatly increases the yield of reaction products, suggesting that these glycosyltransferases exist as a complex. Several synthetic bis-imidazolium salts could inhibit the enzymes. This work identifies potential anti-bacterial targets and a strategy for vaccine synthesis. This project was supported by the Canadian Institutes of Health Research.

26. A novel method to determine phosphohexose mutase activity by anion-exchange chromatography with electrochemical detector

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A novel method has been developed using high-performance anion-exchange chromatography coupled with electrochemical detector (HPAEC-PAD) to direct analysis the interconversion of hexose-6-phosphate and hexose-1-phosphate, so could be used to test the activity of *N*-acetylglucosamine-phosphate mutase (AGM), glucosamine-phosphate mutase (GlmM) and phosphoglucomutase (PGM), which are the members of phosphohexose mutase family. The limits of detection are different for every phosphohexose, in a range from 0.088 to 31.2 μmol. The activity of MtGlmM which tested with this method or traditional coupled method is 1945.75 nmol/min·mg and 76.87 nmol/min·mg respectively. Based on its high specificity, sensitivity and reproducibly, this method will bring convenience to phosphohexose mutase further research.

27. Free *N*-glycans modulate protein folding

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Introduction: Free *N*-glycans (FNGs) ubiquitously occur in developing or growing plant cells as well as mammalian cells. Although it has been believed that these FNGs are produced from misfolded glycoproteins by a sequential action of ENGase and PNGase in the cytosol, the physiological function(s) of FNGs remain to be elucidated. In this study, we found that some FNGs modulated or induced correct folding of denatured proteins. Materials and Methods: Three kinds of FNGs, high-mannose type (HMT, $Man_8GlcNAc_2$), plant complex type (PCT, $Man_3Xyl_1Fuc_1GlcNAc_2$), animal complex type (ACT, $NeuNAc_2Gal_2GlcNAc_2Man_3GlcNAc_2$), were prepared from bean storage glycoproteins or egg yolk glycopeptides. 3Hmut Wil (a mutant of Wil protein that induces AL amyloidosis) was used as a model protein to evaluate the FNGs-function. The state of folding or unfolding of 3Hmut was analyzed by NMR (H-N HSQC) and the formation of amyloid fibril was monitored by Thioflavin (ThT). Results and Discussion: We found that HTM-FNG (~10 mM) can effectively induce the correct folding of denatured 3Hmut and significantly suppress the amyloid fibril formation of 3Hmut. Furthermore, the intrinsic Trp-fluorescence of 3 Hmut was significantly decreased by addition of HMT-FNG (5~15 mM), suggesting that HMT-FNG induced the protein conformation change from denatured state to folded state. These results led us to suppose that if HMT-FNGs generated in the ERAD are transported back to the ER, the FNGs may be involved in the protein-folding/quality control system in the ER.

28. Story of the cytoplasmic peptide:*N*-glycanase (NGLY1)—basic science encounters human genetic disorders

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The cytoplasmic peptide:*N*-glycanase (PNGase) is the enzyme widely conserved throughout eukaryotes. Since the discovery of the enzyme activity in mammalian cells and, 7 years later, the gene encoding the enzyme in yeast, rapid progress has been made in terms of the structural and/or functional study of this enzyme. For example, it has been shown that this enzyme is involved in the degradation of misfolded/non-functional glycoproteins destined for degradation called ERAD (ER-associated degradation). However, initially the biological significance of this enzyme has not been well understood, especially as the PNGase-deletion mutant in yeast did not exhibit any defect in viability/growth. On the other hand, mutation in the gene orthologue of the cytoplasmic PNGase in fruit fly led to serious developmental delay, suggesting the functional importance of this enzyme in higher eukaryotes. In 2012, a patient harboring mutations of PNGase gene (NGLY1) was first reported. Symptom of these patients includes developmental delay, multifocus epilepsy, involuntary movement and liver dysfunction. From this report, it is suggested that an unknown physiological function of PNGase could be contribute to human survival. Our recent study showed that in Ngly1-KO cells, ERAD process was compromised. Interestingly, not only delayed degradation but also the deglycosylation of a model substrate was observed in this cell. The unexpected deglycosylation was found to be mediated by the cytosolic endo-β-*N*-acetylglucosminidase (ENGase), encoded by Engase gene. Surprisingly, the ERAD dysregulation in Ngly1-KO cells were restored by the additional KO of Engase gene. Thus, our study underscores the functional importance of Ngly1 in the ERAD process and provides a potential mechanism underlying the phenotypic consequences of the human NGLY1-deficiency.

Posters

29. ER α-1,2-mannosidase I; studies of the structure and function by site-directed mutagenesis

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Mannose-trimming in high-mannose type oligosaccharides of nascent glycoproteins is performed through secretory pathway in eukaryotes. Removal of α-1,2-linked mannoses highly relates to achieve quality control of glycoproteins in the living cells. The reaction is catalyzed by members of glycosylhydrolase family 47, which include ER α-1,2-mannosidase I (ERManI), Golgi α-1,2-mannosidase IA-C (GolgiManIA-C), and EDEM1-3. We have developed convergent strategy to synthesize a large variety of high-mannose type sugar chains (Chemistry 21, 3224 (2015)). Using synthetic oligosaccharides and

natural glycoproteins, we have revealed that human ERManI (hERManI) removed more mannose moieties when glycoprotein substrates were subjected to denaturing conditions (J. Biochem. 155, 375 (2014)). Recently, knowledge about detrimental mutations in hERManI causing Type II congenital disorder of glycosylation was accumulating. Therefore, we are highly promoted to examine further studies about structure and function of the enzyme. Utilizing the structure (PDB file; 1X9D) of co-crystal of hERManI with substrate-mimic compound (Man-S-Man), we focused on three residues, Ser375 Thr394, and Asp523 which located near substrate-binding cleft in hERManI. Those residues were substituted for the amino acid corresponding to human GolgiManIA. Mutated enzymes were examined for their demannosylating properties using glycoproteins as substrates. Thr394Ile-hERManI showed different profile of demannosylation from the non-mutated; impaired demannosylation of (Glc)Man_9 to (Glc)Man_8B and accelerated demannosylation of Man_8B to Man7A/7C/6. Asp523Gly-hERManI exhibited impaired demannosyalation in bovine lactoferrin, while not bovine ribonuclease B. Ser375Ala-hERManI showed similar properties of demannosylation to the non-mutated. These results indicated that Thr394 and Asp523 played crucial roles in performing enzyme activities of ERManI.

30. Cloning and expression analysis of acidic peptide: *N*-glycanase genes in *Solanum lycopersicum*

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Introduction: Plant acidic peptide:*N*-glycanase (aPNGase), which has the optimum pH at acidic region and active against plant complex type *N*-glycans, releases *N*-glycans from glycopeptides produced by proteolysis of function-lost proteins. As a part of study to elucidate the physiological function of free *N*-glycans associated with plant differentiation and growth, we identified an *aPNGase* gene (*aPNGase-Le*) expressed in tomato fruits and constructed transgenic tomato plants overexpressing the *aPNGase-Le* gene. Interestingly, we found that the transgenic tomato fruits (T1-generation) were softer and smaller than those of wild type tomato, suggesting that the free *N*-glycans may have some function(s) involved in fruit-maturation. In this study, we cloned remaining five putative tomato *aPNGase* genes and analyzed the gene expression profile during tomato fruit maturation. Results and Discussion: Using total RNA extracted from tomato fruits (mature green, turning, and red ripe), we succeeded to clone two other genes (*Solyc03g095850* and *Solyc06g051010*). Based on the expression analysis, it was found that (1) these two putative *aPNGase* genes are expressed in tomato fruits, (2) the expression levels of *Solyc03g095850* and *Solyc06g051010* genes decreased gradually during fruits ripening, while the *aPNGase-Le* gene was constantly expressed. These results suggested that each aPNGase might have specific roles associated with the fruit ripening. Furthermore, it was found that the GFP-tagged product of *Solyc06g051010* was localized in the vacuole of tobacco-cultured cells.

31. Purification and characterization of *Ginkgo biloba* α-1,3/4-fucosidase (α-Fuc'ase Gb) active against Lewis a antigen

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It has been believed that the metabolism of *N*-glycans or *N*-glycoproteins is responsible for the plant growth and fruit maturation. Some plant glycoproteins carry the plant complex type *N*-glycans (PCNGs) containing the Lewis a antigen, although the physiological significance of these plant complex type *N*-glycans remains to be understood. PCNGs sometimes contain two α-fucosyl residues, one is α-1-3-linked fucose and the other is α-1-4-linked one. Only a few of plant α-fucosidases (α-Fuc'ase) involved in the defucosylation from PCNGs has been analyzed, while functional features and molecular identification of many plant α-Fuc'ase associated with the cell wall metabolism have been reported. As a part of study to elucidate the physiological significances of PCNGs, in this study, we focused on purification and characterization of plant α-Fuc'ase active against PCNGs. We used *Ginkgo biloba* seeds as starting materials for purification of α-Fuc'ase, since we already found that the storage glycoproteins in *Ginkgo* seeds carry predominantly PCNGs. For detection of α-Fuc'ase activity, the pyridylaminated PCNGs bearing the Lewis a antigen was used as a

substrate. Purified *Ginkgo* α-Fuc'ase (α-Fuc'ase Gb) with molecular mass of about 120 kDa (SDS-PAGE and Gel-filtration) showed optimum activity around pH 5.5, indicating that the subcellular localization of this enzyme may be in the vacuole or cell wall region. α-Fuc'ase Gb was highly active against the Lewis a antigen in PCNG, modestly active against GlcNAcβ1-4(Fucα1-3)GlcNAc-PA but not against Fucα1-3GlcNAc-PA and Lacto-*N*-fucopentaose I (Fucα1-2$Gal_1GlcNAc_1Gal_1Glc_1$-PA).

32. Human α-1,4-galactosyltransferase (GB3/CD77 synthase): how one amino acid residue may drive promiscuity of a glycosyltransferase

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Human α-1,4-galactosyltransferase (Gb3/CD77 synthase, Pk synthase) synthesizes 2 or 3 carbohydrate antigens of the P1PK (formerly P) blood group system depending on whether position 211 of the polypeptide chain is occupied by Gln or Glu residue, respectively. P1, Pk and NOR antigens are carried by glycosphingolipids. P1 and Pk terminate with Gal(α1-4) Gal disaccharide and have long been thought to be synthesized by the consensus enzyme (encoded by A4GALT gene). The presence or absence of P1 antigen determines the P1 or P2 blood group, respectively. Notably, this blood group polymorphism does not result from nucleotide changes within the open reading frame (ORF) of A4GALT gene but is associated with single nucleotide polymorphisms upstream of the ORF. On the other hand, the rare NOR antigen (whose structure was solved in our laboratory) terminates with unusual Gal(α1-4)GalNAc disaccharide, never before found in mammals. NOR is synthesized by the enzyme variant with 211Q>E substitution caused by 631C>G mutation in A4GALT, which we previously found in NOR-positive individuals. The mutation broadens the enzyme specificity, rendering it able to synthesize NOR in addition to P1 and Pk. While existing evidence for bi- and trispecificity of the consensus and variant enzyme, respectively, is strong, it is all indirect because attempts to obtain recombinant α-1,4-galactosyltransferase and clearly demonstrate its catalytic properties have been futile. Here, using a recombinant catalytic domain of α-1,4-galactosyltransferase obtained in Sf9 insect cells, we present the first biochemical evidence that the consensus α-1,4-galactosyltransferase may synthesize both, P1 and Pk blood group antigens, while its 211Q>E variant is able to additionally synthesize NOR. Thus, our results pinpoint the role of human α-1,4-galactosyltransferase in synthesis of P1PK blood group antigens and the importance of 211 amino acid residue for fine specificity of the enzyme.

33. Site-directed mutagenesis study of Endo-β-*N*-acetylglucosaminidase from *Ogataea minuta*

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Glycoside hydrolase family 85 (GH85) in Cazy database (http://www.cazy.org) refers to the enzymes that hydrolyze the glycoside bond in *N*,*N*'-diacetylchitobiose of *N*-glycan (ENGase). Some of ENGases have been known to show the transglycosylation activity that *en bloc* transfers various types of *N*-glycans onto GlcNAc acceptor other than water. Recently, ENGase from *Ogataea minuta* (Endo-Om) has been identified as a new member of GH85 and the report demonstrated that Endo-Om hydrolyzed high-mannose, hybrid, biantennary and (2,6)-branched triantennary *N*-glycans, while also demonstrating transglycosylation activity (Murakami *et al.*, 2013, Glycobiology). The broad substrate specificity is similar to those of ENGases from eukaryotes, such as *Caenorhabditis elegans* and *Mucor hiemalis*, but different from those of bacterial ENGases, which don't show any activities to complex type *N*-glycans. Their catalytic mechanisms for hydrolysis and transglycosylation activities have been extensively studied; however, the mechanism for oligosaccharide recognition is still unclear. Here, we demonstrate the site-directed mutagenesis studies of Endo-Om to find amino acids involved in *N*-glycan recognition. Trp-295 was selected for the mutagenesis as the structural model of Endo-Om predicted that it was not involved in the catalytic activity but in the interaction with α1,3-mannose of trimannosyl core of *N*-glycan. Indeed, W295F and W295Y showed the ENGase activity to pyridylamino-oligosaccharides, while W295A, W295E,

and W295Q did not show any activities. These results implicate Trp-295 as an essential amino acid for the recognition of *N*-glycan substrate through hydrophobic interaction with the α-1,3-mannose. Most interestingly, W295F mutant showed higher activity than wild-type and W295F. We will demonstrate another mechanism for *N*-glycan recognition using the structural model of W295F mutant.

34. Is site specific glycan microheterogeneity driven by the glycoprotein itself?

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N-glycosylation is characterized by a large diversity of glycan structures. Microheterogeneity of glycans corresponds to the diversity of glycan structures attached to the same glycosylation site, and the mechanism by which it is generated is not entirely understood. We decided to investigate to which extent the protein carrying the glycans influence the microheterogeneity of glycosylation. We hypothesized that, for each enzyme, each glycosylation site represents an original substrate, whose specificity is defined by the combination of the protein 3D structure and its interaction with the glycan. Preliminary studies done in our lab showed that protein disulfide isomerase Pdi1 from *S. cerevisiae*, which contains 5 glycosites, presented a specific glycan profile for each site, when heterologously expressed in insect cells. In particular, we observed a major difference in the glycan processing on the 4th site compare to the other sites, which is probably due to the interaction of Pdi1 with the glycan. To determine if this phenomenon was conserved from species to species or was modified in presence of longer, highly branched or charged structures, we stably or transiently expressed Pdi1 in CHO-S cells. Subsequent quantitative analyses of site-specific *N*-glycan structures abundance were done by LC-MS/MS. Our results show that glycan profiles were different from site to site on an ER-retained and secreted form of Pdi. First, the reticular Pdi presented a pronounced difference in the mannose trimming of the site 4 compare to other sites, similar to the one observed in insect cells. Moreover, on the secreted Pdi, we observed that site 4 presented complex and hybrid structures when sites 1, 2, 3 and 5 presented mainly fucosylated complex glycans, for which the number of HexNAc-Hex repeats was variable from site to site. Taken together these results indicate that the glycan profile is conserved through species and suggest its determination by the protein itself.

35. Synthesis of steroid conjugates for targeted liposomal delivery of RNA-based therapies

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By introducing an RNA molecule into the body as a treatment, RNA-based therapeutics are considered as a promising new technology to overcome challenges of existing medicines. These emerging drugs including small interfering RNA (siRNA) and micro RNA (miRNA) such as antagomiRs which are able to target only specific proteins involved in diseased cells, have shown great potential as treatments for a variety of human diseases. However, their clinical testing has been limited due to challenges with their stability and specificity. In this presentation we describe some of our ongoing efforts to overcome these barriers. Our approach involves the conjugation of cholesterol to a targeting group such as a peptide or carbohydrate, via a PEG linker with or without a fluorescent label. These conjugates are then incorporated into "stealth" (PEGylated) liposomes which encapsulate the antagomiR cargo and are targeted to specific cell types.

36. Detection of glycosyl hydrolase activity on glycan microarrays by MALDI-TOF

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Glycan microarrays are powerful tools for the discovery and characterization of glycan processing enzymes. Recently, we have developed a mass spectrometry microarray platform on indium tin oxide (ITO) slides that allows for the dual readout by fluorescence and/or by mass spectrometry. These glycan microarrays have been employed in the study/characterization of recombinant glycosyltransferases. (Angew. Chem. Int. Ed. 2013, 52, 7477). Based on this preliminary work, we present herein our results in the study of glycosyl hydrolases (GHs), a family of enzymes involved in the synthesis and metabolism of glycoconjugates. Glycosyl hydrolases

are a widespread group of enzymes that selectively break glycosidic bonds between two sugar moieties. These enzymes are found in all domains of life and are implicated in biological processes such as biomass degradation, pathogenesis of virus and bacteria and by the concerted action with glycosyltransferases, are responsible for the post-translational modifications that decorate proteins and lipids. Indium tin oxide slides are functionalized by a lipid bilayer containing carboxylic ligands activated with *N*-hydroxysuccinimide (NHS) groups. Amino functionalized natural oligosaccharides of different lengths and sugar compositions are robotically spotted on top, reacting with the surface through the formation of amide bonds. In this way, micrometer spots containing a single type of oligosaccharide are generated allowing for their independent analysis by MALDI-TOF. Recombinant glycosyl hydrolases such as arabinanases, glucosidases, xylanases and cellulases are studied using this platform. The mayor advantage of this method is the parallel interrogation of several putative oligosaccharide substrates employing minimum amount of samples. We are currently applying these glycan microarrays in the screening of complex biological crudes and lysates for the discovery and characterization of hydrolytic activities presented in bacterial communities.

37. Specificity and structural aspects of UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase from the snail *Biomphalaria glabrata*

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Invertebrates show very complex glycosylation patterns. They combine structural characteristics known from other organisms and decorate their glycans with various additional features. Some snail species are intermediate hosts of parasites and control the intake, the development and the excretion of the parasite at least partly by recognition events based on glycosylation. Snails are therefore a valuable tool to understand the principle rules of glycosylation in general. The mucin-type *O*-glycosylation initiating enzyme UDP-*N*-acetyl-α-D-galactosamine: polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAcT, GenBAnk: KC18251) from the water snail *Biomphalaria glabrata*, the first member of this enzyme family from snail origin, was characterized for its substrate specificity. The ability of the enzyme to glycosylate synthetic and native peptides with different amino acids close to the glycosylation site was quantified. No specific *O*-glycosylation consensus sequence was found, but preferences and no-go criteria could be determined. Especially sites with adjacent proline residues or already glycosylated serine or threonine residues enhanced further glycosylation. Structural modelling of the snail enzyme in comparison with the well known representatives of the family as well as with the scattered data from other molluscs supported the substrate specificity findings. Also the monitoring of the sequence of glycosylation events of a multi-glycosylated peptide by electron transfer dissociation mass spectrometry confirmed the results. This work was supported by the Austrian Science Fund (FWF): project number P22118-B20.

Cell Biology of Protein Glycosylation

Keynote Lecture

38. Encoding the *O*-glycoproteome through Golgi reorganization

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Biological systems process signals largely through protein modifications. A major category of modifications is glycosylation, resulting in addition of glycans mostly to cell surface or secreted proteins. The glycome, the ensemble of glycans, plays a major role in regulating cells' interactions with their environment and coordinating multicellular systems. How information is encoded into the glycome, or how glycosylation is modulated in response to signals remains largely unclear. Glycosylation enzymes operate in the ER and Golgi, a highly compartimentalized membrane-bound environment. Recent work indicates that signals can induce the relocation of *O*-glycosylation enzymes GALNTs from Golgi to the ER. This process, the GALA pathway, results in significant up-regulation of *O*-glycosylation of various proteins, promotes tissue invasion and is frequently activated in cancer cells. GALA illustrates how protein glycosylation can be regulated by Golgi plasticity and suggests that membrane trafficking regulation is an

important mechanism to encode information into the glycome.

Lectures

39. Regulation of hepatic fatty acid synthase properties by *O*-GlcNAcylation

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During meal intake, two metabolic pathways are activated in the liver, the glycolysis and the lipogenesis, to drive the production of fatty acids. The Hexosamine Biosynthesis Pathway (HBP), which end product is UDP-GlcNAc the substrate of OGT (*O*-GlcNAc Transferase) to *O*-GlcNAcylate proteins, is also activated. *O*-GlcNAcylation is a dynamic post translational modification (PTM) that controlled a plethora of protein properties. Disturbance in the *O*-GlcNAcylation dynamism is implicated in several pathologies. Numerous studies link metabolic disorders emergence to *O*-GlcNAcylation mechanisms deregulation. Knowing that there is a close relationship between glucose, *O*-GlcNAcylation levels and activation of the glucido-lipid metabolism, a link between the activation of the glycolytic and the lipogenic enzymes and *O*-GlcNAcylation should exist. More precisely we focused on Fatty Acid Synthase, FAS which produces fatty acids. In this study, *O*-GlcNAcylation levels and FAS expression were analyzed in liver of C57BL6 mice fed a Chow Diet (CD) or High Carbohydrate Diet (HCD), in liver of mice harboring an inhibition of OGA and in primary hepatocytes of mice cultured in different *O*-GlcNAcylation levels. Co-immunoprecipitation experiments showed that OGT and FAS interacted physically but this interaction did not lead to FAS *O*-GlcNAcylation. However, a correlation between FAS expression and *O*-GlcNAcylation level was shown and an increase of *O*-GlcNAcylation levels paralleled the protection of FAS against this degradation. Moreover FAS activity was increased in fasted HCD mice compared to fasted CD mice. Taken together, our results suggest that *O*-GlcNAcylation may represent indirectly a new regulation of FAS protein content and activity in liver under both physiological and physiopathological conditions.

40. Visualizing protein-specific glycosylation inside living cells

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The post-translational modification of proteins with β-linked *N*-acetylglucosamine, termed *O*-GlcNAcylation, is widespread and plays an important role in regulating their structure and function. Dysregulation of protein *O*-GlcNAcylation is related to severe ailments such as type 2 diabetes and Alzheimer's disease. Thus, the imaging of the *O*-GlcNAcylation of specific proteins inside living cells is of great interest. To achieve this, we established an approach by combining metabolic glyco engineering with Fluorescence Lifetime Imaging-Förster Resonance Energy Transfer (FLIM-FRET) microscopy. Thereby, GFP is genetically fused to a protein of interest. A recently developed *N*-acetylglucosamine derivative bearing a cyclopropene-tag is incorporated into the cellular glycome in order to target protein *O*-GlcNAcylation and, in a second step, reacted with a dye-tetrazine conjugate in a bioorthogonal Diels-Alder reaction with inverse electron demand. The glycosylation of a specific protein then leads to FRET between the GFP donor and the acceptor dye. FRET can be detected with high contrast even in presence of a large access of acceptor fluorophores by fluorescence lifetime imaging microscopy. This strategy was successfully employed to visualize the glycosylation of specific proteins inside living cells. First, *O*-GlcNAc-transferase, the key regulator of protein *O*-GlcNAcylation, was chosen as model to establish this methodology by performing Western blot analysis and FLIM-FRET microscopy on fixed and living cells. To exemplify the broad applicability of our technique, we additionally visualized the intracellular glycosylation of the insulin receptor β-subunit and the tumor suppressor p53. This is the first time that the glycosylation state of specific intracellular

proteins has been imaged directly inside living cells. Our new method thus provides a general tool and is suitable for studying the influence of glycosylation on the function and dynamics of proteins.

41. The transmembrane domain of tobacco *N*-acetylglucosaminyltransferase I is the key determinant for its Golgi sub-compartmentation

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The Golgi apparatus is the main site of glycan synthesis and processing in eukaryotes. Golgi-dependent glycosylation steps are governed by the dynamic intra-Golgi localization of sequentially acting glycosidases and glycosyltransferases. The mechanisms and signals that control the functional organization in the Golgi are still largely unknown. Golgi-resident plant *N*-glycan processing enzymes are type II membrane proteins with an *N*-terminal region comprising a short cytoplasmic tail, a single transmembrane domain, and a luminal stem region that directs their non-uniform distribution across the distinct Golgi cisternae. Here, we used a series of chimeric proteins to investigate the contribution of these regions from tobacco *N*-acetylglucosaminyltransferase I (GnTI) for its *cis*/*medial*-Golgi localization and for protein-complex formation in the Golgi apparatus of plants. The individual GnTI protein domains were replaced with the ones from the *trans*-Golgi-located α2, 6-sialyltransferase and the effect on GnTI sub-Golgi localisation and *N*-glycan processing was determined. Using co-localization analysis and *N*-glycan profiling, we show that the transmembrane domain of GnTI is the key determinant for its *cis*/*medial*-Golgi localisation. By contrast, the *N*-terminal cytoplasmic tail is involved in ER-exit and the stem region of GnTI contributes predominately to protein complex formation. Importantly, the distinct sub-Golgi localisation is required to complement the *N*-glycan processing defect of corresponding mutant plants. Currently, we are further investigating amino acid motifs in the transmembrane domain of GnTI that contribute to its sub-Golgi targeting and retention. Our results suggest that a sequence-specific feature in the transmembrane domain of GnTI accounts for its steady-state distribution in the *cis*/*medial*-Golgi in plants, which is essential for efficient *N*-glycan processing *in vivo*. This work is supported by the Austrian Science Fund (FWF) Project P23906–B20.

Posters

42. *O*-GlcNAc is increased in white blood cells after moderate physical exercise

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O-GlcNAc modification (*O*-linked β-*N*-acetylglucosamine) is a unique, dynamic posttranslational modification, which connects carbohydrate metabolism to various intracellular signaling mechanisms. It occurs on more than 1,000 different proteins, including proteins involved in transcription, translation and signal transduction. Based mainly on *in vitro* studies, numerous forms of cellular injury can lead to rapid and dynamic elevation of *O*-GlcNAc levels. This elevation of *O*-GlcNAc modification is considered by many researchers a protective mechanism during stress. In this study our aim was to analyze acute *O*-GlcNAc changes after moderate physical stress. We isolated white blood cells from healthy volunteers before and after physical exercise (20 min. of running). In parallel, we treated Jurkat cells (T-cell derived cell line) for 30 min. with 7 mM lactic acid to mimic tissue acidosis occurring during physical activity. From each samples, we isolated proteins and performed western-blot to detect *O*-GlcNAc positive proteins. In addition, we analyzed a number of routine laboratory parameters from the volunteers. We found that serum lactic acid content of the volunteers increased from 1.72 to 5.17 on average after physical activity. Serum "necro-"enzyme levels, such as creatine-kinase or lactate dehydrogenase increased about 15 % after exercise. Proteins isolated from white blood cells showed elevated level of *O*-GlcNAc modification. Similarly, Jurkat cells treated with lactic acid also increased their overall *O*-GlcNAc levels. Based on our results, we can conclude that even moderate level of physical training changes the *O*-GlcNAc status of white blood cells. We propose that regulation of *O*-GlcNAc modification and its impact on the immune system might be a valid molecular mechanism that contributes to the health benefits of regular physical activity. This work was supported by the Research Fund of the University of Pecs, Faculty of Medicine (AOK-KA 2013/19).

43. Lipoprotein lipase is glycosylated at TRP417

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Lipoprotein lipase (LPL) is expressed in many tissues such as adipose tissues, heart muscles and macrophages. It plays critical roles in lipid metabolism and transport, and mainly hydrolyses triglycerides in triglyceride-rich lipoproteins. Moreover, some reports show that dysfunction of LPL causes dyslipidemia and atherosclerosis. People who have a deficiency in LPL gene also attend to have features of these diseases. On the other hand, the amino acid sequence of LPL contains C-mannosylation consensus sequence Trp-Xaa-Xaa-Trp/Cys motif (Xaa represents any amino acids). C-mannosylation is one of the post-translational modifications, and recently it has been reported that some proteins are C-mannosylated and C-mannosylation affects protein stability and secretion. LPL has one predicted C-mannosylation site at Trp417, but it is not reported whether LPL is C-mannosylated or not. Additionally, the potential site of C-mannosylation (Trp417) is a part of functional domain to capture lipoproteins. Therefore, we assumed that C-mannosylation in LPL affects its enzymatic activity. In this study, we investigated relationship between C-mannosylation and LPL functions. To detect whether LPL is C-mannosylated or not, we established LPL-overexpressing HT1080 cells, and purified recombinant LPL protein from the cell lysate. Using mass spectrometry, we revealed that LPL is C-mannosylated at Trp417. Our fingings suggested that C-mannosylation of LPL at Trp417 may regulate its function and it would be a therapeutic target for LPL deficiency.

44. Glycosylation of granulocyte colony-stimulating factor receptor

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Granulocyte colony-stimulating factor receptor (G-CSFR), also known as colony stimulating factor 3 receptor (CSF3R), is a member of type I cytokine receptor and binds its ligand G-CSF to activate downstream signaling pathway, such as JAK/STAT pathway. This signaling regulates hematopoietic cell maturation, proliferation, survival and differentiation. G-CSFR contains characteristic motif, W-S-X-W-S (X represents any amino acids), which contributes protein folding and this motif is corresponding to the consensus sequence of protein C-mannosylation (W-X-X-W/C). C-mannosylation is one of the glycosylations which has been reported in several proteins; however, it is largely unknown the effect of C-mannosylation on protein functions. Recent studies have shown that some C-mannosylated proteins have thrombospondin type I repeats (TSR1) or W-S-X-W-S motif, and secretion of the some proteins that have TSR1 is regulated by C-mannosylation. G-CSFR has three putative C-mannosylation sites in the extracellular domain (ECD), W253, W318, and W446. Most notably, W318 is corresponded to the first tryptophan residue of the W-S-X-W-S motif. Here we examined whether G-CSFR is C-mannosylated and investigated the effects of C-mannosylation on G-CSFR functions. To determine whether G-CSFR is C-mannosylated or not, we cloned the ECD of G-CSFR cDNA, established a G-CSFR/ECD-myc-his6-overexpressing HT1080 cell line and purified recombinant G-CSFR/ECD from conditioned medium of the cells. Using mass spectrometry, we demonstrated that G-CSFR is C-mannosylated at only W318, but not W253 and W446. Our findings suggested that C-mannosylation of G-CSFR at W318 may regulate its function, and it would play important roles in hematopoietic cell maturation.

45. Glycosylation of C-MPL affects JAK/STAT signaling pathway

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Glycosylation is one of the post-translational modifications and is involved in various biological events including differentiation and proliferation. As 50 % of proteins are proposed to be glycosylated, investigation of association between glycosylation and its biological function is important. C-mannosylation is a unique type of glycosylation that one mannose is attached to the first tryptophan in the sequence Trp-Xaa-Xaa-Trp/Cys (Xaa is any amino acid), but a little is known about C-mannosylated proteins and roles of C-mannosylation. Thrombopoietin (TPO) is a hematopoietic factor that regulates platelet production and differentiating megakaryocyte through JAK-STAT signaling after binding to TPO receptor (c-mpl). c-mpl is type I cytokine receptor and has two Trp-Ser-Xaa-Trp-Ser motifs that are important for TPO binding and are corresponding to the C-mannosylation consensus sequence. In this study, we examined

whether c-mpl is C-mannosylated or not, and the role of C-mannosylation in c-mpl function. Using mass spectrometry, we demonstrated that c-mpl is C-mannosylated at not only Trp269 and Trp474 that are putative C-mannosylation sites, but also Trp272, Trp416, and Trp477 that are not putative. We established C-mannosylation defective c-mpl-overexpressing cell lines in which Trp are substituted to Phe. While JAK-STAT signaling is activated by TPO stimulation in wild type and W416F mutant cell lines, TPO response is not suppressed in other cell lines. These data suggest that C-mannosylation at Trp269, Trp272, Trp474 and Trp477 is important for JAK/STAT signaling pathway on c-mpl. Therefore it is indicated that C-mannosylation of c-mpl is a possible target of diseases of blood system.

46. Glycosylation of R-spondin3 promotes its secretion and activates WNT/β-catenin signaling pathway

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R-spondin3 (Rspo3) is an agonist of Wnt/β-catenin signaling pathway and was reported as an oncogene. We examined Rspo3 functions in terms of post-translational modification. C-mannosylation is an attachment of α-D-mannopyranose to the first tryptophan in the consensus sequence, Trp-Xaa-Xaa-Trp/Cys. Rspo3 has two putative C-mannosylation sites, Trp153 and Trp156 in Thrombospondin Type-1 Repeat. Since all Rspo family proteins shared these two tryptophan residues, it might have crucial roles for their functions. Nevertheless, it has not been shown that whether Rspo3 is C-mannosylated or not. In this study, we have demonstrated the presence of C-mannosylation within Rspo3 and its roles. First, to detect the attachment of mannose to the possible two tryptophan residues, we established an Rspo3-overexpressing cell line and purified recombinant Rspo3 from the conditioned medium of the cells. Using mass spectrometry, we showed that Rspo3 is C-mannosylated at both Trp153 and Trp156 residues. Next, we established C-mannosylation-defective 2WA mutant Rspo3-overexpressing cell line, and analyzed the effects of C-mannosylation on Rspo3 functions. Less amount of 2WA mutant Rspo3 secreted to the conditioned media compared with wild-type Rspo3. Moreover, the activity of 2WA mutant Rspo3 as an agonist of Wnt/β-catenin signaling was attenuated as well. In conclusion, we clarified that C-mannosylation of Rspo3 regulates its functions, not only secretion but also activity as an agonist of Wnt/β-catenin signaling. Since Rspo3 was reported as an oncogene, C-mannosylation of Rspo3 might be a new target for cancer therapy.

Comparative and Evolutionary Glycobiology

Keynote Lecture

47. Glycans and gene flow (reproductive and infectious)

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The tree of cellular life is remarkably "glyco-compartmentalized" as different lineages use different subsets of glycans to decorate the surface of their cells. Are these patterns purely the result of stochastic evolutionary history or do they contribute to evolution in their own right? Phylogenies represent grand summaries of past genetic exchange (gene flow) via both, sexual reproduction and viral infection. We will discuss how glycan diversity and glycan directed immunity (innate and adaptive) contribute to reproductive isolation and to susceptibility to viral infections. Two otherwise common terminal glycans have been lost in the lineage leading to humans. The α-galactosyl epitope was lost over 30 million years ago and the *N*-glycolylneuraminic acid (Neu5Gc) epitope was lost over 2 million year ago. These two losses contributed to major changes in the glycocalyx of human cells compared to most other mammals. Interestingly, other mammalian species have lost either one or the other epitope through shared as well as convergent loss-of-function mutations. Understanding the selective forces that mediated such changes promises important insights into how glycans evolve and how glycans themselves can become drivers of future evolution.

Lectures

48. Experimental evidence for an arms race between defense lectins of prey and glycoepitopes of predatory nematodes

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Many mushroom lectins exhibit toxicity towards the model nematode *Caenorhabditis elegans*. This toxicity was shown to be mediated by binding of the lectin to specific glycoepitopes on glycoproteins or glycolipids in the intestinal epithelium of the nematode. Alterations of the targeted glycoepitope, *e.g.* by loss-of-function mutations in respective

glycosyltransferase genes, were shown to cause resistance of the nematode to the respective lectin. Based on these findings, we hypothesize that there is an arms race between the specificity of defense lectins produced by the prey (plants, fungi or bacteria) and the structure of intestinal glycoepitopes of predatory (parasitic) nematodes which should be manifested by the occurrence of nematodes that are naturally resistant against specific mushroom lectins, possibly due to altered glycoepitopes. In order to assess the natural variability of nematodes with regard to their susceptibility to mushroom lectins and the underlying resistance mechanisms, we took a three-step approach: In a first step, we tested the susceptibility of different bacterivorous nematodes to feeding on bacteria expressing various mushroom lectins. In a second step, we tested binding of the lectins to the different tissues of the nematodes by *in situ* hybridization of permeabilized nematodes with fluorescently labeled lectin. Finally, we assessed binding of the biotinylated lectins to isolated nematode glycoproteins, glycolipids and glycans thereof. The isolated nematode glycans were separated by 2D-HPLC and arrayed on glass slides for analysis. The results suggest that there is a significant degree of natural resistance against mushroom lectins among bacterivorous nematodes which is probably due to different mechanisms including alteration of glycoepitopes. We conclude that here is an arms race between defense lectins produced by the prey and the structure of intestinal glycoepitopes of predatory nematodes which is experimentally easily accessible.

49. The glycophylogeny of marine organisms shows differences in anionic and zwitterionic modifications of glycans

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Marine eukaryotes constitute a huge group of organisms, but their glycomic potential is poorly explored. We have recently begun to analyse in depth a number of example species including two molluscs, an annelid and an echinoderm using a combination of off-line LC-MALDI-TOF MS/MS and on-line LC-ESI-MS/MS. *N*-glycans were released by PNGase F followed by PNGase A and subject to solid-phase extraction prior to fluorescent labelling. Residual glycopeptides were also treated by β-elimination in order to analyse the *O*-glycans. Whereas our previously-published data on the Eastern oyster revealed the presence of sulphated blood group A epitopes of possible relevance to norovirus binding, the olive snail has a highly complex *N*-glycome (with about 100 structures) with a variety of zwitterionic and anionic modifications, including sulphate. The annelid on the other hand has zwitterionic, but no anionic, modifications of its *N*-glycans, whereas the echinoderm *N*-glycans often contain either sialic acid or core difucosylation, a finding in keeping with its phylogenetic juxtaposition between invertebrates and vertebrates. Thus, the glycomic analysis of just four marine organisms demonstrates not only variability in their oligosaccharide structures, but it is obvious that the glycosylation of 'lower' eukaryotes is far from simple and can compete with mammalian glycomes in terms of complexity.

Posters

50. Glycosyltransferase evolution—a view from the search for ascidian glycosphingolipids

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We have conducted studies to elucidate the function of glycosphingolipids (GSLs) via structural characterization from lower animals. We have found that ascidians (chordates) contained undetectable concentrations of ganglioside. We conducted phylogenetic profiling analyses of 173 human glycosyltransferases (GTs) by surveying the genomes of 1341 organisms from the GTOP database. The phylogenetic profiling was given a binary pattern based on whether they contained a homologous GT or not. A clustering analysis of the resulting binary data resulted in a tree diagram with four clades. These clades corresponded to deuterostomes, metazoa, eukaryotes, and others, which were categorized into Classes 1–4, respectively. The glycan biosynthesis pathways were found to be catalyzed first by the GT in Class 3, followed by those in Class 2, and then finally by those in Class 1. Most of the GTs grouped into Classes 1 and 2 were localized to the Golgi apparatus, whereas most of those grouped into Class 3 were localized to the endoplasmic reticulum. We found that ascidians lost sialyltransferases belonged to three (ST6Gal, ST6GalNAc, and ST8Sia) of four families. We also found that the GT families in the CAZy database were composed of GTs that belonged to a single class, except for GT1, GT4, and GT61. GTs related to GSLs were found to be significantly dominant in Class 1. Similarly, those related to *O*-glycan/proteoglycans and *N*-glycans were dominant in Class 2 and Class 3, respectively. This study suggested the following: organisms originally acquired the ability to synthesize *N*-glycans for protein

quality control, which led to the evolution to eukaryotes. The resulting eukaryotes originally acquired the ability to synthesize *O*-glycans to generate mucosal layers, which led to the evolution of metazoan; the resulting metazoa acquired the ability to synthesize GSLs for immune system, cellular differentiation, and signaling, which led to the evolution of higher animals.

51. Chemical characterization of milk oligosaccharides of the common brushtail possum

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Mammalian milk or colostrum contains from a trace to 10 % of carbohydrate in which the disaccharide lactose (Gal(β1-4)Glc) usually predominates over lower concentration of a variety of oligosaccharides; these mostly have a lactose unit at their reducing ends. In the milk of marsupials, however, oligosaccharides usually predominate over free lactose. Marsupials oligosaccharides have been characterized in the tammar wallaby, red kangaroo and koala. The neutral oligosaccharides of milk of the tammar wallaby consist of a major series of galactosyllactoses ranging from Gal(β1-3)Gal(β1-4)Glc to Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-3)Gal(β1-4)Glc and a minor series of branched oligosaccharides containing β(1–6) linked GlcNAc including Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-4)Glc (lacto-*N*-novopentaose 1). The acidic oligosaccharides of milk of the red kangaroo were found to contain non reducing *N*-acetylneuraminic acid or sulphate at OH-3 of non reducing Gal residues; their core structures were similar to the core structures of the neutral milk oligosaccharides of the tammar wallaby. To clarify the homology and heterogeneity of milk oligosaccharides among marsupials, twenty one oligosaccharides of the milk carbohydrate fraction of the common brushtail possum were characterized in this study. Neutral and acidic oligosaccharides were separated from the carbohydrate fraction of mid-lactation milk and characterized by ^{1}H-nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Six of the seven neutral oligosaccharides were identical to those that had been characterized in tammar wallaby milk, while the seventh was a novel hexasaccharide, Gal(β1-3)[Gal(β1-4)GlcNAc(β1-6)]Gal(β1-3)Gal(β1-4)Glc. Most of the acidic oligosaccharides were found to be identical to those that had been characterized in red kangaroo milk.

52. Evolutionary studies of lysosomal β-glycosyl hydrolases in various aquatic organisms

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Lysosomal β-glycosyl hydrolases are enzymes that able to hydrolyze glycosidic bonds in oligo- or polysaccharides and heteroglycosides (proteoglycans, glycoproteins, glycolipids) performing a variety of functions in living organisms: energy, structural, biological recognition and communication *et al*. They are widely used in all organisms, but it is supposed that their activity should differ depending on the species. The aim of the present study was to investigate and compare the activity of 3 lysosomal glycosidases (β-glucosidase, β-galactosidase, β-glucuronidase) in the liver/digestive gland and gills of blue mussels *Mytilus edulis L.* and fish species - pike *Esox lucius L*., whitefish *Coregonus lavaretus L.*, roach *Rutilus rutilus L.*, perch *Perca fluviatilis L*. The activity of β-glucosidase and β-galactosidase was several times higher in the digestive gland and gills of mussels in comparison with fish liver, while the activity of β-glucuronidase was significantly lower. The main strategy of adaptation for bivalves to get isolated from the environment is to close shell valves and minimize the metabolism. The transition to anaerobic energy supply and regulation of metabolic reconstructions require a high glucosidase and galactosidase activity. Fishes use other mechanisms of biochemical adaptation and β-glucuronidase plays a more important role in their metabolism. The research was carried out using the facilities of the Equipment Sharing Centre of the Institute of Biology, KarRC of RAS and supported by State Project №51.3, Grant of the Russian Federation President "Leading scientific school of Russia" 1642.2012.4 and 1410.2014.4; Program of Russian Academy of Sciences "Living nature 2012-2014"; Program of Russian Academy of Sciences "Origin of life 2012-2014"; COOPENOR: "Combined effects of Petroleum and the Environment in bivalves from the Norwegian-Russian Arctic".

Developmental and Stem Cell Biology

Keynote Lectures

53. The role of (poly)sialic acid in morphogenesis and organ function

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Cells from the outside are sugar. This is a standing rule in biology for which exceptions do not exist. Their

unparalleled permutation capacity makes sugars (glycans) the destined elements for cellular communication. Emphasis for this to be the case is provided by the rapidly growing group of congenital disorders of glycosylation, manifesting as severe multi-systemic diseases including dysmorphic and neuropathological symptoms. A sugar tightly associated with developmental processes is sialic acid and its polymeric form, polysialic acid. Using reverse genetics in different animal models, we are investigating the contribution of central (*i.e.* CMP-sialic acid synthetase and CMP-sialic acid transporter) and peripheral (*i.e.* sialyltransferases and polysialyltransferases) elements of the sialylation pathway in ontogenetic programs. Moreover, in collaboration with human geneticists, we use forward genetic approaches to deduce the role of these factors in disease development. In this latter area we profit from the possibility to derive induced pluripotent stem cells (iPSCs) from patient fibroblasts. In the frame of Glyco23, data obtained in conditional animal models and by the use of iPSCs from patients exhibiting defects in enzymes of the sialylation pathway will be presented.

54. Investigation of a novel probe for pluripotent stem cells rBC2LCN and its applications to regenerative medicine

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Human pluripotent stem cells (hPSCs), represented by embryonic stem (hESCs) and induced pluripotent stem cells (hiPSCs), are attracting increasing attention in various research fields. However, their application to regenerative medicine must overcome a critical issue given that they are basically tumorigenic. This inherent problem becomes more significant as the number of transplanted cells becomes larger. In this presentation, our recent findings concerning a novel cell surface glycan marker for hPSCs (rBC2LCN) are described, as well as attempts to its practical application to regenerative medicine. In line with a central concept in the current glycoscience, we assume that cellular glycomes are closely related to not only cell types and states but also their functions. Based on this premise, hESCs and hiPSCs were analyzed by an advanced glycan profiling technology, high-density lectin microarray. As a result, all human iPSC lines (~130) derived from different tissue origins showed essentially the same glycan profiles to one another and hESCs, which were typified by several characteristic structural features, represented by apparent shift from α2-3 to α2-6sialic acid. In addition, a recombinant lectin probe, rBC2LCN, which shows rigorous specificity to H type 1 and 3 glycan structures, was found to serve as an excellent probe for hPSCs. Hence, we focused our efforts on this novel lectin probe to promote realization of regenerative medicine.

Lectures

55. Glycosphingolipids in human embryonic stem cells

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Background: Carbohydrate epitopes are often used as markers for characterization of human embryonic stem cells (hESC) and induced human pluripotent stem cells (hiPSC). In this project, glycosphingolipids from hESCs are structurally characterized. Experimental: Human ESC lines were generated from left-over *in vitro* fertilized embryos. Cells were cultured in mono-layer cultures under serum and feeder free conditions. The glycosphingolipids were isolated from a relatively high number of cells (1×10^9 cells/cell line) allowing separation of the glycosphingolipids into total non-acid and acid fractions, and further separation into sub-fractions. The fractions were structurally characterized by mass spectrometry, ^{1}H NMR and antibody binding. Results: In the non-acid fractions, several novel blood group H, Lex and Ley compounds based on neolacto core chains were characterized, in addition to the already identified lacto- and globo-series glycosphingolipids. Moreover, a blood group A type 1 hexaosylceramide was identified. The acid glycosphingolipid fractions contained several novel hESC acid glycosphingolipids, like the gangliosides sialyl-lactotetraosylceramide and sialyl-globotetraosylceramide, and the sulfated glycosphingolipids sulfatide, sulf-lactosylceramide and sulf-globopentaosylceramide. The distribution of sialyl-lactotetraosylceramide and sulfated glycosphingolipids in hPSC was explored by flow cytometry, immunohistochemistry and electron microscopy. A high cell surface expression of sialyl-lactotetra on hPSC was demonstrated, whereas the sulfated glycosphingolipids were restricted to intracellular compartments. During differentiation of hiPSC into hepatocyte-like cells a rapid down regulation of the sialyl-lactotetra epitope was found. Conclusion: Human ESCs express a complex pattern of glycosphingolipid epitopes, including blood group antigens. The sialyl-

lactotetra carbohydrate sequence is a promising novel marker for human pluripotent stem cells.

56. Unraveling the *N*-glycosylation of induced pluripotent stem cells from a PMM2-CDG patient

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PMM2-CDG, previously known as Congenital Disorder of Glycosylation-Ia, is characterized by low activity of the enzyme phosphomannomutase 2 (PMM2). That results in decreased synthesis of lipid-linked *N*-glycan precursors in the ER and consequently in the absence of one or more *N*-glycan chains on glycoproteins. It is known that *N*-glycans have many crucial roles in the "lifetime" of a protein, from the initial quality control over trafficking, to regulation of its function and stability. Therefore, it is not surprising that in PMM2-CDG many biological functions are compromised, giving rise to a wide range of severe multisystemic clinical conditions. Currently very little is known regarding the pathophysiological consequences of altered *N*-glycan synthesis in PMM2-CDG. However, the discovery that differentiated cells can be reprogrammed to pluripotency and other cell fates opened a remarkable possibility to study the molecular mechanism of the disease from the very beginning—the stem cell level. For this reason, to better understand the impact of reduced PMM2 activity on early human development, induced pluripotent stem cells (iPSCs) from PMM2-CDG patient-specific fibroblasts (PMM2-iPSCs) were generated. We questioned whether *N*-glycomic changes occur already at the embryonic stage. A comprehensive *N*-glycan analysis by multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF) revealed that PMM2-iPSCs bear—as expected—a "normal" *N*-glycan repertoire, with a predominance of high-mannose *N*-glycans. Mannose-specific GNA-lectin staining of PMM2-iPSCs as well as a quantitative xCGE-LIF-based approach unveiled hypoglycosylation of proteins compared to control stem cell lines. Thus, the PMM2-iPSC model helps us to decipher *N*-glycosylation on the stem cell level. Further, it has a potential to extend our understanding of the *N*-glycan-dependent pathogenesis of PMM2-CDG, as well as the role of glycosylation during early human development.

57. Proteomic and glycomic analysis of extracellular matrix produced by murine stromal cells under hypoxic and normoxic conditions

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Stem cell biology relies on the proximity of multiple cell types for differentiation, growth and communication. Extracellular matrices (ECMs) are important in determining the interaction of the cells with their microenvironment via cell signaling and also define the shape and stability of tissues. MS-5 cells represent a continuously growing clone of mouse mesenchymal stromal cells (MSC) and support human hematopoietic stem and progenitor cell survival and differentiation. It has been reported that the environment and conditions under which stromal cells synthesize their ECM influences the differentiation fate of overlying cultured stem cells. We have previously shown that the hypoxic environment influences the differentiation capacities of MS-5 and other continuously growing mouse MSC lines. This aim of this work was to investigate how hypoxia affects the glycosylation of MSC-derived ECM components. Proteomic analysis was carried out on the decellularized MS-5 ECM prepared under normoxic and hypoxic conditions and glycosylation alterations were inferred by differentially regulated glycosyltransferases. Lectin histochemistry, blotting and sialic acid analysis were carried to further investigate these findings. Finally, Western blotting was done to identify proteoglycans and glycoproteins that carried the altered glycosylation. *In silico* analysis showed that one of the most relevant pathways affected by hypoxia was the ECM receptor interaction pathway and the altered glycomic profile focused on modifications to molecules involved in cellular signaling. This work has implications for the maintenance of stemness by stromal cells and for understanding the complex network of communication between MSC and ECM molecules.

58. Glycosylation related genes involved in regulation of pre-adipogensis of murine satellite cells

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The skeletal muscle satellite cells localized between the myofiber and the lamina membrane are considered as adult stem cells. These cells ensure the post-natal growth and the regeneration of skeletal muscle. The satellite cells could be differentiated in myoblast, preadipocyte or osteoblast, this multipotency allows comparing the behavior of a cell type committed in different differentiation pathways. It is well known that the activation and the differentiation of satellite cells are modulated by their environment, which is called "niche", and which includes particularly the glycanic structures of cell components. Glycans present in the extracellular matrix and/or at the cell surface could contribute to the cell differentiation. For instance, it was demonstrated that *O*-GlcNAcylation inhibits skeletal muscle differentiation. In a previous study, we analyzed 383 glycosylation related genes during murine satellite cell differentiation and among them, 31 displayed an important up- or down-regulations. In the present study, we used murine satellite cells and their multipotency to identify glycosylation related genes whose expression level was specifically modified during the pre-adipogenesis. We quantified the transcripts amount of 383 glycosylation genes previously analyzed and revealed that the expression of 56 among them was specifically modified during early adipogenesis. According to the encoded protein function, we analyzed the amounts of *N*-glycans and their sialylation, we showed that *N*-glycans availability and α(2,6) linked sialic acids were significantly decreased during the differentiation of MSC. We also observed the changes in ECM and found a high amount of heparan sulfates in adipogenic differentiating cells only. We evidenced that when MSC differentiation was oriented to early adipogenesis, the myogenic differentiation process was inhibited as a consequence of *N*-glycan degradation and of heparan sulfate increase at the cell surface.

59. T antigen is essential for the maintenance of hematopoietic stem cells in *Drosophila*

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Core 1 β-1,3-galactosyltransferase (C1β3GalT), an evolutionarily conserved enzyme, transfers Gal to GalNAcα1-*O*-Ser/Thr, to synthesize a Galβ1-3GalNAcα1-*O*-Ser/Thr (T antigen). Various studies using C1β3GalT mutants have shown that loss of *C1β3GalT* is associated with a wide range of defects. We have previously reported that *Drosophila* embryonic hemocytes have T antigens, and that the number of embryonic plasmatocytes is reduced in *dC1β3GalT1* mutant. In mice and human, loss of T antigen caused thrombocytopenia. However, the precise roles of T antigen in hematopoiesis were largely unknown. Here we show that the *Drosophila* T antigen, supplied by plasmatocytes, is essential for the regulation of hematopoietic stem cells (HSCs). HSCs are in hematopoietic organs and differentiate into mature blood cells as required. The lymph gland, the larval hematopoietic organ, contains three domains: the posterior signaling center (PSC), the medullary zone (MZ), and the cortical zone (CZ). The MZ consists of HSCs; the CZ contains differentiated blood cells including plasmatocytes; and the PSC is viewed as the stem cell niche. In *dC1β3GalT1* mutant larvae, the formation of proper filopodia from the PSC to the HSCs was disrupted. Then the numbers of HSCs were strongly reduced and the numbers of differentiated blood cells were increased. Rescue experiments revealed that *dC1β3GalT1* is required in the plasmatocytes for the production of a functional T antigen. The loss of filopodia was caused by the accumulation of coagulation factors. Abnormal T antigen altered the characteristics of the hemolymph, allowing the clotting factor, hemolectin, to form a shell around the lymph gland, resulting in the loss of HSCs through impairment of filopodial extension from the hematopoietic niche. This study has identified novel functions for mucin and hemolymph. It also indicates that the signals from the hematopoietic niche to HSCs that are essential for the maintenance of HSCs are transmitted by filopodia.

Posters

60. *O*-GlcNAc transferase is required for fidelity in developmental patterning

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The dynamic posttranslational modification *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) governs cell signaling, protein turnover, and gene expression. The enzymes *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) regulate the addition and removal of the modification to and from Ser/Thr residues, respectively. Proper *O*-GlcNAcylation is critical for organismal development and knockout of OGT and OGA in mammals is lethal. *C. elegans* ogt-1 interacts genetically with players in Hox gene regulation including bar-1 and pal-1, the

homologs of β-catenin and *Drosophila* caudal, respectively. Moreover, *C. elegans* ogt-1 resides on chromosome III directly upstream of the Hox gene cluster, suggesting an evolutionarily conserved role linked to development. We hypothesize that *O*-GlcNAc "fine-tunes" the expression of genes required for the fidelity of body plan development. Utilizing the *C. elegans* model organism, we explored several phenotypes to determine whether loss of ogt-1 and oga-1 influence appropriate development of characteristic hermaphrodite and male body segments. While loss of OGT-1 or OGA-1 activity yielded mutants that lacked gross physiological phenotypes, ogt-1 knockouts are immune-compromised. In addition, hermaphrodites lacking both OGT-1 and BAR-1 activities have an everted gonad, or spew phenotype, suggesting an important intersection between nutrient-responsive OGT-1, Wnt signaling, and vulva precursor cell fate specification. Moreover, while male *C. elegans* lacking either bar-1 or ogt-1 alone have negligible V6 male ray defects, up to 43 % of bar-1; ogt-1 double mutants display development defects in the male-specific sensory organ. Our data highlight that *O*-GlcNAc plays a novel, indispensable role in *C. elegans* developmental patterning. We suggest that the addition of *O*-GlcNAc may play a key role in regulating PcG chromatin proteins required for early patterning of Hox gene repression in *C. elegans*.

61. Catabolism of sialic acid residues during pre-hatching development of medaka embryos

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Sialic acids (Sias) play important roles in early development of medaka, *Oryzias latipes*, which was established as an experimental small fish in Japan. For example, embryos are less lethal in early developmental stages, when the gene expression of the CMP-sialic acid synthetase (CSS), a prerequisite enzyme for *de novo* synthesis of sialic acid residues, was suppressed by the morpholinooligonucleotide injection. Early development of medaka starts with cortical reaction at fertilization. At the cortical reaction, cortical alveoli of unfertilized eggs undergo exocytosis to release their contents into the perivitelline space (PVS). At the same time, vitelline envelope (VE) is elevated and hardened to be changed to fertilization envelope (FE). Hyosophorin is a major cortical alveolus Sia-rich glycoprotein, and is believed to remain at the PVS until hatching after fertilization. Zona pellucida glycoproteins like ZPB and ZPC are sialylated VE components. However, the fate of these soluble and insoluble sialoglycoproteins in early embryos of medaka is largely unknown. In this study, we asked how Sia content changes during development of medaka, with special focuses on Sia content of VE/FE and hyosophorin. After hydrolysis of each sample, the Sias were quantitated by fluorometric HPLC analysis of their DMB derivatives. Most Sia residues were present in FE, and their amount started decreasing at 4 days post fertilization (dpf) to almost zero at 6–8 dpf. The Sia amount of hyosophorin in PVS decreased by about 30 % at 2 dpf, and remained unchanged until hatching at 8 dpf. It is thus concluded that the catabolism of Sia in FE and PVS differently occurs during pre-hatching development. This study was supported in part by the program for leading graduate schools IGER of Nagoya University.

62. *N*-glycosylation changes in the regeneration buds of planarian

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Our previous study showed that planarians contain high mannose-type and pouch mannose-type glycans, some of which include methylated mannose and/or galactosylated core fucose. When planarians were subjected to histochemical analysis using FITC-labeled Con A and RCA-I, most tissues were stained with the lectins. Planarians were then amputated above the anterior pharynx, and the regeneration buds were periodically stained with the lectins. In the early stages (1–4 days) of the regeneration, they were not stained with the lectins but after that stage, the stainings were recovered by 12 days after amputation. Lectin blot analysis of the membrane proteins from the regeneration buds at the 3rd day of the regeneration revealed that the binding of Con A to proteins decreases significantly when compared with that from the head section of the intact animals. These results indicate that *N*-glycosylation levels of proteins decrease significantly or structural changes of *N*-glycans occur in the regeneration buds at the early stages. The biological significance of this phenomenon remains to be elucidated. To investigate further, regenerating planarians were cultured in the presence of tunicamycin (5 μM), and they were dead. Then, planarians were amputated and cultured in the presence of Swainsonine (200 μM), and most of them completed the regeneration. When this treatment was repeated twice, some animals were dead

(18/44 bodies), and others showed abnormal behaviors (5/44), cyclopia (3/44) and headless (1/44). The rest appeared normal. Planarians showing cyclopia or headless were stained with Con A but not with RCA-I, indicating that *N*-glycans with the galactosylated core fucose residue are important for the head formation since the ladder-like nerve system is stained with RCA-I. These results indicate that *N*-glycans are important for regeneration of planarians.

63. Diamond squid (*Thysanoteuthis rhombus*)-derived chondroitin sulfate inhibits osteoclast differentiation and bone resorption activity, and promotes bone formation

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Chondroitin sulfate (CS) exists throughout the body and its structure is widely conserved across in varied species. CS is present in the supportive tissue of the central nervous system, and especially in bone and cartilage. We have reported that diamond squid-derived CS stimulates osteoblastgenesis and improves the bone formation. However, there are very few reports on the CS's effects on osteoclastogenesis. In the present study, we investigated the ability of diamond squid-derived CS on osteoclastogenesis *in vitro*. We examined and compared the effect of osteoclast differentiation and its function. In addition, we investigated that how CS regulate the osteoclast differentiation. Moreover, we injected CS to OVX mouse for 8 weeks by intraperitoneal administration and then measured the concentration of several osteogenesis related factors in serum and evaluated the tibia of the mice histologically. CS significantly inhibited the increase of osteoclast specific marker, TRAP-positive multinucleated cells and had a negative effect on increasing osteoclast size. Pit formation assay revealed that CS also suppressed bone resorption activity. Whereas, chondrotinase ABC (ChABC)-digested CS did not show the inhibitory activity of osteoclastogenesis and its function. Quartz-crystal microbalance analysis clarified that CS had the binding ability to the initiator of osteoclastogenesis, RANKL and interrupted the binding of RANKL to RANK. Furthermore, CS reduced the phosphorylation of ERK in pre-osteoclast cells. These results indicated that CS suppressed both osteoclastogenesis by binding to RANKL and increasing osteoclast size. Moreover, CS inhibited RANKL-induced signal pathway, which resulted in decrease of the bone resorption area. Moreover, 8-week administration of CS to OVX mouse revealed that osteoclastogenesis was down-regulated and osteogenesis in the bone was promoted. These results suggested that CS is a promised substance to promote bone formation.

64. Analysis of zebrafish polypeptide *N*-acetylgalactosaminyltransferase genes during embryonic development

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Mucin-type *O*-glycosylation is one of the most common post-translational modifications of proteins and has diverse physiological functions. The initial biosynthesis of mucin-type carbohydrates is catalyzed by by a group of enzymes, polypeptide α-*N*-acetylgalactosaminyltransferases (GalNAc-Ts), which consist of a large gene family with 20 isozymes in humans. Among them, GalNAc-T8, −T9, −T17, and -T18 consist of a subfamily that have no or very low *in vitro* transferase activities when assayed with typical mucin peptides as acceptor substrates. Although these four isozymes have been reported to be involved in various diseases, their *in vivo* functions have not been reported. To elucidate their functions in zebrafish, we first isolated their galnt cDNAs from total RNA and verified the sequences. The analysis demonstrated that they encode for the zebrafish ortholog genes as judged by the conservation of domain organization and the characteristic replacements as exemplified by substitution of Trp for a Tyr residue in the Gal/GalNAc-T domain. We found that zebrafish has two orthologs (galnt18a and galnt18b) for Galnt18, and an ortholog (galnt8, galnt9, and galnt17) for each of other three isozymes. The galnt8 was expressed in the cephalic mesoderm and hatching gland during early developmental stages, and differently expressed in the head, somatic muscles, and liver in the later stages. The other three orthologs also exhibited the characteristic expression patterns, although their expressions were generally strong in the nervous systems. In addition to the expression in the brain, galnt17 and galnt18a were expressed in the somitic muscles, and galnt18a and galnt18b in the notochord. The information on the isozyme expression patterns, when used together with the gene knockdown/

knockout technology, will help clarify the functions of the isozymes in zebrafish. We are generating zebrafish mutants lacking a galnt gene using methods such as TALEN- and CRISPR/Cas-systems.

65. Sufficient provision of CMP-Sialic acid is necessary for growth and development of medaka fish

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Sialic acid (Sia) residues are expressed on glycan chains of glycoproteins and glycolipids in a developmental stage-specific and an organ-specific manner. However, it has largely remained unknown how significant the spatiotemporal expression of Sia is at animal level. To solve this question, we have tried to generate CMP-Sia synthetase (CSS)-mutated medaka fish using the targeting induced local lesions in genomes (TILLING) method. CSS catalyzes the synthesis of CMP-Sia, which is the only donor substrate of all sialyltransferases, and is prerequisite for the expression of Sia-glycoconjugates. A single gene for CSS is present in the genome of medaka. Like other vertebrate CSS, medaka CSS consists of the N- and C-terminal domains that are responsible for the catalytic activity and the oligomerization of the enzyme, respectively. After we screened the TILL ING library for selecting gametes with mutations in the exons of CSS gene, we found that M1 and M2 mutants, whose CSS contain mutations in N- and C-terminal domains of CSS, respectively, had lower *in vitro* activity. The *in vitro* activity of M1 CSS was greatly decreased to *N*-acetylneuraminic acid (Neu5Ac), while unchanged to *N*-glycolylneuraminic acid (Neu5Gc), indicating that this particular mutation is important for the Neu5Ac recognition. Interestingly, significant abnormalities were observed in M1 mutant embryos, suggesting that the Neu5Ac recognition is important for embryogenesis in medaka. M2 CSS showed half of the *in vitro* activity of wild-type CSS. The M2 mutant fish could not survive beyond 2 months and died as young fry. These results indicate that sufficient supply of CMP-Sia or Sia-glycoconjugates is necessary for normal growth and development. This research was supported in part by the program for leading graduate schools IGER of Nagoya University.

General Glycoscience

Keynote Lectures

66. Siglecs: discovery, classification, evolution and functions

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Sialic Acids have long been known as key components of highly specific binding targets for microbial receptors and toxins. However until the 1990's there were very few examples of vertebrate proteins recognizing sialic acids, and even the known ones such as complement factor H and selectins appeared to mostly detect the negative charge of these terminal monosaccharides. The independent discovery of Sia-binding properties of sialoadhesin and of CD22 and the subsequent cloning of sialoadhesin led to the definition of a new family of cell-type specific vertebrate lectins initially called "sialoadhesins", which were eventually designated as a subfamily of I-type lectins and finally re-designated as the Siglecs. A sub-group of CD33-related Siglecs (CD33rSiglecs) widely expressed on immune cells were then noted to be very rapidly evolving, sometimes showing no clear-cut orthologs between rodents and primates, just "functionally equivalent paralogs". This talk will provide a brief historical background to this rapidly expanding field, and then focus on functions of the CD33rSiglecs and their roles in modulating the innate immune response, often via recognition of sialoglycans as "Self-Associated Molecular Patterns" (SAMPs), as well as via recognition of certain newly discovered Sia-independent ligands. Attention will be given to the more recent discovery of activating counterparts of some inhibitory CD33rSiglecs, which are maintained as "paired receptors" via concerted evolution (repeated gene conversion events) and to the remarkable and frequent microbial mimicry of SAMPs, a highly successful immune evasion strategy. The presentation will include a description and discussion of the high frequency of uniquely human genetic changes in the CD33rSiglecs, events that are either fixed or remain polymorphic in our species. Implications for unique aspects of human evolution and disease will also be considered.

67. Epigenetic regulation of protein glycosylation: implications for complex diseases

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Glycan, the important structural part of a glycoprotein, is synthesized through complex biochemical pathway involving many genes. Therefore, final glycan structure is considerably influenced by external and intrinsic factors where epigenetic mechanisms play mediator role between environment and the glyco-gene expression. Many glyco-genes with a role in normal development are epigenetically regulated, and epigenetic deregulation of glyco-genes was shown to occur in many types of cancer and other complex diseases. By analysing expression and methylation in parallel on several datasets representing different types of cancer we found the intersection of the glyco-genes with altered expression/methylation suggesting that galactosylation, sialylation, fucosylation and mannosylation were highly perturbed in cancer. We have shown as well that epigenetic deregulation of other glycosylation-related genes, such as transcription factors, has an effect on *N*-glycome composition and the disease outcome. Using different epigenetic inhibitors on cells in culture we have shown that many glyco-genes are regulated by DNA methylation and that epigenetic modulation alters glycome composition. Besides studying epigenetic regulation of the plasma *N*-glycome, we focus also on genetics and epigenetics of a single protein glycosylation. Recently, the first genome wide association study of the IgG glycome identified genetic loci other than classical glyco-genes associated with IgG glycosylation. Some of these GWAS hits showed pleiotropy with several inflammatory and autoimmune diseases where IgG glycosylation changes were also reported, suggesting that changes in IgG glycosylation might have functional implications for these diseases. We believe that variation in IgG glycosylation plays an important role in susceptibility for and progression of these diseases and that the pattern of IgG glycosylation is to some extent epigenetically fixed within a clone of B-lymphocytes.

Lectures

68. Deconstruction of the *N*-glycan maturation pathway in the model legume *Lotus japonicus* paves the way for further understanding of protein glycosylation

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Post-translational modifications are covalent attachments to the protein and these modifications often have a large impact on protein/enzyme functionality, activity, and stability. We have identified 19 different *N*-glycan structures from the seed globulin fraction of *Lotus japonicus* using a glycomic approach. From the *Lotus* LORE1 mutant population we obtained knockouts for all genes encoding the 12 enzymes in the *N*-glycan maturation pathway in *Lotus* and for most of these enzyme-genes we have several independent knockout plant lines. The differences in the *N*-glycan structure pattern between the wild type and mutants were determined by MS and using *N*-glycan specific antibodies for immunoblotting. Clear plant developmental phenotypes were observed for three of the *N*-glycan maturation enzyme mutants. Furthermore, differential transcript levels of the *N*-glycan maturation pathway genes were detected between different tissues including seeds and root nodules indicating a different *N*-glycan composition in these tissues. We will elucidate the impact of these differences in the corresponding mutants. Three genes are coding for hexosaminidase in both the *Lotus* and *Arabidopsis* genomes. Two of the *Lotus* hexosaminidase mutants show differentially *N*-glycan structure patterns from the immunoblot experiments and we are currently trying to identify the corresponding glycoproteins. Finally, this *N*-glycan mutant population is used to determine the impact of plant *N*-glycosylation on food allergy. This work was supported by the Danish Council for Independent Research | Technology and Production Sciences (FTP) and the Danish National Research Foundation grant no. DNRF79.

69. Glycoconjugates of antimicrobial peptide as a strategy for improving activity and reducing toxicity

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Antimicrobial peptides (AMPs) or host defense peptides are found widely in nature, where they form part of the innate immune response against bacterial infection. AMPs hold great promise as potential biopharmaceutical drugs for the treatment

of infections. However, clinical applications of AMPs have to overcome a number of significant challenges, in particular pertaining to activity, toxicity, and selectivity towards bacterial membranes. We have designed glycoconjugates displaying an improvement in antimicrobial activity by coupling of multiple copies of AMP monomers to a linear, biocompatible carbohydrate polymer, chitosan, wherein the AMP exerts its activity in a multivalent fashion. We report our findings in grafting the antimicrobial decapeptide anoplin onto chitosan polymers, through azido moieties anchored on the 2-amino groups, by using copper-catalyzed alkyne-azide coupling (CuAAC) chemistry. The generated conjugates consist of a chitosan backbone to which is coupled a number of individual anoplin peptides, which are positioned in parallel from the chitin backbone. Surprisingly, we have found that not only does this approach lead to an enhancement of antimicrobial potency, but at the same time these constructs are highly selective and essentially non-hemolytic. Our results indicate that enhancements in antibacterial activity, *e.g.*, against *E. coli* or *S. aureus*, are promoted by the ability of the chitosan conjugates to form intramolecular, pore-forming clusters of alpha-helical anoplin. The toxicity towards red blood cells (HC50) is reduced by more than two orders of magnitude for the best conjugates.

Posters

70. Epigenetic silencing of HNF1a modifies glycosylation

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The diversity and number of genes regulated by transcription factor HNF1a is very large and one of them is also GnT4a gene coding for glycosyltransferase MGAT4a. The importance of this glycosyltransferase manifests itself in effect of glycosylation on structure, half-life, stability, function and positioning of proteins such as GLUT II, glucose transporter on surface of β cells of Langerhans islets. Impaired function of GLUT II is associated with metabolic disorder, MODY III, which is, on the other hand, linked to methylation status of 4 CpG sites in promoter of HNF1α. Since this transcription factor has a significant role in cell development, there is a prerequisite of quick and fine tuned regulation of its expression that can be achieved priorly by epigenetic modifications. Accordingly, suggested epigenetic cause of GLUT II malfunction, mediated by glycosylation, is highly probable. Still, it has yet to be shown by ongoing analysis via pyrosequencing of PCR amplified, bisulphite-converted DNA isolated from laser microdissected β cells of Langerhans islets obtained from formalin fixed paraffin embedded pancreatic tissue.

71. The Amadori rearrangement: a versatile reaction for the synthesis of neoglycoconjugates

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The Amadori rearrangement - a reaction between aldoses and amines which leads to *C*-glycosyl type 1-amino-1-deoxy ketoses - was investigated as a potential method for the conjugation of carbohydrate moieties to suitable amines without the requirement of any protecting group manipulations. We have demonstrated that this reaction is a versatile synthetic method towards *C*-glycosyl type neoglycoconjugates when considering both, the nature of sugar substrates as well as of amino components. For example, starting from aldoheptoses the corresponding 1-amino-1-deoxyheptuloses have been obtained in a straight forward manner and in excellent yields. Now we are investigating the Amadori rearrangement for its scope and limitation concerning the sugar configuration as well as amino moieties in terms of synthesizing biologically relevant glycoconjugates. Experimental details of this evaluation will be presented.

72. Epigenetic modulation of *N*-glycome from HepG2 cells

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Protein *N*-glycosylation is an important posttranslational modification which affects protein structure and function. A great amount of plasma proteins are synthesized in liver and changes in the plasma glycans are often associated with different types of liver diseases, including hepatocellular carcinoma (HCC). HepG2 cell line is a hepatocellular carcinoma cell line and its secretome is comparable with secretomes of HCC patients. Using different concentrations of DNA methylation inhibitors

(5-aza-citidine, 5-aza-2-deoxycitidine and Zebularine) we have induced hypomethylation in HepG2 cells. Following 72 h treatment with the inhibitors, HepG2 cell population was evaluated for survival, cell cycle stage, and karyotype changes, and glycans were measured from the HepG2 secretome using HILIC and MS. The treatment with all three epigenetic inhibitors resulted in changed DNA methylation and glyco-gene expression level, as well as in *N*-glycome composition of HepG2 secretome. The presented work contributes to our understanding of DNA methylation inhibitor efficiency in altering the *N*-glycan profiles of secreted proteins, but also shows the need for precise dosage of known epigenetic therapies.

73. Enantioselective synthesis of spiroacetal from aldose via tandem asymmetric aldol-aldol reaction

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Stereoselective aldol reaction of acetone with aldose using methyl 2-deoxy- 2-prolinamido-α-D-glucopyranoside as a water compatible prolinamide catalyst afforded higher-carbon ulose in aqueous solution. The tandem asymmetric aldol - aldol reaction has been observed in the reaction of acetone with isopropylidene protected aldose using the catalyst in aqueous solution. Pure α,α'-dideoxynonulose was obtained as a sole product from the reaction of acetone with two molar excess of isopropylidene-D-glyceraldehyde in one pot process. Following acid treatment accomplished the enantiospecific synthesis of (6R)-[5,5]spiroketal as a synthetic precursor for the pheromones of olive fruit fly, whose structure was confirmed by X-ray crystallography. From the tandem reaction with di-*O*-isopropylidene-D-arabinose α,α'-dideoxytrideculose was isolated and derived to enantiomerically pure (6S)-[5,5]spiroketal. The enanntiospecific synthesis of [5,5]spiroketal was accomplished in one pot process for tandem aldol reaction, and consequtive one pot deprotection/spiroacetallization. The characteristic configuration of parent aldose contributed enantiospecific formation of such spiroacetals were configuration.

74. Aberrant methylation and expression of glyco-genes in cancer

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Changes in glycosylation patterns are often associated with malignant cell transformation and tumor progression. Data about epigenetic deregulation of protein glycosylation in cancer are accumulating; however, there is no comprehensive analysis of the impact of epigenetic changes of glyco-genes in cancer. We present an analysis of changes in DNA methylation level and expression level of glyco-genes in different cancer types based on the publicly available expression and methylation datasets. Our analysis revealed that GALNT9 (*N*-acetylgalactosaminyltransferase 9) and MGAT5B (*N*-acetlyglucosaminyltransferase 5) are the genes most frequently demethylated in different types of cancer. On the other hand, among genes with the highest difference in expression between normal and cancer tissue, there were several genes from the family of *N*-acetylgalactosaminyltransferases (GALNT). We conducted a parallel expression and methylation analysis on several unrelated datasets representing hepatocellular carcinoma, breast cancer, melanoma and cervical cancer. The intersect of the genes with altered expression/methylation in different cancer types suggest that the process of galactosylation is highly perturbed. Moreover, the GALNT6 gene appears to be among the top ranked genes on both lists (differential promoter methylation and expression), which suggest its regulation by CpG methylation. The pathways of sialylation, fucosylation and mannosylation were also found to be perturbed in cancer tissue. Some of the involved genes seem to be epigenetically regulated by methylation.

75. Semi-high-throughput isolation and *N*-glycan analysis of human fibrinogen

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Fibrinogen is a glycoprotein that is synthesized in the liver and has a key role in blood clotting. It contains three polypeptide chains (α, β, γ) each in duplicate, linked together by disulfide bonds. In recent years many studies on IgG and plasma glycosylation have been performed, while glycosylation of fibrinogen is not very well studied and understood. A recently developed 8-well (out of 96-well) plate with immobilized antibodies for human fibrinogen was used for fibrinogen isolation from plasma samples of 10 healthy

volunteer individuals, as well as from in house plasma pool (standard). Isolated fibrinogen was desalted by precipitation with cold methanol, denatured and deglycosylated with PNGase F. Released *N*-glycans were labeled with fluorescent dye, 2-aminobenzamide (2-AB), and cleaned-up on hydrophilic GHP plate. Labeled 2-AB glycans were analysed by hydrophilic interaction liquid chromatography (HILIC) on ultra performance chromatography system (UPLC) with acetonitrile and 100 mM ammonium formate as mobile phases. Samples were separated into 24 chromatographic peaks and % area for each peak calculated. Variation in the amount of individual fibrinogen glycans among 10 individuals was shown to be greater than variation of the same glycans in standards, which opens the question about the extent of fibrinogen *N*-glycosylation variability among individuals in larger population and also between different populations. To our knowledge, this is the first analysis of *N*-glycosylation of fibrinogen isolated from human plasma in semi-high-throughput way.

76. Exploring the stability of IgG and plasma glycosylation within healthy individuals

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To explore the stability of IgG and Plasma glycosylation within healthy individuals as precisely as possible it is necessary to derive an experimental design that will take into account variability between individuals, together with the experimental variability of the procedures used for the experiments. Exploring the experimental variability is a tedious task but extremely important. A recommended solution is to use replicates together with the expert knowledge about possible pitfalls in the experimental procedure. This leads to an experimental design where multiple plates and conditions should be used. Variability between individuals can be incorporated into the experimental design by taking into account possible sources of variability within a population. Previous research has found traits like age and gender to be of utter importance together with the possibility of a high influence from the women menstrual cycle. This poster will present the results obtained from such an experiment where the experimental sources of variation were different analysts doing the sample preparation, time between batches of experiments and machine resolution; while sampling of a population was based on age, gender and menstrual cycle. Ten people in total entered the experiment, blood was sampled three times during 10 days and everything was done in triplicates within a plate, with 6 plates for IgG and 6 plates for Plasma samples.

77. Lipopolysaccharides of *Pseudomonas chlororaphis* and *Pantoea agglomerans*: chemical characterization and biological activity

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Lipopolysaccharides (LPS) from two strains of *Pseudomonas chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306 (isolated from soil and cabbage rhizosphere respectively) and seven strains of *Pantoea agglomerans* isolated from a number of plants (tomato, apple tree, oat seeds, cotton) were chemically characterised and its biological activity was studied. LPS of the *P. agglomerans* strains were heterogeneous in monosaccharide composition. Thus, the LPS of *P. agglomerans* 8606 differed considerably from the LPSs of other strains, containing mannose as the predominant monosaccharide (69.8 %), as well as ribose (15.1 %) and xylose (12.6 %), while the content of rhamnose, one of the predominant monosaccharides in other LPS samples, was 2.5 %. Analysis of the fatty acid composition revealed the presence of C12 – C16 acids. In lipids A of all strains tested, 3-OH-C14:0 was the predominant acid (31.7 to 39.1 %, depending on the strain). C12:0 (8.2 to 31.5 %), C14:0 (12.9 to 30.8 %), and C16:0 acids (3.4 to 16.9 %) were also revealed. All LPS tested are characterized by low toxicity however high pyrogenicity and displayed the activity of antigen in homological systems. There were no cross-reactions that indicate the absence of common antigenic determinants. The structure of the *O*-polysaccharides of *Pseudomonas chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306 were established. They are built up from the linear tri- or tetra-saccharide repeating units, respectively. LPS of *Pseudomonas chlororaphis* subsp. *aureofaciens* UCM B-111 and UCM B-306 show high antiviral activity against both plants and human viruses. So the LPSs of both strains inhibited tobacco mosaic

virus infectivity for three species of indicating plants (*Datura stramonium L., Nicotiana tabacum, N. sanderae H.*) in concentrations 0.001–10 mg/ml. Moreover, it was shown for the first time that the LPSs tested are effective agents against 2 type herpes virus. The LPSs inhibited the virus reproduction.

78. Synthesis of the pentasaccharides related to the fucoidan from the seaweeds *Chordaria flagelliformis*

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Sulfated polysaccharides fucoidans from brown seaweeds possess the different types of biological activity such as anticoagulant, antithrombotic, anti-inflammatory, antiangiogenic and other activities. Fucoidan chains are built up mainly of sulfated α-L-fucopyranose residues, but the fine structure of these biopolymers varies depending on seaweed species and growth conditions. Types of glycoside bonds, degree and pattern of sulfation, presence of branches and non-fucose residues were shown to influence on the biological effect of fucoidans. To establish the structure-activity relationship in a group of fucoidans we perform the synthesis of the oligosaccharides related to these biopolymers from different brown seaweed species. Here we report the synthesis of the non-sulfated and selectively sulfated pentasaccharides related to the fucoidan from the seaweeds *Chordaria flagelliformis*. These compounds consist of the (1→3)-linked α-L-fucotrioside backbone, the second unit of which is substituted at O-2 by α-L-fucofuranosyl-(1→4)-α-D-glucuronyl fragment. Stereoselective (1–2)-*cis*-glycosidic bonds formation was achieved using remote acyl group participation strategy. Notably, that stereoselective introduction of α-L-fucofuranosyl residue was reported for the first time. NMR data of the obtained compounds were shown to coincide with the signals of the respective residues in a structure of the fucoidan from *Chordaria flagelliformis*. This fact indicates the similarity of the conformations of the studied oligosaccharides and the respective fragments in the polysaccharides. Therefore, the synthetic oligosaccharides could be considered as model compounds to study the mechanism of biological action of fucoidans. This work was supported by RSF grant 14-23-00199.

79. Molecular basis of sugar recognition by CL-K1 and the effects of mutations associated with 3MC syndrome

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Collectin-Kidney1 (CL-K1) is a multifunctional Ca-dependent lectin with roles in innate immunity, apoptosis and embryogenesis. It binds to carbohydrates on pathogens to activate the lectin pathway of complement and together with its associated serine protease MASP-3. High serum levels in CL-K1 are associated with DIC, where spontaneous clotting can lead to multiple organ failure. Autosomal mutations in the CL-K1 or MASP-3 genes cause a developmental disorder called 3MC (Carnevale, Mingarelli, Malpuech and Michels) syndrome, characterized by facial, genital, renal and limb abnormalities. One of these mutations (Gly204Ser in the CL-K1 gene) is associated with undetectable levels of protein in the serum of affected individuals. In this study, we show that CL-K1 primarily targets a subset of high-mannose oligosaccharides present on both self- and non-self structures, and provide the structural basis for its ligand specificity. We also demonstrate that three disease-associated mutations prevent secretion of CL-K1 from mammalian cells, accounting for the protein deficiency observed in patients. Interestingly, none of the mutations prevent folding nor oligomerization of recombinant fragments containing the mutations *in vitro*. Instead, they prevent Ca binding by the carbohydrate-recognition domains of CL-K1. We propose that failure to bind Ca during biosynthesis leads to structural defects that prevent secretion of CL-K1, thus providing a molecular explanation of the genetic disorder. We have established the sugar specificity of CL-K1 and demonstrated that it targets high-mannose oligosaccharides on self- and non-self structures via an extended binding site which recognizes the terminal two mannose residues of the carbohydrate ligand. We have also shown that mutations associated with a rare developmental disorder called 3MC syndrome prevent the

secretion of CL-K1, probably as a result of structural defects caused by disruption of Ca binding during biosynthesis.

80. The Amadori rearrangement as key step for the synthesis of biologically relevant *C*-glycosyl type neoglycoconjugates

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The Amadori rearrangement is the reaction of aldoses with suitable amines leading to the corresponding 1-amino-1-deoxy ketoses. This reaction allows the conjugation of carbohydrate moieties to amino compounds in a *C*-glycosyl type manner without the need for protecting group manipulations. By careful choice of the reaction components as well as reaction conditions the conjugation product can be obtained in very good yields. We have evaluated D-manno-configured *C*-glycosyl-type glycoconjugates obtained via the Amadori rearrangement as ligands for type 1-fimbriated *E. coli* bacteria by molecular docking and bacterial adhesion studies. Now we present the Amadori rearrangement applied to different aldoheptoses such as D-glycero-D-galacto/D-talo- and D-glycero-D-gulo heptopyranose with a variety of amines for the synthesis of *C*-glycosyl type neoglycoconjugates for new applications. We also want to address the question whether the Amadori rearrangement is suitable as chemical protein modification/ligation method. Synthetic details and biological evaluation of the synthesised compounds will be presented.

81. Peptide-based iminosugar clusters: metal-free synthesis by oxime ligation and glycosidase inhibition activity

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Although it was well-known that the iminosugars (carbohydrate analogues bearing a nitrogen atom instead of the endocyclic oxygen atom) were powerful glycosidase inhibitors, the development of inhibitors based on iminosugar clusters was not explored for a long period of time. However, over the past few years, several studies demonstrated that multivalent iminosugars strongly inhibit the glycosidases. A large number of iminosugar clusters were obtained using the copper-mediated azide-alkyne cycloaddition, the quintessential click reaction. Unfortunately, the cycloadducts are usually contaminated by significant amounts of copper catalyst, which is detrimental to their use as drugs. Therefore, it is important to gain access to iminosugar clusters via metal-free coupling reactions such as the oxime ligation, an efficient tool widely exploited by us and others for the synthesis of complex biomolecules. Free-OH *N*-propyl and *N*-nonyl-deoxynojirimycin derivatives bearing the aminooxy group were prepared in eight steps from methyl D-glucopyranoside (9 % overall yield). The reaction partners, *i.e.* the linear and cyclic decapeptides (KAKPGKAKPG) scaffolds bearing four glyoxyl-aldehyde moieties on the four lysine side-chains, were synthesized by solid-phase peptide synthesis. The couplings, carried out in water at r.t. under mild acidic conditions, gave the corresponding oxime-linked iminosugar clusters that were isolated by preparative HPLC and submitted to inhibition assays against Jack bean mannosidase. The four compounds showed good inhibition activity (K_i=1.3-58.7 μM), the best results being observed for the clusters based on the linear decapeptide platform. Moreover, the clusters featuring the iminosugar units linked to the scaffolds through a longer spacer proved to be stronger inhibitors. This work will help to understand the actual role of the spatial pre-organisation of multivalent iminosugars in the glycosidase inhibition process.

82. Novel functions of UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferase 18 (PPGalNAc-T18): regulation of protein *O*-GalNAc glycosylation in endoplasmic reticulum (ER) and that is essential for cell survival

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O-GalNAc glycosylation is an important protein post-translational modification which plays critical roles in cell-cell communication, host-pathogen interaction, signal transduction, and protein maturation. *O*-GalNAc glycosylation is initialized by members of the ppGalNAc-T family. This family contains 20 members and each of them has an unique substrate specificity and expression profiles. ppGalNAc-T18, known as polypeptide GalNAc transferase-like protein 4 (GALNTL4), only exists in vertebrates and ubiquitously expresses in human tissues. It localizes in ER and lacks the classical GalNAc-transferase activity because of the mutations in UDP-GalNAc binding pocket. In this study, we first found

that interfering ppGalNAc-T18 expression could affect cellular survival and reduce the cell surface *O*-glycosylation level by activating the unfolded protein response (UPR) or damaging the ER folding capacity. Then we found the sodium 4-phenylbutyrate (4-PBA) as a chemical chaperone could rescue the ER folding capacity and glycoprotein trafficking which damaged by the down-regulated expression of ppGalNAc-T18. Moreover, the upregulation of ppGalNAc-T18 could promote the *O*-glycoprotein folding and relieve the UPR triggered by various ER stress inducers (tunicamycin, thapsigargin and brefeldin A) or inflammatory cytokines stimulation. These results indicate that ppGalNAc-T18, an ER-localized glycosyltransferase-like protein, possesses chaperone activity and assists the folding of some *O*-glycoproteins in ER, which further affected ER homeostasis and the *O*-glycosylation synthesis.

83. The mechanism of anticoagulant activity of glycoconjugate isolated from *Echinacae purpurea L.*

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Echinacae purpurae L. (*Astraceae*) is a perennial plant, originated in the North America but distributed in the whole Northern hemisphere. The references of the traditional folk medicine of native American Indians mention a very wide range of *Echinaceae* uses, like hemorrhoids, bee stings, upper respiratory tract infections and the common cold. *Echinaceae* is currently one of the most frequently used medicinal plant, especially for its immunostimulatory and anti-inflammatory effects. The subject of the analysis was the mechanism of previously reported anticoagulant properties of the polyphenolic-polisaccharide conjugate isolated from plant *E. purpurea* isolated by the multi-step method. The study of the mechanism of its action was performed on biosensor, BIAcore system, with use of selected human blood plasma proteins. The interaction of the glycoconjugate with the proteins immobilized on biosensors' surface were examined and compared to the already known anticoagulants. Moreover, the ability to inactivate thrombin and factor Xa via antithrombin and heparin cofactor II, by the colorimetric method, also was studied. Aknowledgments: The studies are a part of the project supported by the European Regional Development Fund and the Polish Government (Operational Programme - Innovative Economy) Grant WROVASC (Integrated Cardiovascular Centre, 2007–2015).

Glycans and Cancer

Keynote Lectures

84. Factors impacting on colorectal cancer risk and survival outcomes: genes, environment and IgG glycosylation

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Cancer of the large bowel (colorectal cancer) is a common cause of cancer death in the Europe. The incidence is increasing and over 1 million people worldwide develop the disease each year. The lifetime risk in high incidence countries is around 5 %, whilst 50 % of patients who develop colorectal cancer worldwide will die from their disease. Furthermore, all affected patients will require some form of therapy. Hence, it is not only a significant personal problem but also a substantial public health issue. Environmental and genetic factors underlie colorectal cancer (CRC) aetiology and great strides have been made in understanding risk factors and the mechanisms responsible for CRC risk. In addition, a number of factors affect survival outcome for those who develop the disease, including patient-specific and tumour-related variables. There is evidence that a number of components that can be measured in blood compartments could be associated with outcome, along with genetic interactions. We have studied a number of genetic and plasma variables to identify biomarkers that might be associated, or even predict, cancer-specific and all-cause mortality following a diagnosis of CRC. As well as a number of candidate genes, we have studied plasma vitamin D and its interaction with genetic variation, and also immunoglobulin G (IgG) glycosylation as a novel prognostic biomarker of CRC. This lecture will discuss the current state of knowledge on factors influencing CRC risk and survival outcomes informed b our studies of a large cohort of Scottish patients with CRC and controls. It will also review the relevant published literature on this common cause of cancer death and how we might tackle disease prevention and to develop biomarkers that might stratify patients using novel prognostic biomarkers of colorectal cancer outcome.

85. Epitope-defined antibodies as new class therapeutic and diagnostic reagents: toward personalized medicine from chemical glycobiology

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Protein structure is directly governed by the genetic information, and almost no changes are seen in the basic structure other than expression level, regardless of the disease onset or its advancement. This means the proteins themselves cannot be a disease-specific epitope when no significant change occurs between the disease and the expression level of the specific gene. It is known that this severe "lack of disease-specific epitope" makes the development of new antibody drugs extremely difficult. The technological innovation in the manufacturing processes of antibody has advanced so much, for example, the humanized antibodies or the improvement of ADCC activity of antibody drugs. Therefore, it's not an exaggeration to say that the biggest issue in the development of new antibody drugs is all summed up in "finding new disease-specific epitopes." CA15-3 and KL-6 as serum biomarkers of breast cancer and interstitial pneumonia are a sort of glycopeptide fragments shed from cancer cell surface MUC1. These molecules are nothing other than good "disease-specific epitopes" which are expressed dominantly in the cancer cells. They directly reflect specific structural information of the cell surface glycoproteins existing in the affected area of patients with breast cancer or with pneumonia. Needless to say, they are prospective targets for antibody drug development. Toward personalized medicine, our goal is to establish a promising strategy for the direct drug discovery system from disease-relevant antigenic determinant (QuaDRADTM) based on the specific posttranslational modification of the target glycoproteins. QuaDRADTM, our new glycotechnology platform composed of glycoblotting-based glycomics and robust synthetic glycopeptide library, allowed for the development of potential epitope-defined antibodies (ED-Abs) targeting cancer-relevant glycopeptide epitopes in MUC1, namely SN-101 and SN-102, showing potent inhibitory effects on HER2 negative breast cancer cell growth.

86. *O*-GlcNAcylation of RACK1 promotes hepatocellular carcinogenesis

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Hepatocellular carcinoma (HCC) is among the most common and aggressive cancers worldwide, and our previous research has indicated that ribosomal RACK1 promoted the chemoresistance and growth in HCC. However, how ribosomal RACK1 is regulated remains little understood. In this study, we found that ribosomal RACK1 was *O*-GlcNAcylated at the site of Serine 122. *O*-GlcNAcylation of RACK1 enhanced its protein stability, ribosome binding and association with PKCβII, leading to increased phosphorylation of eIF4E and cap-dependent translation of potent oncogenes in HCC cells. *O*-GlcNAcylation of RACK1 at Serine 122 promoted tumor growth, metastasis, survival and angiogenesis *in vitro* and *in vivo*. RACK1 S122A transgene also attenuated hepatocellular carcinogenesis in DEN-induced mouse model. We also found that hypoxia induced *O*-GlcNAcylation of RACK1 and its aggregation in stress granules, resulting in the dissociation of RACK1 from HIF-1α and increased HIF-1α protein stability. Our results indicate that *O*-GlcNAcylation of ribosomal RACK1 enhances hepatocellular carcinogenesis, and provide evidence to link *O*-GlcNAc modification to translational regulation and HCC development.

Lectures

87. Protein *N*-glycosylation is an excellent target for developing glycotherapy for breast cancer

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Breast cancer progression requires a symbiotic relationship between angiogenesis and cancer cell proliferation, each following the core principles defining the hallmark of cancer. Glycosylation has been claimed as a universal feature of helping cancer cells escaping immune surveillance, facilitate tumor invasion, and increased malignancy with increased tumor burden and poor prognosis. In particular, asparagine-linked (*N*-linked) protein glycosylation is increasingly being recognized as one of the most prominent biochemical alterations involved in tumorigenesis and metastasis. It has also been suggested that inhibition of hybrid and complex-type *N*-glycans synthesis inhibits he formation of capillary tubes. In contrast, inhibition of

only complex-type *N*-glycans, but not hybrid-type *N*-glycans, does not inhibit the tube formation. To provide an experimental support we inhibited the *N*-glycan synthesis using *N*-acetylglucosaminyl 1-phosphate transferase inhibitor tunicamycin and observed both capillary endothelial cells and triple negative human breast cancer cells undergo cell cycle arrest followed by apoptosis. When tested in breast tumors in nude mice tunicamycin also reduced the tumor growth. The tumors exhibited reduced microvessel density and low mitotic index. The expression of Ki67 and VEGF was also reduced in tumor tissue. In fact, breast cancer cells MDA-MB-231, MDA-MB-468, MCF-7, BT-20, ZR-75 and BT-474 expressing *N*-glycans specific to their phenotypic characters all were susceptible to tunicamycin. The molecular mechanism supported the development of ER stress and induction of unfolded protein response signaling. We have used western blotting, QRT-PCR, Raman Spectroscopy, cDNA microarray, immunofluorescence microscopy, as well as glycomics. The work was supported in part by grants U54-CA096297 from NIH and Susan G. Komen for the Cure BCTR0600582 (to D. K. B.), and NIH/NIMHD 2G12MD007583 (to K. B.).

88. Glyco-gene regulation in breast and colon carcinoma cells during induced apoptosis

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Most of the cancer cells in human bodies in their lytic stages die through necrosis. Through the pores of the necrotic cancer cells, degraded products of the cancer cells come out and cause the death of many normal cells. Unlike necrosis, apoptotic cell death of normal cells is less damaging according to scanning electron microscopy studies. On the other hand, cancer cells to achieve immortality avoid apoptosis. The steps of apoptosis cell death in cancer cells are either blocked or deleted at the crucial points. However, apoptosis in colon and breast carcinoma cells could be induced by certain chemicals (which could be used as a new generation of anti-cancer drugs). Success of these chemicals to be used as new generation of anti-cancer drugs depends on the proper drug delivery system. To study glyco-gene regulation, we used four clonal metastatic cancer cells of colon and breast cancer tissue origin (Colo-205, SKBR-3, MDA-468, and MCF-3). The glyco-genes for *in vitro* biosynthesis of GD3, SA-Le^x and SA-Le^a (which contain *N*-acetylglucosamine, sialic acid, and fucose) in these cells were modulated differently at various phases of induction. Apoptosis inducers, L-PPMP (inhibitor of glucosylceramide biosynthesis), Betulinic Acid (a triterpinoid isolated from bark of certain trees and used for cancer treatment in China), Tamoxifen (a drug in use in the west for treatment of early stages of the disease in breast cancer patients), and *cis*-platin (an inhibitor of DNA biosynthesis used for testicular cancer patients) modulated GSL: glycosyltransferases differentially. Transcriptional expressions of all these glyco-genes were quantitated by Micro-arrays (containing over 340 glyco genes attached to the glass cover slips). A differential transcriptional regulation was observed during short induction (2–6 h). However, after longer induction (24–48 h) these enzymes were down-regulated (less than 90 %) when tested for posttranslational activity measurement or posttranscriptional mRNA content.

89. MALDI imaging mass spectrometry of glycans in formalin- fixed paraffin-embedded ovarian tumours enables tissue differentiation

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Ovarian cancer is the most fatal gynaecological malignancy in adult women with a 5-year overall survival rate of 30 %. Glycomic and glycoproteomic profiling studies have reported extensive glycosylation pattern alterations in ovarian cancer. Therefore, further investigation of these glycoylation changes may unearth novel biomarkers for early stage diagnosis. A novel method for investigating tissue-specific *N*-linked glycans is using Matrix assisted laser desorption/ionization

(MALDI) imaging mass spectrometry (IMS) on formalin-fixed paraffin-embedded (FFPE) tissue sections that can spatially profile glycoforms in tissue-specific regions. In this study, tissue regions of interest (*e.g.* tumour, stroma, adipose and necrotic) were isolated from FFPE late stage patients (n=3) with serous ovarian cancer. *N*- and *O*-linked glycans were structurally characterized through enzymatic peptide-*N*-glycosidase F (PNGase F) release of *N*-glycans, followed by β-elimination of *O*-glycans. The released glycans were analyzed through porous graphitized carbon liquid chromatography (PGC-LC) and collision induced negative mode fragmentation analysis. The glycan repertoire identified through this analysis was further used to spatially profile the location and distribution of *N*-glycoforms on FFPE ovarian cancer sections. High resolution MALDI-IMS revealed tissue-specific *N*-glycoforms, for example, high mannose glycans were predominantly expressed in the tumour region while complex glycans were significantly abundant in the underlying stroma. Therefore, tumour and non-tumour tissue regions established clear demarcation based on their *N*-glycoform distributions. MALDI-IMS and LC-ESI-MS/MS were used as complementary techniques to generate high resolution images and structural information of tissue-specific *N*-glycoforms. Furthermore, *O*-glycoforms were characterized for the first time in FFPE ovarian cancer patients.

90. Loss of Fut8 inhibits chemically induced hepatocellular carcinoma and tumorigenesis by down-regulating multiple cellular signaling

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Up-regulation of core fucosylation catalyzed by α1,6-Fucosyltransferase (Fut8) has been observed in hepatocellular carcinoma (HCC). Here, to explore the role of Fut8 expression in hepatocarcinogensis, we established the chemical induced HCC models in the male wild-type ($Fut8^{+/+}$), hetero ($Fut8^{+/-}$) and knockout ($Fut8^{-/-}$) mice by using diethylnitrosamine (DEN) and pentobarbital (PB). In the $Fut8^{+/+}$ and $Fut8^{+/-}$ mice, multiple large and vascularized nodules were induced with an increased expression of Fut8 after DEN and PB treatment. However, the formation of HCC in $Fut8^{-/-}$ mice was almost completely suppressed. This potent inhibitory effect of Fut8 deficiency on tumorigenesis was also confirmed by the abolished tumor formation of Fut8 knockout HepG2 cells using xenograft tumor model. Further, loss of the Fut8 gene resulted in attenuated responses to EGF and HGF in the HepG2 cell line, which provides the possible mechanisms for the contribution of Fut8 to hepatocarcinogensis. Taken together, our study clearly demonstrated that core fucosylation acts as a critical functional modulator in the liver and implicated Fut8 as a prognostic marker, as well as a novel therapeutic target for HCC.

91. High-throughput analysis of plasma samples and determination of changes in glycomic patterns in the immunoglobulins of patients after image-guided tumor ablation

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An overview of high-throughput methods for very fast and reproducible sample preparation is presented, followed by high-throughput glycomic analyses in order to explore the changes in IgG and IgM glycosylation in patient plasma after image-guided tumor ablation. The samples were taken before, immediately after, and subsequently after 2–3 h, 2–4 weeks, 3 and 6 months following the therapy, separated by use of chromatographic and affinity and pseudo affinity chromatographic methods for enrichment and separation of immunoglobulins IgG and IgM. IgG antibodies were isolated and the concentration of IgG at each time point was monitored using affinity chromatography with an immobilized recombinant protein A packed column. These antibodies were than analyzed for typical changes in glycosylation and their specificity against tumor antigens. The IgM antibodies were also isolated by use of a combination of affinity and anion-exchange chromatography. Antibody concentration at each time point was also monitored. Both IgG and IgM antibodies were analyzed for typical changes in glycosylation. The changes in glycan profile of were determined by HILIC followed by LC-MS/MS. In order to find trends in changes that could be correlated with ablation and cancer type and patient outcome, only week correlation could be found. Overall, it seems that both IgG and IgM glycosylation patterns may be more indicative of risk factors for inability of the immune system to prevent growth and

spread of certain cancers, rather than products of immune response to cancers or direct markers for their existence.

92. Intrinsic hepatocyte de-differentiation is accompanied by upregulation of mesenchymal markers, protein sialylation and core α-1,6-linked fucosylation

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Hepatocellular carcinoma (HCC) is a heterogeneous disease that most often arises in a background of long-term liver disease and inflammation. Despite the relationship between chronic liver disease and the development of HCC, it has been difficult to predict or diagnose progression to HCC at stages early enough to initiate effective therapy. We have been using glycomic- and glycoproteomic-based strategies to identify HCC biomarkers. We have documented several consistent changes in the glycoproteomic profile of individuals with HCC, including increased fucosylation and increased branching of *N*-linked glycans on selected serum proteins and in HCC tissue. We now show that the activation of cancer-associated cellular signaling pathways in primary rat hepatocytes can increase core fucosylation and induce additional glycoform alterations on hepatocyte proteins. Specifically, we show that increased levels of protein sialylation and α-1,6-linked core fucosylation are observed following activation of the β-catenin pathway. Activation of the Akt signaling pathway or induction of hypoxia also results in increased levels of fucosylation and sialylation. Importantly, we show that increased core fucosylation is directly associated with de-differentiation of hepatocytes and with the appearance of markers indicative of a transition of cells from an epithelial to a mesenchymal state. We believe that this knowledge may help in the use of this glycan modification in the management of those with HCC.

93. Ganglioside-dependent membrane organization controlling the adhesion and motility of human ovarian cancer cells

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Altered signaling downstream of integrin receptors is common in tumors, representing an important factor determining the aggressiveness of the tumor phenotype. With this regards, the glycolipid composition of the membrane microenvironment of integrin receptors is of great importance. We have generated human ovarian cancer cell lines characterized by different expression levels of GM3 synthase (ST3GAL5), the first biosynthetic enzyme of a- and b-series gangliosides. High GM3 synthase expression resulted in elevated ganglioside levels, reduced cell motility and increased adhesion to fibronectin, and enhanced expression of caveolin-1. Caveolin-1 phosphorylation and activity of c-Src were crucial for the ganglioside-dependent modulation of adhesion/motility in ovarian cancer cells. Treatment of A2780 cells with exogenous gangliosides markedly increased the caveolin-1 phosphorylation. Conversely, ganglioside depletion in high GM3 synthase-expressing clones by D-PDMP treatment markedly reduced caveolin-1 phosphorylation. On the other hand, D-PDMP treatment or transient silencing of caveolin-1 in high GM3 synthase-expressing cells led to c-Src activation, while gangliosides administration in low GM3 synthase-expressing cells reduced c-Src kinase activity. Ganglioside depletion deeply affected the membrane microenvironment of integrin receptor subunit, strongly reduced the diffusion speed of caveolin-1 in the plasma membrane, and increased the lateral interaction between caveolin-1 and integrin receptor subunits. Our data suggest that high ganglioside levels in the plasma membrane of ovarian cancer cells allow the formation of a ganglioside/caveolin-1 complex that dissociate Src signaling from integrin receptors, keeping cells in a high adhesion/low motility state that contributes to a less aggressive phenotype.

94. Serum IgG galactosylation as a marker for ovarian cancer

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CA-125, the most frequently-used biomarker for ovarian cancer detection, cannot provide accurate diagnosis due to the poor specificity as it may also increase in many benign gynecological conditions. Thus, reducing the false-positive outcomes is urgently needed. Decrease in terminal galactosylated *N*-glycans of serum IgG has been found in various

malignancies compared to healthy controls. In the previous study, we demonstrated that the alteration of IgG galactosylation has potential in distinguishing between false-positive subjects and ovarian cancer patients. However, our previous study has been done at small study size (n=58) and single-centre study design. Here, we further designed a large-scale and multicentre validation study to assess the diagnostic accuracy of IgG galactosylation as a serum protein marker for ovarian cancer. The degree of IgG galactosylation was measured from the relative intensities of IgG digalactosyl (G2), monogalactosyl (G1) and agalactosylated (G0) *N*-glycans according to the formula G0/(G1+G2*2). This ratio was found significantly higher in malignant group (n=150) than in benign group (n=272). ROC analysis demonstrated an improved specificity from 65 % (by CA-125 test alone) to 84 % while maintaining sensitivity at 90 % by incorporating quantitative analysis of IgG galactosylation to the current assay. Therefore, IgG galactosylation could complement measurement of CA125 in the diagnosis of ovarian cancer and improve differential diagnosis of ovarian cancer.

95. Immunogenicity study of Globo H analogues with modification at the reducing or non-reducing end of the tumor antigen

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Globo H-based therapeutic cancer vaccines have been tested in clinical trials for the treatment of late stage breast, ovarian and prostate cancers. In this study, we explored Globo H analogue antigens with an attempt to enhance the antigenic properties in vaccine design. The Globo H analogues with modification at the reducing or non-reducing end were synthesized using chemoenzymatic methods, and these modified Globo H antigens were then conjugated with the carrier protein diphtheria toxoid cross-reactive material (CRM) 197 (DT), and combined with a glycolipid C34 as an adjuvant designed to induce a class switch to form the vaccine candidates. After Balb/c mice injection, the immune response was studied by a glycan array and the results showed that modification at the C-6 position of reducing end glucose of Globo H with the fluoro, azido or phenyl group elicited robust IgG antibody response to specifically recognize Globo H (GH), Gb5 and stage-specific embryonic antigen 4 (SSEA4). However, only the modification of Globo H with the azido group at the C-6 position of the non-reducing end fucose could elicit a strong IgG immune response. Moreover, the antibodies induced by theses vaccines were shown to recognize GH expressing tumor cells (MCF-7) and mediate the complement-dependent cell cytotoxicity against tumor cells. Our data suggest a new potential approach to cancer vaccine development.

96. Computer modeling and biacore assay predict the interactions between cancer associated Globo-H ceramide and TRAX, thereby enhancing angiogenesis

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Globo-H ceramide (GHCer) is the most prevalent glycolipid overexpressed on a majority of epithelial cancers. To investigate whether GHCer binds to TRAX, and whether the binding of GHCer interferes with the interaction of PLCβ1 on TRAX and thus enhance angiogenesis, the binding mode of the TRAX-GHCer complex was first predicted through molecular docking studies. This molecular docking of GHCer against TRAX was performed using Dock 5.1.1 to generate possible protein-ligand binding modes. Subsequently, our newly developed HotLigsoftware was applied to analyze intermolecular interaction and predicted the binding site for the carbon chain of sphingosine of GHCer to be the hydrophobic region of α4, α5 and α6 in TRAX. Furthermore, based on the x-ray crystallographic structures of human TRAX and PLCβ3, and the known association between TRAX and the C-terminal region of PLCβ1, we predicted the major binding region for PLCβ1 on TRAX to be distributed around a concave region formed by α4, α5 and α6 in TRAX, which coincided with exactly the same hydrophobic groove in TRAX (α4, α5 and α6) for its binding to sphingosine of GHCer. On the other hand, the hexasaccharide portion of GHCer interacted with a hydrophilic polar-amino-acid region of TRAX, which overlapped with the other predicted minor PLCβ1-binding region. To validate the binding of GHCer to TRAX, Biacore system with immobilized TRAX protein on sensor chip CM5 was used. It was observed that at concentrations ranging from 9.77 nM to 5 μM, GHCer binds TRAX with a fast association rate, followed by slow dissociation. The K_D of the GHCer binding with TRAX was estimated to be about 4.09×10^{-8} M, by fitting the binding curves with BIAevaluation 4.1. Therefore, these data confirm our previous observation that GHCer binds to TRAX, thereby perturbed the association of TRAX and PLCβ1, with consequent release of PLCβ1 to trigger Ca^{2+} mobilization.

97. Silencing of GnT-V decreases β-1,6-GlcNAc structure on anti-tumor drug transporter hENT1 and potentiates the chemosensitivity of gemcitabine in bladder cancer

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Altered *N*-glycosylation plays a key role in cancer progression and metastasis. *N*-acetylglucosaminyltransferase V (GnT-V) controls the formation of multiantennary *N*-glycans. However, relatively little is known about the role of GnT-V in chemosensitivity to gemcitabine in bladder cancer. Here, we report that GnT-V protein expression was significantly elevated in human bladder cancer tissues and cells compared with the normal tissues, and positively correlated with pathological grade and lymph vascular invasion. Downregulation of GnT-V *in vitro* increased gemcitabine sensitivity in bladder cancer cells with time and dose dependent way, promoted the cell cycle arrest at G0-G1 phage and apoptosis. GnT-V knockdown combined with gemcitabine treatment in bladder cancer cells attenuated tumor cell proliferation, angiogenesis *in vivo* and prolonged animal survival time significantly compared with gemcitabine treatment alone. Furthermore, silencing GnT-V caused dramatic decrease of β-1,6-GlcNAc structure on anti-tumor drug transporter hENT1, and apparently enhanced uptake of gemcitabine in bladder cancer cells. Together, these results indicate that reduction of β-1,6-branched *N*-glycan in bladder cancer cells contribute to gemcitabine chemosensitivity enhancement, which is related to modulation of hENT1 transport activity, providing new potential strategy to elevate antitumor drug sensitivity during cancer chemotherapy.

Posters

98. E-selectin ligands in breast cancer cells

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Breast cancer, the most common cancer among women, is characterized by a metastatic pattern. Tumour cell migration and metastasis share many similarities with leukocyte trafficking, including the rolling over endothelium followed by firm adhesion and transendothelial migration. The cancer cell interaction with endothelial selectins is of uttermost importance for hematogenous metastasis. Being E-selectins constitutively expressed by marrow microvessels, it's imperative to investigate their ligands in cancer cells. Our work aims to elucidate the role of E-selectin ligands in breast cancer metastasis, in order to reveal mechanisms involved in the initial steps of breast cancer metastasis and to contribute to find means of circumventing bone metastasis. Breast cancer tissue express high levels of E-selectin ligands. We have established primary breast cancer cells from the breast cancer patients. These cells are able to bind to E-selectin in flow conditions, simulated by the rotation on an orbital shaker, and this binding is dependent of sialic acid and fucose residues. By western blot, we verified that they all express approximately 200, 150 and 70 KDa glycoproteins reactive with E-selectin/Ig chimera and HECA-452 clone (which recognize sialyl Lewis X tetrasacharide). Potential E-selectin ligands, such as PSGL-1, CD43, CD18, MUC1 and MUC16, were tested regarding their presence in the breast cancer cells. However we verified that none of these cells expressed these glycoproteins. HCELL, a sialofucosylated glycoform of CD44 and a potent E-selectin ligand described in hematopoietic stem cells, was also not expressed by the cancer cells. Currently, we are analyzing which are the glycoproteins that play a role as E-selectin ligands in these primary breast cancer cells, through mass spectrometry analysis. The identification and functional analysis of E-selectin ligands in breast cancer will reveal potential targets to circumvent avoid breast cancer metastasis.

99. Glycoprotein B7-H3 as a marker for oral cancer: overexpression and aberrant glycosylation in tumor growth and immune response

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The incidence and mortality rate of oral cancer continue to rise, partly due to the lack of effective early

diagnosis and increasing environmental exposure to cancer-causing agents. To identify new targets for oral cancer for early diagnosis and therapy, we employed a glycosylation probing platform to label and investigate the function of the sialylated glycoproteins differentially expressed on oral cancer cells. Of several glycoproteins identified in this study, B7-H3 was found to be most significant as it was overexpressed in oral squamous cell carcinoma (OSCC), and the extent of its expression correlated with larger tumor size, advanced clinical stage and low survival rate in OSCC patients. In addition, knockdown of B7-H3 suppressed proliferation of tumor cells, and restoration of B7-H3 expression enhanced tumor growth. We also observed differences in the glycoforms of B7-H3 between cancer and normal cells; higher fucosylation, higher branching and terminal α-galactosylation of *N*-linked glycans were observed in the cancer cells but not normal cells. Moreover, B7-H3 derived from Ca9-22 had better interaction with the lectin receptors DC-SIGN and Langerin than B7-H3 derived from normal cells. Overall, this study demonstrates that B7-H3 overexpression with characteristic aberrant glycosylation correlates with tumor growth and immune response, and can therefore be considered as an oral cancer marker.

100. A water-soluble polysaccharide isolated from *Capsosiphon fulvescens* inhibits the growth of HT-29 human colon cancer cells via induction of apoptosis in xenograft tumor

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Capsosiphon fulvescens (CF) is a green alga, which is widely ditributed in the Southern coastal area of Korea. A water-soluble polysaccharide named CF-P1 was extracted and purified from the CF mainly by acidic water extraction (0.01 N HCl), ethanol precipitation and DEAE-cellulose column chromatography. Previously, we reported that this polysaccharide has an *in vitro* anti-cancer acivity against HT-29 human colon cancer cells through induction of apoptosis. The induction of apoptosis by CF-P1 was shown to be mediated by induction of mitochondrial dysfunction. In the present study, we examined whether CF-P1 is equally active against HT-29 colon cancer cell xenograft tumors in a mouse model. Administration of CF-P1 (200 mg/kg, 400 mg/kg) through IP (Intraperitoneal) injection to BALB/c-nu mice bearing HT-29 cells human tumor xenografts dose-dependently reduced both tumor volume and weight by approximately 25 and 10 %, respectively, at 400 mg/kg. The histological analysis of tumor tissues with H&E (hematoxylin and eosin) staining showed that CF-P1 obviously decreased the cell numbers in tumor tissues and TUNEL assay showed apoptotic cell death of tumor cells by DNA fragmentation. On the other hand, GOT (glutamate oxaloacetate transaminase) level in the serum was not influenced by administration of CF-P1, suggesting that CF-P1 was not toxic to liver at the concentration used (400 mg/kg). Taken collectively, these results clearly demonstrated that a water-soluble polysaccharide isolated from *C. fulvescens* effectively inhibits the tumor growth by induction of apoptotic cell death *in vitro* and *in vivo*, suggesting that it can be a good candidate for the development of a potent anti-cancer agent against human colon cancer.

101. Mass spectrometric characterisation of oncofetal glycan epitopes displayed on polylactosamines in ovarian cancer

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Ovarian cancer (OC) is the most deadly gynaecological disease in many countries. The 56 % 5-year mortality rate is due to late detection and lack of effective treatment after the tumour becomes chemotherapy resistant. Therefore, there is a great need for biomarkers and effective targeted therapy for OC. The Antibody Discovery Platform (ADP) at BTI has developed a novel in-house monoclonal antibody that not only discriminates between cancer and normal cells, but it also kills cells directly via oncosis. Characterisation by glycan array suggested that the antibody targeted oncofetal glycans. Oncofetal glycans hold promise for targeted therapy because of the link between cancer and cell developmental stages. Additional characterisation revealed that antibody-antigen detection was sensitive to endo-β-galactosidase, indicating that antigens were displayed on polylactosamines on OC. Glycan epitopes presented on polylactosamine repeats protrude into the extracellular space and may represent another level of therapeutic specificity. However, extended polylactosamines greatly increase the mass of the glycan and hence the energy

required for comprehensive fragmentation in MS/MS. This difficulty in generating informative fragments makes terminal glycan linkages on polylactosamines difficult to elucidate on MALDI-TOF-MS/MS. To overcome this difficulty, the total glycopeptides from six OC cell lines and one ovarian surface epithelial cell line were digested with endo-β-galactosidase, hydrolyzing the internal galactose residues of polylactosamines. Released glycan termini were isolated by reversed phase chromatography and permethylated in order to increase sensitivity and facilitate unambiguous sequencing. High-sensitivity advanced mass spectrometry of the permethylated glycans identified H and Type 1 polylactosamine epitopes as the most likely antigens. These particular epitopes have not been well-explored as OC biomarkers, and are promising potentials for targeted therapy.

102. The mechanism underlying anti-cancer bioactivity of natural glycans

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Natural glycans are main and important compounds in soluble components in alternative herbal medicine. Although they are relative less toxic but with broad spectrum of therapeutic properties, which functional targets molecules they bind to, how these macromolecules function in cancer cells, whether glycans could be absorbed after oral administration remains a controversial scientific question and the underlying mechanism was largely unknown. By using quartz crystal microbalance and Surface Plasmon Resonance techniques, we show that bioactive glycans may target galectin-3 or epidermal growth factor (EGF) to inhibit tumor cells growth, bind to bone morphogenetic protein-2 (BMP2) and its receptors to block tumor angiogenesis, or target detectin-1 on cell membrane to stimulate immune system to impede tumor cells growth *in vivo*. Interestingly, these glycan can be taken in by intestine mucosa through clathrin/dynamin/Eps15/rab5-dependent, and cell membrane receptor mediating way. In addition, wnt/β-catenin, NF-κB and EGFR signaling also play important roles in the process. These findings provide novel insight into the mechanism underlying glycans against cancer and pave the way to support the development of new oral glycan-based drugs.

103. Biological impact of simple *O*-glycans in gastric cancer using glycoengineered cells

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Altered glycosylation is a common feature of cancer and aberrant expression of sialylated structures, such as sialyl-Tn (STn), at the surface of cancer cells has been extensively reported and correlated with an invasive phenotype in various cancer models. In fact, STn expression has been shown to be an independent indicator of poor prognosis in gastric cancer. Our aim is to study the biological impact of simple *O*-glycans GalNAc (Tn) and NeuAc-GalNAc (STn) expression in gastric cancer cells. Glycoengineered gastric cell line models lacking core-1 synthesis due to the knockout of COSMC were used to evaluate *in vitro* and *in vivo* the role of truncated *O*-glycans (Tn and STn) in the gastric cancer cell biology. Cytoskeleton changes, signaling pathways activation, increased cellular migration and invasion were observed. Furthermore, changes in angiogenesis and tumourigenesis were evaluated *in vivo* using both chorioallantoic membrane (CAM) assay and nude mice model. Cancer cell lines expressing high levels of STn glycoforms revealed a more aggressive phenotype, suggesting that expression of immature *O*-glycans and absence of further extension of *O*-glycans, has a crucial impact in key cellular signaling pathways controlling cell behavior, mediating motility, invasion, and therefore driving tumor cell aggressiveness and metastization capacity.

104. Identification of SLeX expressing proteins in gastric carcinoma: possible role in cancer cell invasion and metastasis

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Sialyl-Lewis X (SLeX) is a sialylated glycan antigen expressed on the cell surface during malignant cell

transformation and is associated with cancer progression and poor prognosis. In a previous study, we demonstrated that overexpression of ST3Gal IV in MKN45 gastric carcinoma cells induces the biosynthesis of SLeX in glycoproteins. The expression of SLeX antigen led to an increased invasive phenotype both *in vitro* and *in vivo* characterized by the activation of c-Met and downstream signaling activation. In the present work, we aimed to identify, by mass spectrometry, the SLeX expressing glycoproteins and validate the identified protein using immunoprecipitation and proximity ligation assay (PLA) technics. In addition, we assessed the expression of the identified protein with SLex antigens in a series of gastric carcinoma tissues using *in situ* PLA and correlate with clinicopathological features. The mass spectrometry results exposed a promising glycoprotein as a major carrier of SLeX in gastric cancer cells. Further, we were able to validate this result by immunoprecipitation and proximity ligation assay. The *in situ* PLA analysis of a series of gastric carcinoma tissues demonstrated that 80.6 % of the cases display SLeX antigens in the identified protein, and this expression was associated with the pattern of tumor growth and venous invasion of the tumors. In conclusion, we identified a very promising glycoprotein as carrier of SLeX antigen in gastric carcinoma cells, and demonstrated a co-expression and presence of these molecular complexes in gastric carcinoma tissues. The identification of the SLeX expressing protein opens a new avenue for the understanding of the underlying mechanism of gastric cancer cell dissemination and metastasis and may contribute to the development of new therapeutic strategies.

105. Enhanced expression of the β-4-*N*-acetylgalactosaminyltransferase 4 gene impairs tumor growth of human breast cancer cells

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Aberrant glycosylation of proteins and lipids is one of the characteristic features of malignantly transformed cells. The GalNAcβ1→4GlcNAc (LacdiNAc) group expressed on the non-reducing termini of *N*- and/or *O*-glycans is not a common structure in mammalian tissues and cells, but its expression level also changes in several cancer cells. To date two β-4-*N*-acetylgalactosaminyltransferases (β4GalNAcTs), β4GalNAcT3 and β4GalNAcT4, have been shown to be involved in the synthesis of the LacdiNAc group, and only β4GalNAc-T4 is expressed in human mammary gland. We previously found that the expression level of the LacdiNAc group on *N*-glycans decreases as human breast cancers progress. To investigate biological significances of this disaccharide in human breast cancers, we transfected the FLAG-tagged β4GalNAcT4 cDNA into MDA-MB-231 cells (one of the human breast cancer cell lines), and obtained several clones showing enhanced expression of the gene. Clones 1 and 2 showed 15 and 9 times more transcript than mock-transfected cells. The FLAG-β4-GalNAcT4 protein and its product, the LacdiNAc group, were detected in clone 1 and 2 cells. No change was observed in their growth rates while significant decreases in colony forming and invasive abilities were observed for clone 1 and 2 cells. When clone 1 cells were transplanted subcutaneously into nude mice, no tumors were formed while tumors were formed with mock-transfected cells. These results strongly indicate that the expression of the LacdiNAc group on *N*-glycans is quite important for the suppression of malignancies of the MDA-MB-231 cells.

106. Detection of mucin 1 possessing a 3′-sulfated core1 in recurrent and metastatic breast cancer

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MUC1 is a highly glycosylated type I membrane glycoprotein with a molecular weight of approximately 300 kDa. It consists of a short N-terminal region, a center, a transmembrane region, and a C-terminal cytoplasmic region. There is mounting evidence that the glycan structures of MUC1 derived from various malignant cancers, including breast cancer, are differentially modified. We previously reported that MUC1 purified from the culture medium of human breast cancer YMBS cells contains 3-*O*-sulfated or 3-sialylated core1 and extended core1. Therefore, we used a galectin-4 sandwich immunoassay system (named Gal4/MUC1) in this study to determine whether MUC1 possessing a 3′-sulfated core1 (3Score1-MUC1) can be detected at some level in early-stage and/or metastatic breast cancer patients in whom CA15-3 cannot be detected. Since breast cancer is the most frequent

cancer threatening the lives of women between the ages of 30 and 64. The cancer antigen 15–3 assay (CA15-3) has been widely used for the detection of breast cancer recurrence; however, its sensitivity and specificity are inadequate. Using the Gal4/MUC1 assay method, we found that 3Score1-MUC1 was profoundly expressed in the blood streams of patients with recurrent and/or metastatic breast cancer. The positive ratio of the Gal4/MUC1 assay was higher than that of the CA15-3 assay in both primary (n=240) and relapsed (n=43) patients, especially in the latter of which the positive ratio of Gal4/MUC1 was 86 % whereas that of CA15-3 was 47 %. Furthermore, serum Gal4/MUC1 levels could more sensitively reflect the recurrence of primary breast cancer patients after surgery. Therefore, the Gal4/MUC1 assay should be an excellent alternative to the CA15-3 tumor marker for tracking the recurrence and metastasis of breast cancer.

107. Role of Sdc1 in the tumorigenicity of the stem cell-like subpopulation within colon cancer cell lines

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Syndecan-1 (Sdc1), a transmembrane heparan sulfate proteoglycan binds growth factors, cytokines and ECM components thereby regulating cell motility, proliferation and invasion. During colon cancer development, complex changes occur in the expression pattern of Sdc1 during progression from well differentiated to undifferentiated tumours. Loss of Sdc1 is associated with change in phenotypic plasticity with an increase in invasiveness, metastasis and dedifferentiated cells. Empirical evidence showed that this change in phenotypic plasticity allows cancer cells to dynamically enter into a stem-cell-like state. Therefore, we investigated the ability of Sdc1 to modulate cancer stem cell properties using the human colon cancer cell lines Caco2 and HT-29. We demonstrate that siRNA mediated depletion of Sdc1 increased the stem cell phenotype based on *in vitro* sphere-forming assays and flow cytometry-based assays (side population (SP), ALDH and CD133). Mechanistically, loss of Sdc1 activates β1 integrin with an increase in focal adhesion kinase phosphorylation (pFAK), suggesting that Sdc1 may be linked to integrin-induced actin remodeling. Importantly, with Sdc1 knockdown Wnt signaling is enhanced which in turn induces TCF4 expression promoting the FAK:WNT signaling axis. Specifically, we demonstrated an increase in SP, CD133 and colonospheres, which could be blocked using a FAK specific inhibitor. We conclude that loss of Sdc1 co-operatively enhances activation of integrins, focal adhesion kinase and WNT, which then generates signals for increased invasiveness and cancer stem cell properties.

108. The C-type lectin CLEC10A specifically recognizes glycoproteins in primary breast cancer relevant for prognosis

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In breast cancer and other malignancies, alterations of glycan structures and changes in the expression of genes controlling glycosylation were frequently observed; implicating that aberrant glycosylation plays a pivotal role in tumor development and progression. Recently, we introduced a novel strategy, named protein domain histochemistry (PDH), using the extracellular domain of human glycoreceptor CLEC10A as a probe to monitor the glycosylation state on formalin-fixed paraffin-embedded tissues (FFPE). CLEC10A belongs to the superfamily of C-type lectins, specifically recognizes terminal *N*-acetylgalactosamine-(GalNAc) structures as well as Sialyl-Tn antigen and is expressed on dendritic cells and alternatively activated macrophages. As tumor-associated cells of the immune system are gaining increasing importance in the regulation of tumor development and progression, we applied CLEC10A as a "reader" of the state of glycosylation in human breast cancer and investigated the value of PDH on breast cancer patient outcome. In total, 146 primary breast carcinomas of the Hamburg breast cancer study cohort treated by standard therapy protocols were retrospectively investigated by PDH with 8-year follow-up. Moderate and strong positivity for CLEC10A staining was observed in 36 % of breast cancer specimens while 64 % of tumors were weakly positive or stained negative. Kaplan Meier analysis demonstrated that positive

staining by CLEC10A was significantly associated with increased disease-free interval and overall survival (OS). Multivariate Cox regression analysis proofed positive staining by CLEC10A as an independent prognostic marker for OS. Protein pulldown experiments of tumor extracts using recombinant CLEC10A followed by mass spectrometry led to the identification of several cancer-associated glycoproteins representing potential CLEC10A binding substrates and may serve as valuable tumor markers in breast cancer in the future.

109. Glycosite-specific biomarker discovery for gastrointestinal (GI) cancers via targeted glycoproteomic analysis of serum haptoglobin

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Glycosylation plays a central role in many biological processes including carcinogenesis. While general profiling of *N*-glycome has already revealed potential biomarkers for various types of cancer, localization of the aberrant glycosylation is desirable in order to improve the specificity of the diagnosis. Gastrointestinal (GI) cancer biomarker discovery remains as a challenge due to it's worldwide incidence, but to the limitation of current diagnostic method. Here we have developed the platform for quantitative, site-specific profiling of serum glycosylation for purposes of protein- and site-specific GI cancer glyco-biomarker discovery. It is combining global lectin blotting of serum glycoproteins, protein-specific immune-affinity purification, multi-specific proteolysis, and automated high-throughput sample preparation. Haptoglobin was selected as the primary protein of interest and digested using multi-specific proteolysis in order to produce site-specific glycopeptides. After enrichment by PGC-SPE, glycopeptides were separated and analyzed by chip-based nano-LC/MS and -LC/MS/MS. In sample preparation we used highly reproducible automated high-throughput system. This platform was then applied to large clinical colon cancer samples as well as gastric cancer samples. Significant alterations in site-specific glycosyation have been observed in haptoglobin extracted from GI cancer patients. Statistical tests established critical differences between control and cancer serum samples. We found site-specific approach improves AUC scores, or diagnostic capability. Our strategy was successful in developing glycosite-specific biomarker to generate a higher level of diagnostic specificity, suggesting the importance of localization of the altered glycosylation. This study showing strong correlation between GI cancer and glycosylation can give insight into application of glycoproteomics in GI cancer research.

110. The alteration of protein glycosylation profiles of hepatocellular carcinoma cells treated with sorafenib

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Sorafenib known as a multikinase inhibitor has shown the great therapeutic efficacy of hepatocellular carcinoma (HCC). Clearly, it is a need for studying the effect of sorafenib on the protein glycosylation profiles of HCC cells owing to deep understanding the tumor cell behavior. After human HCC cells MHCC97L, MHCC97H were treated by Sorafenib with various dosage, the inhibitory efficacy on cell proliferation was determined by the Cell Counting Kit-8. The lectin microarray with 50 kinds of tumor associated lectins was employed to identify the alternation of glycosylation profiles after Sorafenib treatment. Proliferations of both HCC cells could be definitely inhibited by Sorafenib treatment. Total proteins were extracted from both treated HCC cells and then lectin microarray experiment was performed. Comparing with the untreated cells (controls), glycoproteins binding affinities of treated cells to BPL, DBA, EEL, HAL, HPL, JAC, MPL and VVL were enhanced; however, binding affinities to CAL, LEL, LPL, MAL-I, PHA-L, RCA60, SNA and STL were reduced (S/B ≥ 2, $p<0.05$). It implied that glycoproteins from Sorafenib treated MHCC97L and MHCC97H cells had increased GalNAc structure and decreased GlcNAc, Sia and β-1,6-branched *N*-linked oligosaccharides. This alteration of protein glycosylation profiles was due to the inhibition of cell signaling by sorafenib such as Ras signal transduction pathway. The potential significance of our study is that may lead to find the novel glycol-biomarker to monitor the effectiveness of sorafenib and design the glycan related anti-HCC drugs for clinic.

111. Histidine-rich glycoprotein regulating cell proliferation by inhibiting ERK1/2 phosphorylation and depending on its *N*-glycosylation status in hepatocellular carcinoma

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Histidine-Rich Glycoprotein (HRG) as significantly downregulated glycoprotein during neoplastic transformation of WB F344 hepatic oval like cells was screened out by iTRAQ labeling followed by 2DLC-ESI-MS/MS analysis. The expression of HRG was significantly lower in HCC tissues. Overexpression of HRG in Huh7 hepatoma cell line showed decreased cell proliferation, cell migration, and colony forming ability, and increased cell apoptosis and adhesion. HRG could inhibit cell proliferation via bFGF-ERK1/2 signaling pathway by reducing ERK1/2 phosphorylation. On the other hand the functional expression of HRG was also dependent on the glycosylation status in its N-terminal especially in the glycosylation site, Asn 63, and Asn 125. Glycosylation of HRG may play a key competitive role in the interaction between HRG and HS for binding and activating bFGFR. These findings provide novel insights into the molecular mechanism of HRG involved in the regulation of HCC.

112. Therapeutic antibodies for the treatment of STn expressing cancer cells

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Immune response allied to the recognition of cancer-specific antigens has motivated the development of targeted therapies, like antibody-based immunotherapies. This type of therapy involves the manipulation of the immune system to eradicate tumor cells with potentially higher specificity, long-term efficacy and lower toxicity. In this line of though, we aim to develop anti-STn monoclonal antibodies (mAbs) to further integrated on the development of novel trifunctional bispecific antibodies (TrAbs) targeting simultaneously STn^+ cancer cells and immune cells to promote tumor regression. The cell-surface carbohydrate antigen sialyl-Tn (STn) is expressed in several types of cancer and absent in normal healthy tissues. This antigen is associated with tumor invasiveness and metastasis. Recently it was shown that STn^+ cancer cells are prone to cause immune tolerance. Therefore, STn is considered a potential target to avoid tumor-induced tolerogenic mechanisms, promoting the control of cancer progression and dissemination. Hybridoma technology was used and optimized in order to produce anti-STn mAbs. For that, different STn-based antigens, namely OSM and membrane extracts from STn expressing MDA-MB-231 cell line, were used to immunize mice. Reactivity of mice's serum against proteins containing STn and STn^+ breast cancer cell lines was assessed by ELISA and flow cytometry, respectively. Furthermore, the produced hybridoma cells were screened by ELISA and flow cytometry for specific STn recognition. Hybridoma analysis revealed that some hybridoma cells were able to produce antibodies with reactivity susceptible to sialidase treatment, suggesting that produced mAbs react against sialylated structures, probably STn. Further analysis and approaches are in development in order to obtain stable hybrid cells producing the desired anti-STn mAbs. Subsequent steps are antibody purification and characterization to ultimately develop innovative TrAbs to be used in immunotherapy.

113. Glycophenotype of breast cancer stem cells treated with glucosylceramide synthase and phospholipase C-gamma 2 inhibitors

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Metastasis, tumor relapse and resistance to therapy remain the principal causes of death for breast cancer patients. The emerging paradigm posits that tumor progression is driven by a small subpopulation of cancer cancer stem cells that exhibit the ability to self-renew

and the ability to regenerate the phenotypic heterogeneity of the parental tumor. Many cancer cellular functions have been discovered to be regulated by phospholipase C-gamma 2 (PLC) activation, suggesting that it represents an important therapeutic target for development of anticancer drugs. Here, we investigate the influence of a newly developed, small molecule PLC gamma inhibitor, with or without glucosylceramide synthase inhibitor (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, D-PDMP) therapy, on the growth, survival and glycophenotype (CD15s and GM3) of breast cancer stem cells (CSCs: CD44+CD24-). MDA-MB-231 breast cancer cells were incubated with glucosylceramide synthase inhibitor (D-PDMP) and/or PLC gamma inhibitor. The viable cells were determined by the MTT assay. Flow cytometric analysis of cells positive to anti-CD44 and glycoantigens, and negative to CD24 was performed 48 h after inhibitor treatment. Treatment of the MDA-MB-231 cells with the PLC gamma inhibitor decreased the number of total viable cells. Additional decrease was achieved after combined inhibitor treatment. Percentage of CSCs was significantly decreased only after PLC inhibitor alone treatment. CSC GM3 geometric mean fluorescence intensity (GMI) was increased after PLC inhibitor alone and combined inhibitor treatment. CSC CD15s GMI was slightly decreased after PLC inhibitor alone treatment but significantly after combined inhibitor treatment. PLC gamma inhibitor alone was the most effective against CSCs, but observed decrease of CSC CD15s GMI after combined inhibitor treatment indicates possible lower metastatic ability of dually treated cells.

114. Does aberrant *O*-glycosylation affect the function of MUC1 and osteopontin during tumour progression?

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Changes in *O*-glycosylation from core2 to core1 *O*-glycans have been observed in breast cancer. Nevertheless little is known about how these changes affect the function of individual proteins that are key to the transformation and metastatic process. We have therefore investigated how changes in *O*-glycosylation affect the function of two *O*-linked glycoproteins MUC1 and Osteopontin (OPN), during cancer progression. MUC1 has been shown to interact with EGFR, a membrane receptor that upon binding of EGF is phosphorylated and activates signaling pathways that lead to cell growth, resistance to apoptosis and cell motility. Moreover EGFR itself can translocate to the nucleus where it binds to several gene promoters during which time it interacts with the cytoplasmic tail of MUC1. Using a breast carcinoma cell line expressing sialylated core1 glycans and an isogenic cell line expressing core2 glycans, genome wide transcriptional changes in response to EGF stimulation were compared. The two cell lines clearly show a different gene expression signature; of particular interest are genes known to regulate cell division and the tumour microenvironment. Systemic instigation occurs when the presence of a growing tumour fosters the growth of a secondary tumour or a metastasis in a different organ. Osteopontin, a phosphorylated glycoprotein is necessary but not sufficient for systemic instigation. We are studying the glycosylation of OPN by instigating and non-instigating cells. Glycomic and glycosyltransferase analysis of these cell lines show differential expression of several glycosyl and sialyltransferases. Moreover, biochemical analysis of OPN purified from the two cells lines showed that OPN from instigators has lower levels of sialic acid and phosphorylation than OPN from non-instigators. These complementary studies will provide important information on how changes in *O*-linked glycosylation affect the malignant process.

115. Differential role for HS3ST2 in modulating breast cancer cell invasiveness: a molecular mechanism mediated by protease expression via map kinase and WNT pathways

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Heparan sulfate proteoglycans present ubiquitously in all cells have been shown to be involved in the process of breast cancer metastasis partially due to their heterogeneous sulfation patterns which allow for specific binding of a multitude of ligands. HS3ST2, involved in the 3-*O*-sulfation modification of the HSPGs is known to be silenced by hypermethylation in breast cancer. The aim of this study was to elucidate the role of HS3ST2 in breast cancer cell behavior in human breast cancer cell lines MDA-MB-231 and MCF-7. To address the potential role of this modification we reintroduced, we investigated the phenotypical changes. HS3ST2 transfected MCF-7 cells became less invasive while MDA-MB-231 cells showed a highly significant increase in invasiveness & motility is accompanied by significantly increased expression of several matrix metalloproteinases (MMPs) as well cadherin 11 & E-

cadherin. Treatment of MDA-MB-231 cells with TIMP-1, a protease inhibitor hampered invasion suggesting role of MMP's in increased invasiveness. In addition, both the cell lines became sensitive to chemotherapeutic drugs due to the dysregulated ion transporters. HS3ST2 overexpression in MDA-MB-231 lead to increased basal and FGF-specific signaling through p44/42 MAPK pathway which depend on the presence of heparan sulfate. Increased MAPK activation was accompanied by a significantly increased expression of the transcription factor TCF4 and β-catenin. MAPK inhibition with MEK1/2 inhibitor downregulated the expression of TCF-4 and also reduced invasion in MDA-MB-231 cells providing a clue that increased MAPK signaling also plays a role in the invasion having a cross talk with Wnt pathway. This study provides the first *in vitro* evidence of the involvement of HS3ST2 in breast cancer cell invasion. Increased activation of the MAPK signaling pathway and of TCF4 in the presence of HS 3-*O*-sulfation emerge as novel mechanistic aspects leading to increased expression of proinvasive gene products.

116. Glycome and sialoproteome characterization of gastric cancer cells overexpressing sialylated terminal glycans

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Terminal sialylation of glycans, such as α-2,3- and α-2,6-linked sialic acid to galactose, precludes further elongation of the glycosylation chain. These sialylated structures may include the expression of cancer relevant epitopes such as sialyl lewis X (SLeX). We have previously established cellular clones derived from MKN45 gastric carcinoma cells transfected with the sialyltransferase ST3GAL4. This cell line model overexpressing sialylated terminal glycans showed *in vitro* and *in vivo* an increased invasive behavior through the activation of c-Met and downstream signaling pathways. This aggressive behavior resembles the phenotype observed in patients with gastric cancer overexpressing SLeX. In the present study we have evaluated at the structural level the glycome and the sialoproteome of this gastric cancer cell line utilizing state-of-the-art analytical methods such as LC-ESI-MS/MS. Our results showed that the overexpression of ST3Gal-IV leads to a broad range of glycomic changes on both *N*- and *O*-glycans. The alterations included reduced extension of *O*-glycans, reduction of bisecting and increase of branched structures on *N*-glycans, and a shift from α-2,6- towards α-2,3-linked sialylated *N*-glycans, which was also observed by UPLC and lectin blotting. The sialoproteomic analysis further identified 96 proteins with significantly more abundant sialylated *N*-glycosylation. These included proteins such as β1-Integrin, Insulin-receptor, c-Met, and others, known to play a role in adhesion, cell signaling and immune response and to be key players in malignancy. These results demonstrate that alterations of single glycosyltransferases can have broad glycosylation effects modifying several important proteins in cancer development and progression.

117. CLEC-2 suppresses gastric cancer metastasis

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The C-type lectin-like receptor 2 (CLEC-2) is a membrane receptor with a single YxxL motif expressed in platelet and immune cells and plays crucial roles in platelet activation and immune response. However, the expression pattern and biological function of CLEC-2 in epithelial cells remains largely unknown. Here, we found CLEC-2 was highly expressed in normal stomach mucosa and frequently down-regulated in gastric cancer. Activation of CLEC-2 suppressed cell migration and invasion as well as epithelial-mesenchymal transition in gastric cancer cells in vitro. These effects were dependent on the spleen tyrosine kinase (Syk). Syk associated with the immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic tail of CLEC-2, and induced the inactivation of AKT/GSK3β pathways. Inhibition of phosphated-Akt abolished CLEC-2 depletion-mediated epithelial-mesenchymal transition, as well as tumor metastasis *in vitro* and *in vivo*. We also found a strong positive

correlation between CLEC-2 and Syk expression in gastric cancer. Syk directly promoted the CLEC-2 protein stability in gastric cancer, and the mRNA levels of Syk and CLEC-2 were co-regulated by DNA methylation. Taken together, these findings indicate that CLEC-2 functions as a suppresser of gastric cancer metastasis through Syk signaling, and suggest CLEC-2/Syk as a novel marker for predicting prognosis and therapeutic treatment in gastric cancer patients.

118. Cell surface α-2,6-sialylation modulates the adhesion of hepatocarcinoma cells to lymph nodes via CD22

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The alterations of cell surface sialylation play a key role in the adhesion, invasion and metastasis of tumor cells. Enhanced α-2,6-sialylation on *N*-glycans results from overexpression of the Golgi enzyme α-2,6-sialyltransferase (ST6Gal-I). Hca-F and Hca-P cells are murine hepatocarcinoma cell lines with high and low potential of lymphatic metastasis, respectively. Our previous study showed that *N*-glycosylation was involved in the lymphatic metastasis of Hca-F cells. However, the role of cell surface sialic acids in liver carcinogenesis and progression remains poorly understood. Here, we showed that the expression levels of α-2,6-linked sialic acids on Hca-F cells were higher compare to Hca-P cells. Knockdown of ST6Gal-I by shRNA transfection decreased the expression of α-2,6-linked sialic acids, and inhibited the adhesion of Hca-F cells to lymph nodes *in vitro* and *in vivo*. The adhesion ability was found to be mediated by CD22 which preferentially binds to α-2,6-linked sialic acids. This is the first study suggesting a new mechanism of tumor lymphatic metastasis caused by alterations in cell surface α-2,6-linked sialic acids expression via CD22. (This work was supported by grants from the National Natural Science Foundation of China 31470799).

119. Knockdown of ST6Gal-I inhibits the growth and invasion of osteosarcoma MG-63 cells

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The up-regulation of α-2,6-sialyltranferase 1 (ST6Gal-I) has been observed in several malignant tumors including colon, ovarian and liver cancers, where its expression correlates with the invasion and metastasis of these tumors. However, the roles and molecular mechanisms by which ST6Gal-I mediates the growth and invasion of osteosarcoma cells still remains poorly unknown. In this study, we investigated the expression of ST6Gal-I in osteosarcoma MG-63 and Saos-2 cells which have different metastatic potential, and found that ST6Gal-I was highly expressed in MG-63 cells compared to Saos-2 cells. Down-regulation of ST6Gal-I by shRNA in MG-63 cells significantly inhibited their malignant behaviors including in cell proliferation and soft agar colony formation, as well as migration and invasion properties. In addition, we found that ST6Gal-I knockdown inhibited the expression levels of N-cadherin, vimentin, a-SMA, MMP-2, MMP-9 and VEGF. Together, our results suggest a role for ST6Gal-I to promote the growth and invasion of osteosarcoma cells through modulation of EMT-related molecules, and might be a promising marker for the prognosis and therapy of osteosarcoma.

120. Decreased expression of HNF4α/MIR-122 axis in hepatitis B virus-associated hepatocellular carcinoma enhances potential oncogenic GALNT10 activity

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MicroRNA-122 (miR-122), a mammalian liver-specific miRNA, has been reported to play crucial roles in the control of diverse aspects of hepatic function and dysfunction, including viral infection and hepatocarcinogenesis. In this study, we explored the clinical significance, transcriptional regulation and direct target of miR-122 in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC). Reduced expression of miR-122 in patients with HBV-associated HCC was correlated with venous invasion, and poor prognosis. Furthermore, UDP-*N*-acetyl-α-D-galactosamine polypeptide *N*-acetylgalacto-saminyltransferase-10 (GALNT10) was identified as a *bona fide* target of miR-122 in hepatoma cells. Ectopic expression and knockdown studies showed that GALN T10 indeed promotes proliferation and apoptosis resistance of hepatoma cells in a glycosyltransferase-dependent manner. Critically, adverse correlation between miR-122 and GALN T10, a poor prognosticator of clinical outcome, was demonstrated in hepatoma patients. Hepatocyte nuclear factor 4α (Hnf4α), a liver-enriched transcription factor that activates

miR-122 gene transcription, was suppressed in HBV-infected hepatoma cells. Chromatin immunoprecipitation (CHIP) assay showed significantly reduced association of Hnf4α with the miR-122 promoter in HBV infected hepatoma cells. Moreover, GALNT10 was found to intensified *O*-glycosylation and following signal activation of epidermal growth factor receptor (EGFR). In addition, in a therapeutic perspective, we proved that GALNT10 silencing increases sensitivity to sorafenib and doxorubicin challenge. In summary, our results reveal a novel Hnf4α/miR-122/ GALNT10 regulatory pathway that facilitates EGFR activation and hepatoma growth in HBV-associated hepatocarcinogenesis.

121. Caveolin-1 regulates core-fucosylation and α-1,6-fucosyltransferase (FUT8) expression level in hepatocarcinoma via WNT/β-catenin signaling

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Caveolin-1 (Cav-1) is a major structural protein of caveolae and is implicated in lipid transport, signal transduction and tumor progression. Previous results indicate that Cav-1 expression up-regulates *N*-glycosylation and α-2,6-sialylation on mouse hepatocarcinoma cell surface. Herein, we reported that core-fucosylation and α-1,6-fucosyltransferase (Fut8) expression level reduced in heart, kidney and liver tissues of Cav-1 gene knockout mice. When hepatocarcinoma was induced by chemicals in Cav-1 gene knockout mice and wild type mice, respectively, the protein level of β-catenin along with Fut8 expression decreased significantly in the hepatocarcinoma tissues in Cav-1 KO mice, compared with wild-type mice. Conversely, exogenous Cav-1 expression elevated Fut8 expression by enrichment of β-catenin protein in the nuclei of mouse hepatocarcinoma hepa1-6 cells which exhibited no Cav-1 expression. Further mRNA level, ChIP and promoter activity assay in human hepatocarcinoma SMMC-7721 cells indicated that FUT8 gene expression at transcriptional level is significantly increased by Wnt-1, beta-catenin and Cav-1 expression. These results suggest that Caveolin-1 may up-regulate core-fucosylation and α-1,6-fucosyltransferase expression level in hepatoma carcinoma via Wnt/β-catenin signaling. This work was supported by grants from the Major State Basic Research Development Program of China (2012CB822103) and National Natural Science Foundation of China (31170774).

122. Serum fucosylated GP73 as a potential glycobiomarker of hepatocellular carcinoma complementary to AFP-L3

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Serum GP73 is a functional resident Golgi type II membrane protein with three potential *N*-glycosylation sites. In this study, we analyzed glycan patterns of serum GP73 to evaluate its diagnostic value for distinguishing hepatocellular carcinoma (HCC) from liver cirrhosis (LC). Antibody overlay lectin microarray and lectin blot were performed to observe fucosylated glycan of GP73, α1-6/α1-3 fucose recognized by *Aleuria aurantia* lectin (AAL) increased significantly in LC than that in HCC patients. Thus, AAL ELISA assay using ELISA Index was utilized to measure fucosylation alterations of GP73 on its protein level (Fuc-GP73). ELISA Index of 26 LC and 23 HCC patients was obtained and the area under the ROC curve (AUC) was 0.801 with a sensitivity of 73.9 % and a specificity of 65.4 %. Meanwhile, the AUC of AFP-L3 with the same serum specimens was 0.701, the sensitivity and specificity were 69.6 and 69.2 %, respectively. In addition, combining Fuc-GP73 and AFP-L3 greatly improved the diagnostic accuracy (AUC=0.834) and the diagnostic values were 87.0 % sensitivity at 73.1 % specificity. These data indicated that Fuc-GP73 could act as the glycobiomarker candidate and the combination of Fuc-GP73 and AFP-L3 could definitely have the diagnostic value for discrimination of HCC from LC patients in clinic.

123. Discovery of *N*-glycans involved in ovarian cancer metastasis

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Generally, ovarian cancer has the highest mortality rate within all gynecological malignancies. This is caused by the fact that

the majority of early-stage cancers are asymptomatic, resulting in over three-quarters of all diagnoses being made at a time when the disease has already established regional or distant metastases. Inhibition of peritoneal metastasis is of great significance for improving the prognosis of patients with ovarian cancer. The role of glycosylation modification playing in ovarian cancer metastasis has also been indicated in several studies. However, exact *N*-glycan changes associated with metastasis and the role of certain types of glycan structures on the metastasis of ovarian cancer remained unclear. So as to further elucidate the influence of metastasis-related *N*-glycan on ovarian cancer metastasis and the mechanism, in our study, we employed a quantitative glycomics approach based on metabolic stable isotope labeling to compare the differential *N*-glycosylation between an ovarian cancer cell line SKOV3 and its high metastatic derivative SKOV3-ip. We revealed the structure and quantitative information of *N*-glycans in the cancer cells. Intriguingly, the *N*-glycans with bisecting GlcNAc were all significantly decreased in SKOV3-ip compared to SKOV3. This alteration in bisecting GlcNAc glycoforms as well as its corresponding association with ovarian cancer metastatic behavior was further validated at the glycotransferase level with lectin blotting, real-time PCR and western blotting. The strategy combining metabolic stable isotope labeling quantitative glycomics with molecular biology technology provides a reliable method to find the specific changed glycans in the process of metastasis of ovarian cancer. Our study illustrated metastasis-related *N*-glycan alterations in ovarian cancer cell *in vitro* for the first time, which is a valuable source for the research on the mechanism of metastasis of ovarian cancer.

Glycans in Signaling and Cellular Communication

Keynote Lectures

124. Sialic acids and cancer: metabolic engineering as a potential strategy for therapy

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Sialic acids represent negative-charged terminal sugars of glycoproteins or lipids on the cell surface of vertebrates. One hallmark of cancer cells is hypersialylation. Sialic acids protect cancer cells from the immune system and are known to promote cell migration (= metastasis). Furthermore, they contribute to chemo- and radiation resistance. Modulation of sialylation poses a challenge for cancer therapy, especially for neuroblastoma therapy, due to the high heterogeneity and therapeutic resistance of neuroblastoma cells. Here we report that metabolic sialic acid engineering modulates cell adhesion and migration and that it is an effective strategy to fight cancer progression and supports cancer therapy.

125. Role of glycans in gastric cancer: functional and biomarker applications

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Glycosylation alterations are frequently found during gastric carcinogenesis and gastric cancer progression. *Helicobacter pylori* attachment to human gastric tissue is mediated by bacterial outer-membrane adhesins that recognize glycan antigens expressed by gastric epithelial cells. *H. pylori* infection induces alterations in the expression of host glycosyltransferases causing modifications in the gastric cell glycophenotype, and leading to an increased bacteria adhesion. Other major glycosylation alterations observed in gastric cancer, include (1) specific modifications on *N*-glycosylation of E-cadherin, crucial in gastric cancer cell invasion; (2) the increased expression of sialyl-Lewis X that activates the c-Met (a Tyrosine Kinase Receptor) and its downstream signaling pathways; and (3) the expression of immature truncated *O*-glycans, specifically sialyl-Tn (STn) antigen, which induces modifications in gastric cancer cells associated

with aggressive phenotypes. The aberrant glycosylation of proteins with STn constitutes a source of cancer biomarkers. Advances towards the identification of these biomarkers are going to be presented, and their clinical applications are going to be discussed.

Lectures

126. Genetic disruption of the A4GALT (α-1-4-galactosyltransferase) gene depletes globosides and P1 in IGROV1 ovarian cancer cells

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Glycosphingolipids (GSLs) of the globo (Gb3) and neolacto series (P1) have been recently addressed in the context of ovarian cancer. In order to study their molecular function, we utilize CRISPR Cas9 mediated genome editing to delete A4GALT, a glycosyltransferase required to terminate both of these GSLs, to further deplete the globosides and P1. We designed two single guided RNA and cloned in pSpCas9 (BB)-2A-GFP in order to delete the open reading frame of A4GALT. GFP positive single cells were isolated using FACS and assayed by genotyping PCR, DNA sequencing and semi quantitative PCR to identify bi-allelic A4GALT knockout cells (ΔA4GALT). Changes of GSL expression were monitored by flow cytometry and confocal fluorescence microscopy. Growth curve and colony formation was measured by using trypan blue exclusion cell counting and crystal violates staining, respectively. Flow cytometry revealed a distinct expression of cell surface GSLs on tested cell lines with IGROV1 being positive for P1 (30–50 %) and Gb3 (60–90 %), which was therefore selected for genome editing. Bi-allelic ΔA4GALT cells (3/86) were obtained and characterized (deletion of 1335 bp and truncated transcript). Consequently, ΔA4GALT cells showed depletion of globosides (Gb3 and SSEA3) and P1 while lactosylceramide, paragloboside and GM1 remain unchanged compare to wild type. The ΔA4GALT cells showed reduced cell growth after 48 h and significantly lower colony formation ($p<0.01$) compared to parental wildtype. In conclusion, we generated a stable A4GALT knockout depleted for globosides and P1 showing phenotypic changes compared to its parental counterpart. We are currently establishing a stable rescue system re-expressing A4GALT, which will be used along with the wild type and knockout to perform next generation sequencing and *in vivo* experiments in order to elucidate the biological function of A4GALT and associated GSLs in an ovarian cancer model.

127. Soluble CD52 glycan sequesters the DAMP protein HMGB1 to mediate T-cell suppression through SIGEC-10

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T cells use a variety of mechanisms to maintain immune homeostasis. We have reported that, upon activation, T cells release the GPI-anchored glycoprotein CD52, which suppresses T-cell function via the inhibitory sialic acid binding Ig-like lectin (Siglec)-10 receptor on T cells. Here, we determine the structural requirements for suppression by soluble CD52. Binding of CD52-Fc and other proteins was measured in microtiter plates and by surface plasmon resonance. Native proteins associated with CD52 were identified by immunoprecipitation-blotting and mass spectrometry (MS). CD52 glycan structure was determined by MS/MS (Gang Wu, Stuart M Haslam and Anne Dell, Imperial College London, UK). The terminal CD52 glycan is a multi-antennary sialyated polylactosamine. It had no biological activity in serum-free medium due to absence of the damage-associated molecular pattern (DAMP) protein, high-mobility group box 1 (HMGB1), normally present in serum. HMGB1 or its pro-inflammatory box B domain, but not its anti-inflammatory box A domain, bound to the CD52 glycan to promote its binding to Siglec-10. Upon ligation of Siglec-10, its cytoplasmic immunoreceptor tyrosine-based inhibititory motifs (ITIMs) bound SHP1 phosphatase, resulting in impaired phosphorylation of the T-cell receptor (TCR)-associated tyrosine kinases Lck and Zap70 and inhibition of TCR signalling. In innate immune cells (monocytes, macrophages, dendritic cells), HMGB1 was also a requirement for CD52 glycan to suppress phosphorylation and nuclear translocation of the NFkB complex, and transcription of cytokines. In conclusion, soluble CD52 glycan exerts a concerted immunosuppressive

effect by first sequestering HMGB1 to abrogate its pro-inflammatory effects, followed by binding to inhibitory Siglecs.

128. MytiLec, an R-type lectin from mediterranean mussel regulates cancer cell growth through glycan-dependent signaling

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MytiLec, a 17 kDa lectin isolated from the Mediterranean mussel was found to have a novel triple tandem primary structure and affinity to an a-galactoside, Gb3 (Gala1-4Galb1-4Glc-Cer). Crystallographic study showed that MytiLec possesses the b-trefoil conformation which is a characteristic of Ricin B-chain type (R-type) family lectins, though amino acid sequences were very much different. Due to the absence of QXW sequence, the hallmark of R-type lectins in MytiLec, it can be suggested that possibly conformation-based classification should get more importance than primary structure-based classification in case of R-type lectin family. The lectin specifically killed Gb3-expressing Burkitt's lymphoma Raji cells dose- and glycan-dependently. After the administration of FITC-labeled MytiLec to Gb3-expressing HeLa cells, it was observed that similar to Ricin, MytiLec gets incorporated into cells, translocated to the trans-Golgi network through endosomes to eventually kill those. Whereas Ricin goes inside the cell by its B (lectin) subunit and kills cells by the toxic A (*N*-glycoside hydrolase) subunit, MytiLec was able to kill cells despite of having only the lectin subunit. The lectin was also applied to four cultured cell lines that expressed Gb3 glycans and each cell line showed different degrees of dose-sensitivity against the protein. It was found that cell regulatory molecules like ERK/MEK and p38MAP kinase/JNK were phosphorylated by MytiLec whereas it might have induced the cell cycle arrest at G1 and S phase through the interaction of p21 and CDK2/CDK6 complexes. Additionally, caspase-9 and caspase-3 molecules were activated indicating an apoptotic pathway to trigger cell death.

129. Plant lectin domains in the nucleocytoplasmic compartment: role in signaling and defense

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Plants are continuously exposed to stress, including abiotic stresses from the environment or biotic stresses caused by pathogens and herbivorous insects. Following the exposure to stress situations plants will respond by changing their glycoprotein profiles and altering the expression levels of particular proteins with a lectin domain. The whole group of plant lectins comprises an elaborate collection of proteins capable of recognizing and interacting with specific carbohydrate structures and can be considered as part of a multi-layered surveillance system. Due to the vast diversity in protein structures, carbohydrate recognition domains and glycan binding specificities, plant lectins constitute a very diverse protein superfamily. In the last decade, new types of plant lectins have been identified and characterized, in particular lectins expressed inside the nucleus and the cytoplasm of plant cells often as part of a specific plant response upon exposure to different stress factors or changing environmental conditions. For instance, EUL-related lectins are ubiquitous in the plant kingdom and are typically expressed after plant exposure to stress situations like drought and salt stress. Interestingly, Nictaba-related lectins are expressed in very specific cells and some might function as chromatin remodelers. Depending on the plant species under study different triggers enhance the expression of the lectin. Detailed studies of the carbohydrate specificity of the lectins revealed the promiscuity of their carbohydrate recognition domain. An overview of the plant lectin motifs used in the constant battle against pathogens and predators will be presented and their contribution to plant signaling pathways will be discussed.

Posters

130. Galectin-1 ligands in human trophoblast

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Galectins have been long proposed to participate in several reproductive processes, including implantation and

placentation. Our previous finding showed that galectin-1 functions as a molecule important for trophoblast invasion *in vitro*, partially through recognition and interaction with β-galactoside containing ligands at the cell surface or in the extracellular matrix. So far, the only two identified ECM ligands of galectin-1 in the human placenta are oncofetal fibronectin and laminin, while membrane associated mucin(s) have been recently shown to bind gal-1. Interactions of several glycoproteins expressed in trophoblast – MUC1/mucin(s), but also integrins, with galectin-1, using immunocytochemistry, co-immunoprecipitation, non-denaturing electrophoresis was studied here. The results obtained showed overlapping localizations of galectin-1, with MUC1/mucin(s) as well as with integrins at the migration front of trophoblast cells. Furthermore, in galectin-1 immunoprecipitates both MUC1/mucin(s) and integrins were detected. Binding of galectin-1 to trophoblast mucins and integrins was sensitive to a carbohydrate inhibitor of galectin binding—lactose. Influence of galectin-1 interaction with mucin or integrin on migratory and invasive capacity human trophoblast was tested *in vitro*. The results obtained in this study suggest that under physiological conditions, both, mucin-galectin-1 and integrin-galectin-1 partnership could have functional consequence for trophoblast invasion.

131. Immobilization of bacterial exopolysaccharide provides a novel platform for protein interaction studies

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In the legume-rhizobium symbiosis, bacterial exopolysaccharides (EPS) are pivotal for development of infected root nodules in which nitrogen fixation takes place. In this study, the direct binding of plant protein exopolysaccharide receptor (EPR3) to EPS is assessed through site-selective chemical modification and immobilization of EPS, followed by kinetics studies using the biolayer interferometry methodology (ForteBio). This represents a novel approach to immobilization of rhizobial carbohydrates as well as binding studies with protein. Carbohydrates were conjugated to biotin following an approach analogous to the one described by us (Broghammer *et al*. 2012, PNAS), in which the octasaccharides are first modified with a heterobifunctional aminooxy-thiol linker, *O*-(2-(2-(2-(2-tritylsulfanylethoxy)ethoxy)ethoxy)ethyl)hydroxylamine. Following deprotection, they are then reacted with *N*--(2-(2-(2-(biotinylamido)ethoxy)ethoxy)ethyl)-2-iodoacetamide, to provide glycoconjugates with an oligo(ethylene glycol) (OEG) spacer and a biotin moiety. After HPLC purification and HR-FT-ICR-MS confirmation, the resulting compounds were immobilized on streptavidin-functionalized biosensors (ForteBio), and interaction with ectodomain EPR3 was measured. This yielded a dose- and time-dependent response, enabling a kinetics calculation with a result of a K_D of 2.7±0.2 μM. This clearly demonstrated for the first time, that EPR3 is able to directly bind bacterial exopolysaccharide, and is in full agreement with the plant- and genetics studies performed by Kawaharada *et al*. (2015, submitted), a research project to which this work contributed.

132. Characterization of novel highly fucosylated *N*-glycans extracted from human saliva using structure-specific LC/MS and LC/MS/MS

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Salivary secretions are a highly complex mixture such as water, electrolytes, proteins, lipids, and carbohydrates that contribute to the many biological roles and functions of saliva. Glycoproteins, which are involved in the maintenance of oral health and protect from the external environment, are one of the major bio-molecular components in saliva. However, the glycome of saliva have not been well characterized due to the complexity of the glycosylation and limitations in analytical tools. Recently, the salivary glycome have been demonstrated to identify markers of human disease such as Ocular rosacea. Here, we have characterized specific glycans isolated from human saliva by mass spectrometry. *N*-glycans were released by PNGase F. Released glycans were purified and fractionated by solid phase extraction using a porous graphitized carbon cartridge. Glycan compositions were assigned based on accurate masses obtained from MALDI-TOF MS and quantitative analysis of each glycan were further performed by nano LC-chip/Q-TOF MS. Highly Fucosylated complex/hybrid type glycans consisting of $Hex_{3-7}HexNAc_{3-7}Fuc_{1-7}$ are found as a major glycan type

from saliva in contrast with other human fluid such as serum and milk oligosaccharide. The novel fucosylated *N*-glycans having the 3 fucose to the outer arm Hex-HexNAc residue were elucidated by tandem MS for the first time. In addition, we successfully characterized *N*-glycans from 31 human corpse saliva. And those glycans found in corpse saliva were qualitatively and quantitatively compared with *N*-glycans released from live human saliva. Human saliva glycome may be applied to disease marker discovery and forensic field.

133. MUC1-mediated signaling enhances expression of urokinase-type plasminogen activator, L

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Mucin 1 (MUC1) is overexpressed in various human malignant tumors and its expression is correlated with a poor prognosis. The MUC1 protein is translated as a single polypeptide, which is cleaved in the endoplasmic reticulum, yielding N-terminal ectodomain (MUC1-ND) and C-terminal domain (MUC1-CD) that form a heterodimeric complex bound through noncovalent interactions. MUC1-CD anchors the MUC1-ND to the cell surface. MUC1 is involved in potentiation of growth factor-dependent signal transduction, which contributes to the growth and survival of cancer cells. However, the mechanism by which MUC1 promotes cancer cell invasion remains unclear. Microarray analysis revealed that expression of urokinase-type plasminogen activator (uPA) was elevated in MUC1-overexpressing cells. Furthermore, up- and down-modulation of MUC1 expression was clearly correlated with the change of uPA expression. An immunochemical study showed that the distribution of uPA coincided with that of MUC1in various human cancer tissues. The MUC1 C-terminal domain was associated with NF-κB p65 in MUC1-expressing cells. ChIP assays demonstrated that MUC1-CD existed with NF-κB p65 on the uPA promoter. Luciferase assays indicated that the uPA transcriptional activity was correlated with the level of MUC1 expression and that this MUC1-enhancing effect on the uPA transcription was abolished by introduction of mutations into the NF-κB binding sites on the uPA promoter. These results indicate that formation of the MUC1-CD and NF-κBp65 complex enhanced nuclear translocation of NF-κB p65 and subsequent occupancy of NF-κB binding region on the uPA promoter, leading to elevated transcription of uPA. We also demonstrated that uPA induced by MUC1 enhanced the matrix metalloproteinase (MMP)-2 and −9 activities, and consequently promoted cancer cell invasion. Thus, a MUC1 co-operating NF-κB signaling pathway plays a critical role in cancer cell invasion in MUC1-expressing cells.

Glyco(bio)informatics

Keynote Lectures

134. Utilizing the semantic WEB in GlyTouCan

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Since April, 2014, the Japanese government granted a group of glycoscientists funds to develop an international glycan structure repository using Semantic Web technologies which would enable the integration of various informatics resources. This glycan repository, named GlyTouCan (http://www.glytoucan.org), is a freely available, uncurated registry for glycan structures that assigns globally unique accession numbers to any glycan independent of the level of information provided by the experimental method used to identify the structure(s). That is, any glycan structure, ranging in resolution from monosaccharide composition to fully defined structures including glycosidic linkage configuration, can be registered as long as there are no inconsistencies in the structure. GlyTouCan is now released and fully based on Semantic Web technologies, whereby links to other life science databases can be traversed computationally. In other words, other related information, such as protein and lipid information, which pertain to a particular glycan can be retrieved online semi-automatically. Currently, GlyTouCan provides links to other major glycan databases such as GlycomeDB and BCSDB, and other databases will also be linked once they are also made available on the Semantic Web. Moreover, the glycan structure representation called WURCS is used as the main format for storing glycans, thus ensuring uniqueness of even ambiguous glycan structures while representing them as linear text strings. This allows for efficient searching of the repository for existing structures because a simple text comparison can be used. GlyTouCan is also being supported by the MIRAGE project, recommending that its accession numbers be used when reporting glycomics

experiments that include identified glycan structures. Thus, in the future, not only can GlyTouCan serve as a central registry, but it can serve as a portal to search for glycan-related publications as well as other biological information.

135. Data connectivity, the UniCarbKB way!

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UniCarbKB is an international effort that aims to further our understanding of structures, pathways and networks involved in glycosylation and glyco-mediated processes by integrating structural, experimental and functional information. The knowledgebase offers access to a growing, biocurated overview of information associated with biological fluids, tissues and proteins at the site-specific. Despite the great value of providing researchers access to a comprehensive collection of glycan structures and experimental datasets, an increase in accumulated knowledge necessitates the development of creative bioinformatic resources, which facilitate data discovery. Developers often face hurdles in designing sophisticated resources with the capability to handle complex and diverse data collections. Over the last few years UniCarbKB has aligned its efforts with the GlycoRDF, MIRAGE and the GlyTouCan initiatives to build a robust infrastructure. The availability of standards and ontologies is opening new and exciting avenues for connecting and interrogating large volumes of accessible data that is allowing UniCarbKB to meet its aims to: (i) better organise data to enable user-friendly interaction and querying by; (ii) building a platform that supports the inclusion of data mining tools to connect disparate resources and; (iii) integrate functional data through cross-linking with sugar-binding information. Here, we will give examples of new approaches and tools for exploring and correlating structural and experimental data including updates to the suite of UniCarb-databases. Furthermore, the utility of semantic technologies to connect glycan-related knowledgebases with other omics resources to enhance data discovery and inference of protein and glycan biological function will be highlighted. In addition, we will introduce concepts for describing structures and strategies for efficiently data mining GlycoRDF affiliated databases and the adoption of structure identifiers.

Lectures

136. Network-based inference on the IgG glycosylation pathway

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Immunoglobulin G (IgG) is a major player in the human immune response and is one of the most studied glycoproteins. However, the molecular mechanisms that regulate IgG glycosylation are still not fully understood: the known IgG glycosylation pathway might still be incomplete and, to the best of our knowledge, an experimental validation on IgG is not feasible. Therefore, in this study, we analyze LC-MS measurements of plasma IgG Fc glycans to statistically infer possible unknown enzymatic reactions in the IgG glycosylation pathway. First, we calculate a glycan correlation network using Gaussian Graphical Models (GGMs), which are based on partial correlations and allow to eliminate spurious effects due to the presence of confounders. We show that glycan pairs that have a significant partial correlation correspond to the substrate and the product of a single enzymatic reaction in the pathway. We exploit this finding to build a rule-based approach for pathway inference. Since we observe a tight association between glycan GGM and glycosylation pathway, we expect to be able to infer additional unknown reactions that might occur. We cluster all possible untested reactions into six enzymatic rules, according to the features of the involved glycoforms, for example the presence of fucose or bisecting *N*-acetylglucosamine. We then evaluate whether the addition of these new reaction rules to the known glycosylation pathway associates stronger with the calculated GGM than the known pathway alone. Remarkably, we find evidence that

two among the six hypothetical rules considered are likely to occur. Statistical significance of the results is tested via bootstrapping and all findings are replicated in four cohorts. Moreover, results of GWAS show that there is a strong association between loci containing genes encoding for enzymes involved in the IgG glycosylation pathway and the glycoforms that we assume to be possible substrates for such enzymes, further supporting our hypothesis.

137. Modelling the conformation of bacterial polysaccharide antigens

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While effective carbohydrate conjugate vaccines are licensed for diseases caused by *Streptococcus pneumoniae*, *Neisseria meningitidis*, and other bacteria, there exists a paucity of information on the conformation of the saccharide antigen component of the vaccines. Within these vaccines, specific antigens elicit immune responses that provide cross protection against infection by closely related (non-vaccine) strains. However, in some cases this relationship does not hold, despite similarity of primary structures. Conformational information can provide a mechanistic understanding for antigen cross-protection, or the lack thereof, observed in the clinic. Bacterial conjugate vaccines are structurally complex, comprising saccharide components linked to a variety of carrier proteins. To be effective, the integrity of the polysaccharide antigen must be maintained in the conjugate in order to elicit an optimal immune response. Experimental elucidation of the conformation poses a number of challenges. We have developed an effective approach to investigating saccharide conformation, involving systematic molecular modelling corroborated by key inter-residue distances calculated from NMR NOESY experiments. We have developed a toolkit of ancillary software to support this work, including the (publicly available) CarbBuilder software to enable rapid building of an initial 3D model from the primary structure of a saccharide. We apply our approach to a range of saccharides in important infectious bacteria. For example, our simulations of *S. pneumoniae* serotypes 19F and 19A saccharides reveal marked differences in repeat unit, chain conformation and dynamics that may account for the limited antibody cross-protection observed between these serotypes. In addition to prediction of conformational patterns and hydrodynamics, our method allows us explore the effect of substituents, such as acetylation, on the conformation of saccharide antigens, which may be important for immunogenicity.

138. Higher order structure and encoding of mass spectrometry data for high throughput human Fc IgG *N*-glycosylation analysis

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By continuous progress in mass spectrometry (MS) instrumentation, most importantly through increase in mass resolving power and scan speed, MS technology is generating more and more raw data. For high throughput MS, it is of crucial importance to properly structure and format generated data. Currently, MS experiments generate data encoded in binary proprietary formats, not accessible without producer's library. Raw data is usually converted using ProteoWizard toolkit into XML based MS open formats, such as mzXML or mzML. These formats, including proprietary ones, are developed around individual MS experiment. When these tools are used in high-throughput setting, the main disadvantage is that data is spread into thousands of individual files, and is loosely structured (different number of scans per sample, different number of measurements in individual mass spectra, etc.). To enable high-throughput MS glycan analysis we developed an approach to restructure and reformat MS data into multidimensional (sample, scan, mass-over-charge and intensity) arrays encoded using hierarchical data format (HDF) library, designed for storage and organization of large amounts of numerical data. For this we used pyTables library that is build around HDF, but brings some additional advantages, such as BLOSC meta-compression, optimized for speed. Using this approach, we are able to efficiently analyze human Fc IgG *N*-glycosylation in relatively high throughput manner (cohorts of several thousand samples analyzed with a rate of roughly 100 samples per day).

139. An E-workflow for glycoanalyitical mass spectrometric data

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MS and tandem MS techniques provide substantial high-quality information about glycoproteins, and are considered leading techniques for glycoprotein characterization. MS experiments produce large volumes of information. However, despite the routine use of this technique there is not yet a uniformly accepted means to share, exchange, analyze and assure quality of data. Here we present a workflow to address all these deficits. In order to be able to share MS raw data we adopted the proteomic software Proteios, an open source web-based tool to facilitate the exchange of, to track, and to analyze glyco MS spectra available in the national Swestore data storage. For high-throughput processing of MS raw data, Glycoforest was utilized. This platform clusters quality glycan spectra within single LC-MS/MS run to produce unique consensus spectra for each glycan. The glycan structures are then annotated by comparing the consensus to spectra from previously identified glycans or by manual annotation. Data from this pathway can then be prepared for deposition at UniCarb-DB. UniCarb-DB data is an openly accessible data repository that meets the need of a verified collection of manually annotated LC MS/MS spectra data with associated structure to guide structural assessment. Moreover, the status of the repository is to remain open, in order to support the development of improved software to automate spectra characterization. Proteios, Glycoforest and UniCarb-DB, are stand-alone tools with, whose intercommunication creates a semi-automatic pathway for glycomics research.

140. Modeling IgG glycosylation changes in aging

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Most membrane and extracellular proteins are glycosylated, which makes the attached oligosaccharide an integral part of the glycoprotein, thus altering its structure and function. Glycosylation of IgG antibodies is of particular interest. The glycan part critically affects the effector function of the antibody. The glycosylation pattern is a result of a complex interplay of many enzymes connected into an intricate network. In order to understand how this network changes with age, we used the IgG glycome data from several hundreds of individuals. We built a correlation network of glycan structures based on our extensive dataset. Our model uses covariance regression with Markov chain Monte Carlo simulations, with independent fitting of the mean and the covariance for each glycan structure. This strategy required careful model selection, evidence of Markov chain convergence and visualization of the covariance matrix in a network form, which represents the basis for biological interpretation. We found good clustering into different IgG classes when the model was applied to the integral dataset, with strong positive correlations within individual IgG classes and mostly negative correlations between different classes. To gain a deeper insight into the aging process, we shifted our focus to the correlations within individual IgG classes. Our preliminary results have identified several key glycosylation enzymes that might serve as control points for the whole network and point to their role in changes in the glycome observed during aging, affecting the IgG effector function and thus having a possibly important role in both the autoimmune diseases and cancer.

Posters

141. CSDB: Carbohydrate Structure Database merged from bacterial, plant and fungal parts

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The Carbohydrate Structure Database (CSDB) has been designed for accessing published data on glycans and glycoconjugates of prokaryotic, plant, fungal and protista origin. It stores primary glycan structures, detailed bibliography, taxonomy (up to strains), assignments of NMR spectra, analytical methods, cross-references, and other information (medical, biosynthetic, etc.) if available. In 2015, two parallel databases, Bacterial (BCSDB) and Plant and Fungal (PFCSDB) ones, have been merged to CSDB. Main features of CSDB are: 1) coverage on prokaryotic glycans close to complete; 2) high data quality achieved by automated and manual expert verification; 3) manually verified bibliographic, taxonomic, and NMR spectroscopic annotations; 4) automated data exchange with other databases using dedicated formats and GlycoRDF ontology within Resource Description Framework; 5) free access via the Internet (http://csdb.glycoscience.ru/). As of 2015, CSDB contains ca. 10900 bacterial glycan structures from ca. 5900 organisms published in ca. 4400 articles and ca. 5300 plant and fungal glycan structures from ca. 1400 organisms published in ca. 1800 articles. The coverage

is above 80 % for bacteria and archaea glycans published up to now; ca. 1000 new records are added annually. For plant and fungi, the coverage is ca. 40 % and is expanding rapidly. Most data are derived from manual curation based on retrospective literature analysis. Many structures published before 1996 were imported from CarbBank, appended with missing data and verified manually against the original publications with error correction. Merging the two databases simplified cross-project interactions and improved coverage-dependent services built on the CSDB platform: empirical and database-driven ^{13}C and ^{1}H NMR spectrum simulation, NMR-based structure ranking, taxon coverage and fragment distribution analysis tools, and clustering of taxa based on similarities in their glycans.

142. Normalization and batch correction methods for high-throughput glycomics

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Glycomics is rapidly emerging field in high-throughput biology that aims to systematically study glycan structures of a given protein, cell type or organic system. As it is characteristic for any other high-throughput method in biology (microarrays, next-generation sequencing, mass spectrometry), accuracy of high-throughput glycomics methods is highly affected by complicated experimental procedure. Standard experiment requires highly trained personnel, complicated sample collection and preparation procedure, large set of chemicals and calibrated machines. Standard study includes 1000 to 2000 samples, experiment can take several months and during that time many experimental conditions can vary. As a consequence, differences in experimental procedure represent huge source of variation and need for normalization and batch correction arises naturally. We compared most popular normalization and batch correction methods, from microarray and metabolomics field, on several glycomics datasets. We evaluated them based on variation of standards and correlation of replicates. According to standard variation and replicate correlation measures, every normalization and batch correction method performs relatively well, showing that use of any preprocessing method decreases experimental variation and increases the statistical power of the analysis.

Glyco(bio)technology

Keynote Lectures

143. Trageted drug delivery by carbohydrate mimetic peptide overcomes blood brain barrier in mouse brain tumor models

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The presence of blood–brain barrier (BBB) presents challenges to chemotherapeutic approaches to brain malignancies: chemotherapeutic drugs injected intravenously into patients do not pass from the circulation into brain stroma due to tight adherens junctions between endothelial cells. Previously, we identified a carbohydrate mimetic peptide designated IF7 that homes to the tumor vasculature through Annexin 1 (Anxa1) in an unprecedented manner, in terms of efficacy and specificity. We showed that IF7 conjugated anti-cancer drugs suppressed growth of colon, melanoma, breast, prostate and lung tumors in mouse models. As IF7 is transported across endothelial cells through the transcytosis pathway, we hypothesized that IF7 could cross the BBB to deliver anti-cancer drugs to brain stroma. Anxa1 is overexpressed in brain malignancies, suggesting that IF7-driven chemotherapy could be effective to treat brain malignancies. To assess tumor targeting and the ability of IF7 to penetrate brain tumors, we generated glioma tumor models by injecting rat C6 glioma cells stably expressing firefly luciferase (C6-Luc cells) into mouse brain. Growth of C6-Luc tumor was monitored by a Xenogen IVIS imager as in a similar manner we have done previously for subcutaneously injected HCT116-Luc tumors. To compare drug dosages required to suppress brain versus subcutaneous tumors, we generated a two-tumor model, in which a single NOD-SCID mouse received C6-Luc cells both in brain and under the skin. Tumor growth both in brain and under the skin was suppressed by IF7-SN38 treatment, although brain tumors were more effectively antagonized than were subcutaneous tumors. A comparable two-tumor model using melanoma B16-Luc cells in C57BL/6 mice as a model of brain metastasis confirmed the effect of IF7-SN38. Thus, IF7 is extremely efficient brain tumor targeting reagent, representing clinically relevant targeted drug delivery for brain malignancies in humans.

144. Chemical glycobiology of *N*-glycans: tools and applications

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Current methods for glycan profiling in biopharmaceutical development are either too slow, too expensive, too inaccurate, too low throughput or combinations of those. Also, for the development of glycans as diagnostic disease markers quantitative, sensitive and highly reproducible methods are scarce. We have developed synthetic methodology to produce in a economically viable manner libraries of C13 enriched glycans as internal standards for glycan quantification by mass spectrometry. Pre-formulated solutions of these standards have been further developed into a kit to quantify in absolute terms and with very good reproducibility mAb-glycosylation by MALDI-tof MS. A second topic of the talk will deal with the application of glycan arrays to screen anti-glycan antibody responses towards parasite infection, to study the binding selectivities of lectins and to monitor hydrolase activity by matrix-free LDI-MS.

Lectures

145. Production of mucin type *O*-glycans in *Nicotiana benthamiana* plants

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Despite their recognized importance for therapeutic proteins the production of structurally defined glycans is still a challenging issue for currently used expression systems. In terms of glycosylation, plants offer certain advantages compared to other organisms as the *N*-glycosylation pathway of plants can easily be modified towards the generation of homogenous human-type *N*-glycans and a typical mammalian mucin-type *O*-glycosylation pathway does not exists at all. In this project, we focus on the *de novo* generation of tailored *O*-glycan structures on recombinant glycoproteins produced in plants. For the generation of sialylated *O*-glycans, we introduced an entire mammalian biosynthetic pathway into *Nicotiana benthamiana*, comprising the coordinated expression of the genes for (i) biosynthesis, (ii) activation, (iii) transport, and (iv) transfer of Neu5Ac to *O*-linked GalNAc. When the reporter glycoprotein and the human GalNAc-transferases T2 and T4 were retained in the endoplasmic reticulum of the plant cell, all five potential *O*-glycosylation sites of a peptide derived from the cancer associated mucin-1 (MUC1) were modified with GalNAc residues. The incorporated GalNAc residues on the MUC1 peptide could be further elongated *in vivo* by co-expression of glycosyltransferases for STn and core 1 as well as core 2 *O*-glycan biosynthesis. Our study demonstrates that *N. benthamiana* are amenable to *O*-glycosylation engineering and are a valuable platform to produce glycoproteins with defined *N*- and *O*-glycans for therapeutic use and structure-function studies.

146. Combination of enzyme modules for the *in vitro* synthesis of hyaluronic acid

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Hyaluronic acid (hyaluronan, HA) is a linear polysaccharide from the glycosaminoglycan family consisting of repeating disaccharide units of D-glucuronic acid (GlcA) and D-*N*-acetylglucosamine (GlcNAc). It occurs in the extracellular matrix of vertebrates and has evolved one of the most used natural biomaterials due to its unique physico-chemical properties. We report here on the *in vitro* synthesis of HA using a combinatorial biocatalysis approach. Recombinant enzymes from different origins and pathways were interchangeably combined in an enzyme module system with *in situ* UDP regeneration. The two nucleotide sugar modules provide short metabolic access to the activated monosaccharides UDP-GlcA and UDP-GlcNAc. The third module comprises a hyaluronan synthase (HAS) which subsequently uses the UDP sugars as substrates for HA polymerization. The establishment of the system was accomplished by use of multiplexed capillary electrophoresis. This high throughput analytical device allows a fast and efficient characterization of the single enzymes, modules and combinations thereof. This work is supported by the DBU (Deutsche Bundesstiftung Umwelt) within the project HYALURONAN-Polymer (AZ 30812–32).

147. Metal ion responsive neoglycoconjugate self-assemblies: controlling the nano-scale properties of biopharmaceuticals

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There are currently 157 protein and peptide drugs. Chemical modifications to improve the properties of natural peptides are growing in importance to allow them to be efficiently used in the clinic. A variety of modifications are known, but most commercialized modifications have focused on polyethyleneglycol (PEG) chains. Here we describe the synthesis of conjugates of insulin and a plant-derived complex carbohydrate, homogalacturonan, as neoglycoconjugates of a biopharmaceutical protein. Carbohydrates from plant cell walls include pectins, from which one can prepare homogalacturonans. Homogalacturonans are extremely widely used in the food industry as a gelating agent, with a propensity to bind metal ions. These carbohydrates have a remarkably low immunogenicity. They undergo an association mediated by divalent metal-ions, such as Ca^{2+}. This leads to 'egg-box' formation where four carboxylates from the oligosaccharide form a complex with the metal-ion. They have only seen very little application for biopharmaceuticals. Crucially, their metal ion mediated self-assembly has not been utilized in a biopharmaceutical context. We present the development of insulin analogs covalently linked to oligogalacturonic acid. As the insulin hexamer formation is induced by formulation with Zn^{2+} and phenol, a self-assembling system is created, which increases in size by titration of Ca^{2+} ions. The system combines the native self-assembly of insulin with the 'egg-box' formation of homogalacturonans, to construct higher order self-assemblies, which can be dynamically tuned by the Ca^{2+} concentration. The oligomeric state of insulins define their property as either fast- or long-acting. Our neoglycoconjugates presents a new option to control the properties of insulin, *e.g.* for subcuteneous depot formation and slow release.

148. Analysis of human milk oligosaccharides and infant formula galacto-oligosaccharides

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Human milk oligosaccharides (HMOs) have been shown to play important roles in health and disease of infants. Over 200 different HMOs have been indicated and more than 100 have been identified. However, it should be noted that not every woman synthesizes the same ensemble of HMOs. Notable differences between individuals in the type of fucosylation of HMOs, reflecting differences in Secretor and Lewis blood group determinants, has led to the distinction of 4 milk groups. Making use of a series of HMO samples, a rapid ^{1}H NMR analysis method, based on the ^{1}H NMR structural-reporter-group concept for analyzing glycoprotein glycans, has been developed that identifies the presence/absence of α2-, α3-, and α4-linked Fuc residues in HMO mixtures. The data obtained afford the essential information to attribute different HMO samples to a specific milk group. Besides the interest in human milk and the humanization of cow milk, artificial baby food products with excellent prebiotic functions have conquered the food market. A typical example is the galacto-oligosaccharide mixture (GOS), synthesized from lactose by enzymatic trans-galactosylation. Depending on the microbial β-galactosidase used, species-specific ensembles of linear/branched components, varying in type of glycosidic linkage and polymerization degree, are created. GOS produced by Bacillus circulans β-galactosidase has found wide application as ingredient in infant formula, and making use of a developed ^{1}H NMR structural-reporter-group concept, we have characterized it in detail. The generated ^{1}H NMR data library has made it possible to collect very easily the main characteristics of other GOS products, prepared with different β-galactosidases. In view of reported functions of both GOS and sialylated HMOs, taking into account the low amounts of sialo-oligosaccharides in cow milk, the α3-sialylation of GOS using bovine sialo-glycoconjugate donor substrates and *Trypanosoma cruzi* trans-sialidase will be presented.

149. NMR characterization of bioconjugate vaccines

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Glycoconjugate vaccines are the most effective way to protect against bacterial diseases caused by encapsulated pathogens. They are structurally diverse and complex comprised of polysaccharide or oligosaccharide fragments, linked through a variety of coupling chemistries to different carrier proteins. Their potency cannot be reliably predicted by biological assays and therefore their development, quality control and licensure rely mostly on the physicochemical characterization of the starting polysaccharide and protein, intermediates and the conjugate vaccine produced. The structure and

identity of the saccharide antigen is traditionally determined on the starting polysaccharide by use of immunochemical and chemical assays, and NMR spectroscopy. A subset of this testing is later performed on the conjugate to confirm the structural integrity of the saccharide antigen in the vaccine. Following the success of *Haemophilus influenzae* type b vaccines, multivalent conjugate vaccines against meningococcal and pneumococcal bacteria have been licensed with vaccines against other pathogens in development. GlycoVaxyn AG has developed a proprietary technology that enables the manufacture of bioconjugate vaccines in *Escherichia coli*. The target saccharide is coupled to a specific asparagine residue in an engineered protein consensus sequence through an enzymatic process resulting in the reproducible production of *N*-linked glycoproteins in living bacterial cells under mild conditions. Unlike traditional vaccines based on the parent polysaccharide, the saccharide antigen structure must be determined on the intact glycoprotein. In addition to chemical assays, the sensitivity of high field NMR spectroscopy permits elucidation of the structure of the saccharide repeating unit including substituents that may be important for immunogenicity. The NMR characterization of vaccines prepared against *Shigella* and *E. coli* pathogens using the bioconjugate technology platform will be presented.

150. Antibodies produced in plants: glycan-engineering and modulation of functional activities

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Serum proteins circulate as a particularly highly heterogeneously glycosylated mixture of otherwise homogeneous protein backbones. It is well known that these proteins modulate their effector function and PK values through sugars. However, our limited technology in producing complex proteins, such as immunoglobulins, with defined glycan structures hamper in depths studies. Here we introduce a plant based expression platform enabling extensive engineering of glycans towards human like structures. The procedure is based on the simultaneous delivery of appropriate cDNA constructs for the expression of (a) target proteins (*e.g.* antibody heavy and light chain) with (b) human glycan modifying enzymes into plant leaves. Harvesting of recombinant proteins 1 week post construct delivery allows high speed and flexibility. Major achievements include the introduction of particularly complex structures such as polysialylation and the production of functionally active slialylated pentameric IgMs in tobacco (unpublished results). These activities required the coordinated expression of up to 13 different foreign genes in plant cells. Importantly, IgG antibodies carrying an in planta engineered glycan-profile exhibit enhanced activities and are used for the production of the currently most promising substance for EBOLA treatment. The system provides a viable approach to the generation of glycoproteins with defined glycoforms thus facilitating to elucidate glycan associated functional activities.

Posters

151. Serum asialohaptoglobin, a new biomarker for liver cirrhosis—visual detection by novel plasmonic sandwich ellsa

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Cirrhotic condition of liver has long been attributed to the preface to liver cancer. In our current endeavor, the desialylation status of the serum acute phase protein, haptoglobin has been recognized as the novel biomarker for liver cirrhosis. The reliability of this new bio-marker has been evaluated with 30 liver cirrhosis patients having history of earlier viral hepatitis C (HCV-LC). A novel enzyme linked lectinosorbent assay has been developed coupled with plasmon mechanism of gold nanoparticle aggregation as the colorimetric read out which can visually distinguish the cirrhotic liver patients from the normal healthy controls. The assay can be useful for rapid, high-throughput, point-of-care, visual detection and monitoring of liver cirrhosis, which even an untrained person can execute without specialized instrument. This method employs Sambucus nigra agglutinin (SNA) to detect the extent of α-2,6-sialylation of serum haptoglobin, the new biomarker for liver cirrhosis.

152. The use of RNA-SEQ to identify a transcript encoding an *N*-acetylglucosamine-binding lectin from the edible kurokawa (*Boletopsis*) mushroom

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Lectins are a ubiquitous class of proteins that bind to specific sugar moieties in a non-catalytic and reversible manner. They play important roles in both biology and biotechnology. Because of their ability to bind specific epitopes within oligosaccharides, lectins have been adopted for use in techniques such as glycoprotein affinity purification, glycan microarrays and imaging. Most lectins used in molecular techniques are derived from plants where they are abundantly produced and readily purified. However, there is a need for identification of novel lectin specificities to improve the sensitivity and selectivity of these methods. In recent years, more attention has been paid to identifying and characterizing lectins from yeasts and fungi. One interesting fungal lectin (BLL) derives from the Japanese edible mushroom "Kurokawa" (*Boletopsis leucomelaena*). BLL is small (~15 kDa), aglycosylated, and selectively binds terminal *N*-acetylglucosamine (GlcNAc) on partially or fully agalactosylated biantennary complex-type *N*-glycans. However, despite its useful specificity, the gene encoding BLL has not been previously identified and *B. leucomelaena* is difficult to source for the isolation of native BLL. In the present study, we have utilized RNA-Seq to identify a transcript encoding an ortholog of BLL from *B. grisae* (termed BGL), a close North American relative of *B. leucomelaena*. A strand specific transcript library was constructed, sequenced on an Illumina platform, and raw sequence data was assembled using Trinity software. Over 43,000 transcript sequences were obtained and queried via blastx against signature peptides derived from the purified BLL and BGL proteins. A transcript harboring a putative BGL-encoding sequence was identified and verified through heterologous protein expression and determination of the ability of recombinant BGL to bind GlcNAc using ITC.

153. Malonic acid suppresses *O*-glycan degradation during hydrazine treatment of glycoproteins

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The mucin-type *O*-glycan (*O*-glycan) seems to play an important role in the maintenance of gastrointestinal tissue homeostasis by mucins. To characterize the function of the *O*-glycans, hydrazine treatment was frequently used for preparing the *O*-glycans because the method provided *O*-glycans retaining the reducible GalNAc at their reducing-end, which is available for fluorescent labeling. Because the HILIC and reducing-end analyses of the *O*-glycans showed that the degraded products were produced by "peeling" during the hydrazine treatment of the rat gastric mucins (RGM) despite being under highly anhydrous conditions, a further examination has been performed using porcine gastric mucins (PGM) and bovine fetuin as the model glycoproteins. In the present study, it was found that malonic acid suppressed the degradation of the *O*-glycans produced from PGM by hydrazine treatment in both the gas- and liquid-phases. Although this is paradoxical because the release of an *O*-glycan from a protein occurs under alkaline conditions, malonic acid seems to prevent the degradation by acting as an 'acid' because other weak acids also prevent the degradation. Furthermore, the malonate·2Na salt did not suppress the degradation. The suppressive effect for the degradation by malonic acid was also observed during the hydrazine treatment of fetuin. RGM prepared from the corpus mucosa of the stomach was used for evaluating this method. In the absence of malonic acid, most of the major *O*-glycans obtained by the hydrazine treatment were degraded as previously described (2). In contrast, almost all the major *O*-glycans obtained by the hydrazine treatment in the presence of malonic acid consisted of intact ones, although some degradation was still observed. In conclusion, hydrazine treatment in the presence of malonic acid allows the glycome analysis of the mucin glycoproteins.

154. *O*-glycan preparation for liquid chromatography analysis using "BlotGlyco® sample preparation kit for *O*-glycans"

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Glycosylation is a critical post-translational modification of proteins. The attached glycans can be classified into *N*-glycans and *O*-glycans according to their glycan-peptide linkage. Although the analysis of *N*-glycans is well established, the analysis of *O*-glycans is still a challenge. In particular, the technology of liquid chromatography (LC) analysis of *O*-glycans has been left behind due to two main reasons. First, there was lacking of compatible method for releasing *O*-glycans with free reducing termini and preventing degradation in *O*-glycans release. This degradation was referred to as peeling of *O*-glycans. Second, *O*-glycans should be purified after releasing from peptides for acquiring accurate detection. In this step, BlotGlyco™ beads that possess high-density hydrazide group is known as an useful approach for glycan purification. It enables selective and

comprehensive trapping of whole oligosaccharides and highly effective in impurities elimination. After the purification, *O*-glycans can be derivatized with 2-aminobenzamide (2AB). The 2AB-derivatized *O*-glycans are desalted with cleanup column. The protocol is different from our BlotGlyco™ kit for *N*-glycan. In this study, we compared between the conventional methods with hydrazinolysis and our "BlotGlyco™ Sample Preparation Kit for *O*-Glycans kit" by using bovine fetuin as a model sample. The results show that hydrazinolysis method has proper recovery amounts of *O*-glycans but the peeling rate is detected in high level. The peeling causes poor repeatability and effects glycan profiles in LC analysis. On the other hand, by using our BlotGlyco™ kit, the results indicate that the peeling rate was suppressed to the half level by compared with the hydrazinolysis method. Moreover, BlotGlyco™ kit has easy and simple operation protocols. And also, by using this kit, the hazardous/toxic reagents are unnecessary. Therefore, our BlotGlyco™ kit is suitable for practical use in LC analysis.

155. Microwave-assisted biocatalysis employing a thermophilic galactosidase from *Pyrococcus woesei*

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Microwave-catalyzed reactions have been employed as "green strategy" in organic chemistry due to the reduced quantities of solvents and selective conversions in shorter reaction times. In recent years, this approach also represents an innovative tool in biocatalysis. Apart from lipase-catalyzed reactions, studies on enzymatic syntheses have mainly focused on glycoside hydrolases as model proteins. Evaluation of the specific nonthermal microwave impact on temperature-sensitive reactions requires a permanent cooling system as well as online-monitoring of crucial reaction parameters (temperature, microwave irradiation, pressure, stirring rate), which is given by Discover®CoolMate™ synthesis system. In our previous studies mesophilic β-galactosidases were used for the stereo- and regioselective synthesis of nucleotide activated oligosaccharides under microwave irradiation. As a result, stability of the product formation accompanied by reduced hydrolysis was declared. Moreover, the hydrolytic performance of a thermophilic β-glucosidase (CelB) from *Pyrococcus furiosus* could be increased far below its temperature optimum using microwave energy. We here report our results on the activating influence of microwave irradiation compared to conventional heating on the hydrolysis as well as a synthetic approach for the production of glycoconjugates using a hyperthermophilic glycoside hydrolase. Following this reaction engineering strategy, the recombinant *Pyrococcus woesei* β-galactosidase represents an especially suitable biocatalyst. A higher product yield was achieved under microwave-assisted reaction at similar ambient temperature. Financial support by the DFG International Research Training Group 1628 "SeleCa" is gratefully acknowledged.

156. A lectin-device is effective for trapping and clearance of HIV-1

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Viral infection often causes pandemic disease. Antiviral reagents have therefore been investigated in details to prevent the viral infection. Otherwise, it may be effective to palliate the infectious disease by reducing the number of the causing viruses, as the removal of viruses by dialysis treatment contributed to improve the symptom of an AIDS patient. Carbohydrate-binding proteins may also be potent candidates for trapping the viruses by binding to viral surface glycoproteins. Here we prepared anti-HIV algal lectin-immobilized devices by a combination of our original techniques and investigated them for the ability of trapping HIV-1. We selected four families (type I~IV) of high-mannose *N*-glycan-specific algal lectins, and tried to prepare their modified recombinants, which were designed to immobilize with high orientation and density to the monolithic silica gel consisted of macro- and mesopores. The lectin-immobilized devices were also examined for biocompatibility assessment including hemolysis, blood clotting, complement activation, and bradykinin production. As the results, we succeeded in the preparation of the modified recombinants of type I (OAA from *Oscillatoria agardhii*) and type II (BCA from *Boodlea coacta*). rOAA1 was immobilized in the orientation-controlled manner to the monolithic gel with different pore sizes of macropore (100 μm) and mesopore (up to 200 nm), and packed into both spin columns and stainless columns. The immobilized amount (0.1~7 mg/ml gel) could be adjusted by controlling the surface area of mesopore when prepared the gel. The rOAA1-device with small-pore sized mesopore mostly trapped the HIV-1 added, whereas that with

large pore sized one, which was prepared to remove the viruses directly from whole blood, did not trap HIV-1. The rOAA1-immobilized device was safety for compatibility assessment examined. Thus, rOAA1-device should be useful to remove the viruses bearing high-mannose glycans on their surfaces.

157. Bifunctional fluorescent linker for glycan labeling, mass spectrometric analysis and glycan arrays

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In the past few decades, glycan-protein interactions have been found to participate in numerous relevant biological processes, such as cell-cell communication, pathogen-cell recognition, fertilization and cancer metastasis. In order to study these interactions, glycan arrays have become the technique of choice, due to its high-through capacity using small quantities of glycan samples. Thus far, many of the glycan microarrays have been prepared by chemical and/or chemoenzymatic synthesis. However, the large repertoire of glycans in nature is still not completely covered on the microarray. Therefore, a fluorescent linker that allows both the structural characterization and the biomolecular interaction analysis can be extremely useful to study the biological function of natural glycans that have not been previously characterized. Here, we describe the synthesis of a bifunctional fluorescent linker that can be employed for fluorescent labeling of free glycans, structural characterization by mass spectrometry and preparation of glycan arrays. The linker is based on three key elements: (1) a methoxyamine functionality for the chemoselective labeling of glycans with preservation of the natural ring structure, (2) a fluorescent 6-amino-2-naphthoic acid to increase sensitivity during purification and, (3) an amino-pentanoic spacer for immobilization and preparation of glycan arrays. As a proof-of-principle, the histo-blood group antigens were fluorescently labelled, structural characterized by mass spectrometry, immobilized onto a commercial available NHS-functionalized microarray support and interrogate with several carbohydrate-binding proteins.

158. An efficient and optimized purification procedure for fetuin-glycan binding lectin from the mushroom fruiting body of *Hericium erinaceum*

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Mushroom lectins harboring carbohydrate binding specificity are a powerful tool to detect diverse glycoconjugates. However it is difficult to obtain the reproducible quality and quantity of glycan structure-specific binding lectins from a natural resource. In this study, I optimized the purification for a fetuin-glycan-specific binding lectin from the mushroom fruiting body of *Hericium erinaceum*. The mushroom fruiting body lysate was directly applied to an affinity chromatography using fetuin-immobilized agarose. The one step purification showed at least three main proteins containing other minor proteins on SDS-PAGE. To optimize the purification of the glycan-binding proteins, ion exchange chromatography using DEAE-Sepharose, affinity chromatography using fetuin-agarose and size exclusion chromatography using Superose 12 were sequentially applied. The conditions on the purification procedure of the mushroom lectins including concentration in ion exchange chromatography and the galactose concentration in affinity chromatography were optimized. Finally, two distinct proteins, HEL1 and HEL2, with molecular weights of 15 and 37 kDa, were separately isolated from *H. erinaceum*. In the purification steps, interestingly, HEL2 indicated strong binding properties to ion-exchange column and fetuin-agarose column rather than HEL1. The purified two lectins showed hemagglutination activities against porcine erythrocytes. Interestingly, HEL2 showed protease activity, however HEL1 did not show any catalytic activity. A hemagglutination inhibition assay of monosaccharides indicates that these lectins showed different binding specificities. These optimized purification procedures could be useful to separate an individual lectin bound to specific glycan structures in fetuin glycoprotein. Acknowledgement: This work was supported by "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009783)" Rural Development Administration, KOREA.

159. Carbohydrate binding specificity of a core 1 *O*-linked glycan-specific lectin from the mushroom *Hericium erinaceum*

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Mushroom lectins harboring carbohydrate binding specificity are powerful tools to detect glycoconjugates with *N*-linked or *O*-linked glycans. In this study, we purified a novel core 1 *O*-linked glycan-specific lectin from the fruiting body extract of a mushroom *Helicium erinaceum* by ion-exchange chromatography, affinity

chromatography on fetuin-agarose, gel filtration chromatography. The purified lectin was designed as HEL1 (*H. erinaceum* lectin 1). Tricine-PAGE, MALDI-TOF mass spectrometry, and N-terminal amino acid sequencing indicated that the native lectin has an identical form with a molecular weight of approximate 15 kDa and a lectin-like structure sequence. Isoelectric focusing of the lectin showed bands near pI 5.4. Agglutination assay displayed HEL1 was more effective toward porcine's erythrocyte rather than other animals red blood cells. Glycan microarray analysis showed that HEL1 interacts with Galβ1-3GalNAc present in Thomsen-Friedenreich antigen or core 1 *O*-linked glycan structure such as Peanut agglutinain (PNA). Comparing the glycan binding affinities in glycan microarray, both HEL1 and PNA bound core 1 *O*-linked glycan. Interestingly, HEL1 can bind a broad range of glycan structures with an extension on the β1-3Gal linked to the GalNAc as well as a terminal α-2,3-linked sialic acid which is not bound by PNA. These HEL1 binding affinities could be useful to apply the cancer research and diagnostic assay involving core 1 and extended core 1 glycan structures. Acknowledgement: This work was supported by "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009783)" Rural Development Administration.

160. Structural characterizations of *N*-acetylglucosamine-terminating neolacto-series glycosphingolipids using the monoclonal antibodies specific to *N*-acetylglucosamine residues (MAC-1) and its SCFV properties

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We have reported the establishment of mAbs specific to lactortriaosylceramide (MAC-1) and have investigated of GSLs with GlcNAcβ1-3 epitope in some cancer cell lines, in which antigens have been distributed comparatively. The established mAbs have also been shown to react with not only Lc3Cer, but also with GlcNAcβ1-3-terminal GSLs prepared from glycosidase treated gangliosides fractions despite branching or lactosamine chain lengths, and human transferrin with terminal GlcNAc residue. During the course of the study, we noticed that several bands exhibit positive reactions to the mAbs, in addition to Lc3Cer and that they are present comparatively in the neutral GSL fractions of bovine erythrocytes. Here we report that MAC-1 reactive GSLs with GlcNAcβ1-3 epitope are preferentially present in bovine erythrocytes and their detailed strucutures were characterized by several analytical procedures after purification. Structures of MAC-1 reactive GSL-1, −II, −III and –IV had the similar *N*-acetylgluscosamine-terminating neolacto-series glycosphingolipids structure at the non-reducing terminal. GSL-1 to -IV have assigned as the structure below: GSL-I, GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-1Cer (nLc5Cer); GSL-II, GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-1Cer, IV6GlcNAc-nLc5Cer; GSL-III, GlcNAcβ1-3(Galβ1-3GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-1Cer, IV6 Galβ1-4GlcNAc-iso-nLc5Cer; GSL-IV, GlcNAcβ1-3(Galα1-3Galβ1-3GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4Glc-1Cer, IV6Galα1-3Galβ1-4GlcNAc-iso-nLc5Cer. Based on the antigen epitope findings above, we are currently investigating and comparing similar series of the mAbs and their specificities using variable region analysis by RT-PCR method. Use of thus generated scFv by phage display technology will be useful in further characterization of defined specificity of similar carbohydrate antigen epitopes.

161. Microarrays and biosensors—assessing adaptive immune responses to glycan antigens

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Glycans play key roles in immune recognition. The body's ability to recognize "non-self" glycans can sometimes raise protective IgG and IgM antibodies against tumor associated cancer antigens. To study these intriguing serum responses to glycan antigens, we are creating highly sensitive glycan microarrays and biosensors that promise to reveal binding affinities of the serum antibody repertoire. Glycans from both synthetic and natural sources were conjugated with a bi-functional *p*-nitrophenyl anthranilate and subsequently reacted with ethylene diamine in a single reaction, yielding a fluorescent, NHS-reactive glycan library. HILIC-UPLC confirmed the purity (58–100 %) and concentration (46.25–502 μM) of the glycan library. Tagged glycans were then printed (spot pitch 500 μm, concentration 12.5 μM) onto NHS-functionalized glass. Putative anti-cancer, monoclonal IgG and IgM antibodies derived from whole-cell immunization experiments in mice were hybridized with the

glycan array and detected using fluorescent probes. Our results show that these antibodies bind primarily to fucosylated blood group glycans such as H-type I antigen. One antibody showed broad specificity for Galβ1-3GlcNAcβ1-3Gal backbone structure. Separately, a novel, low-cost, ellipsometry system is being developed to observe the real-time binding of lectins and antibodies to glycans spotted onto functionalized silicon substrates. Different surface functionalization and glycan attachment strategies were tested for their ability to present glycans for binding interactions, and we present results from: 1. BSA-based glycoconjugates prepared off-line; 2. Neutravidin + biotin-based glycoconjugates prepared *in situ*. We present some strategies to enhance the yield of biotin-tagged glycans, and show that Neutravidin is an inexpensive substrate suitable for creating highly sensitive glycan arrays with low background noise.

162. Glyco-engineering of yeast using YlMpo1 expression for increase of mannose-6-phosphate glycans

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Therapeutic enzymes for treatment of lysosomal storage diseases require the attachment of *N*-glycans containing mannose-6-phosphates essential for lysosome targeting. The mannosylphosphorylated mannose structure (mannose-1-phosphate-6-*O*-mannose) of *N*-glycans in yeast can be converted to mannose-6-phosphate structure (phosphate-6-*O*-mannose) by uncapping the outer mannose residue. In the traditional yeast *Saccharomyces cerevisiae*, both ScMNN4 and ScMNN6 genes are required for efficient mannosylphosphorylation. ScMnn4 protein has been known to be a positive regulator of ScMnn6p, a real enzyme for mannosylphosphorylation. On the other hand, YlMpo1p, a ScMnn4p homologue, mediates mannosylphosphorylation in *Yarrowia lypolytica* without the involvement of ScMnn6p homologues. We show that heterologous expression of YlMpo1p can perform mannosylphosphorylation in *S. cerevisiae* without the help of ScMnn4p and ScMnn6p. Moreover, mannosylphosphorylation of *N*-glycans enhanced by YlMpo1p overexpression is much higher than that with ScMnn4p overexpression, and this is highlighted further in Scmnn4- and Scmnn6-disrupted mutants. We applied the strategy of mannosylphosphorylation enhancement by YlMpo1p overexpression to a glyco-engineered *S. cerevisiae* strain (Scoch1 and Scmnn1 double-disrupted mutant) in which the synthesis of yeast-specific immunogenic glycans is blocked. When compared to ScMnn4p overexpression, a great increase of bi-mannosylphosphorylated glycan is observed. Through an *in vitro* process involving the uncapping of the outer mannose residue, this bi-mannosylphosphorylated structure is changed to a bi-phosphorylated structure with high affinity for mannose-6-phosphate receptor. The superior ability of YlMpo1p to increase bi-mannosylphosphorylated glycan in yeast shows promise for the production of therapeutic enzymes with improved lysosomal targeting capability.

163. Serum supplementation affects the glycosylation pattern of recombinant therapeutic proteins expressed in human TE671 cells

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Although mammalian cell culture is still technically complex and expensive, the ability to produce desired posttranslational modifications, in particular human-like glycosylation, is of paramount interest. Since glycans have fundamental influence on serum half-life, biological activity or especially product immunogenicity, we chose a human system for recombinant protein expression. In order to overcome the limited number of available human hosts and exploring cell-type specific differences, the rhabdomyosarcoma cell line TE671 was investigated as a novel platform to produce glycosylated biopharmaceuticals. The optimization of cell transfection and serum-free expression succeeded for two therapeutical proteins: the serine protease inhibitor α-1-antitrypsin (A1AT) and the hematopoietic cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF). *N*-glycan analyses of both purified proteins by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry provided fundamental insights into the TE671 glycosylation potential. Besides protein specific pattern, strong distinctions were observed depending on the medium conditions of the respective TE671 cell cultivations. Presence or absence of fetal calf serum and the use of different serum-free media determine the *N*-glycosylation profiles of A1AT and GM-CSF regarding sialylation, fucosylation and structural heterogeneity. Our data impressively show that cell culture conditions can profoundly affect the final glycosylation

pattern of recombinant glycoproteins of biotechnological interest, necessitating a deeper understanding of crucial impact factors and standardized expression protocols.

164. A novel fusion protein for enrichment of *N*-linked glycopeptides

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Glycoprotein characterization presents many challenges due to the complex nature of *O*-linked and *N*-linked glycan modifications. We have developed a novel fusion protein for isolating glycopeptides or glycoproteins that possess the common $Man_3GlcNAc_2$ core of *N*-linked glycans. The immobilized protein-based reagent is capable of unbiased binding and enrichment of a diverse array of complex *N*-linked glycopeptides as determined by isothermal titration calorimetry experiments as well as HPLC and mass spectrometry studies. We propose that this mass spec – friendly method may be employed to characterize the glycan profile of complex samples and accordingly, may enable biomarker discovery.

165. Incorporation of artificial sugars into the glycans of heterologously expressed proteins in CHO cells

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The glycosylation pattern of recombinant therapeutic proteins plays a major role for their pharmacokinetic and pharmacodynamic properties. For some applications a glycosylation of authentic human proteins with inherently low immunogenicity is aspired, for others immune reactions are welcome. The latter one can be achieved *e.g.* with artificial glycosylation, which can be promoted by chemical modifications of the monosaccharides itself or by the use of reactive functional groups allowing covalent binding of further compounds, *e.g.* by click chemistry. The most prominent sugars used in these techniques are sialic acids and fucose. We have developed different methods to incorporate artificial sugars into the glycans of target proteins by metabolic oligosaccharide engineering. This allows the specific alteration of the glycan pattern and the coupling of functional groups. As an expression system we have chosen CHO cells, the most common cell line in recombinant glycoprotein expression. We will present data about the generation, structural and functional analysis of glycoengineered proteins.

166. Automated glycan sample preparation system based on BlotGlyco technology

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In this study, we established two novel automation systems for glycan sample preparation based on BlotGlyco™ technology; one is an especially-designed robot and the other is based on a general-purpose liquid handling platform. Both systems are based on 96-well format for high-throughput operation. Those systems performed as well or better than skilled operators in reproducibility and purification efficiency. It makes a significant contribution especially to quality control of therapeutic glycoproteins, CHO cell line screening and process development. Glycosylation is a highly complex modification influencing the functions and activities of therapeutic proteins such as antibody. The difficulty of glycan analysis of glycoconjugates mainly depends on the complicated procedure of glycan sample preparation, especially purification and labeling, hence automating glycan sample preparation is vital to fulfill the contemporary needs of the biopharmaceutical industries in the viewpoint of reproducibility and prevention of human error. Currently, glycans are analyzed by using various methods such as HPLC and MS, however, traditional sample preparation methods require several days to purify and label glycans because of their extremely tedious multistep. Previously we reported about a new method to easily prepare glycan samples based on chemoselective ligation between glycan and polymer support, namely, BlotGlyco™. It is a polymer bead that possess high-density hydrazide group, which enables selective and comprehensive trapping of glycans and effective removal of impurities. Also, various labels are easily attached to glycans by using the beads for different purposes such as MALDI-TOF MS, HPLC and LC-MS. All processes are completed within 6 h that is significantly faster than traditional methods. The combination of BlotGlyco™ and automation technology enables high-throughput and comprehensive glycan analysis of protein pharmaceuticals or more complicated samples.

167. Glycan arrays using natural and synthetic glycans

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Anti-carbohydrate antibodies are present when foreign glycans are detected by the immune system and thus can be a problem if recombinant glycoproteins, such as antibodies, are used as therapeutic agents. It is also of importance that the recombinant antibodies have the correct glycosylation in order to prevent clearance from the serum. Using glycans from natural sources can be problematic because of the low amounts generated. This work demonstrates how glycans can be generated synthetically using enzymes to generate physiologically important epitopes and how these can be used on arrays to investigate binding between glycans and anti-carbohydrate antibodies, lectins and their binding partners. Using enzymes to extend glycan structures after an initial glycoconjugate has been chemically linked to a new linker allows up to 100 % conversion of the glycoconjugate and retention of closed ring form reducing end of the sugar. Thereby smaller amounts of material than for chemical elongation are needed. These reactions can be done in solution and then purified before spotting onto a microarray or they can be done directly on the microarray surface. The use of microarrays allows high throughput screening of binding of the glycans with their binding partners. Another approach has been to use the AEAB (2-amino-*N*-(2-aminoethyl)-benzamide) linker, to connect natural glycans purified from natural sources, though the reducing end of the glycan is then present in the open ring form. However, this linker can also be used to attach purified glycans from natural sources to NHS-coated glass slides. These glycans can then also be probed using anti-carbohydrate antibodies and lectins.

168. Glycobiology in *Xanthomonas campestris*: optimization of the metabolic flux towards xanthan biosynthesis

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Xanthomonas campestris pv. campestris (Xcc) is a Gram-negative, phytopathogenic bacterium with high industrial relevance. It is the causative agent of the black rot disease in cruciferous crops. Furthermore it is the producer of the thickening agent xanthan. Xanthan is an exo-polysaccharide that consists of repeating units formed by D-glucose, D-mannose and D-glucoronic acid (2:2:1) attached with pyruvate or acetate. It was discovered in the 1950s and is used in the food industry, pharmaceuticals and also in oil drilling. In 2014 the worldwide production of xanthan was around 160.000 tons. Mutants of selected genes in metabolic pathways with a putative influence on xanthan production are screened for xanthan. Furthermore the phenotypes of these mutants are analysed to understand the impact of the mutated genes. Particularly mutations in the nucleotide sugar and Lipopolysaccharide (LPS) metabolism might have a positive effect on xanthan, since both, LPS and xanthan depend on nucleotide sugars as precursors. It could be shown that inhibition of the gene *wxcB*, a putative kinase essential for the O-antigen in Xcc, leads to an increased xanthan production. However, other mutants from the same metabolic networks do not lead to a better precursor availability for xanthan, but increase the amount of resources used for LPS. Strains with mutations in *wxck* or *wxcN*, respectively, share a phenotype with an increased polysaccharide length in the LPS. Both genes play a crucial role for the O-antigen side chain biosynthesis, indicating that the O-antigen side chain might be important for the O-antigen chain lenght phenotype.

169. A new bacterial lectin that recognizes core α1-6 linked fucose in *N*-glycans

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We report a new fucose-binding lectin from *Streptomyces* bacteria identified by analysis of available metagenomic DNA sequences. The identified protein SL2-1 belongs to a new group of bacterial fucose-specific lectins, with no similarity to the known bacterial fucose-binding proteins, but related to some core fucose-binding eukaryotic lectins. The 16 kDa protein was produced recombinantly in *E. coli* and purified by affinity chromatography on fucose-Sepharose. Recombinant SL2-1 shows high stability and precise glycan-binding specificity. In a glycan array screen, SL2-1 binds only to core α-1,6-fucosylated *N*-glycans, but not to the core α-1,3-fucosylated *N*-glycans, or other α-1,2-, α-1,3-, α-1,4-fucosylated oligosaccharides. Furthermore, the minimal binding epitope of SL2-1 was determined to be α-1,6-fucosylated chitobiose. Application of the lectin for detection and analysis of the core fucosylation of *N*-glycans will be discussed.

170. *N*-glycosylation is important for the activity of glycoside hydrolases

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Polysaccharides such as chitin, alginate and fructosan are abundant in nature. A convenient strategy to utilize these polysaccharides was hydrolysis the polysaccharides to soluble oligosaccharides or monosaccharides. The enzymatic method is considered as the best method because it's low cost and pollution. Several high-activity enzymes like chitinase, alginate lyase, inulinase were identified and characterized in my lab. Some of these enzymes showed better activity than the commercial enzymes. *N*-glycosylation is a common post-translational modification which has a crucial role in protein function. Most of our identified polysaccharides degrading enzymes are *N*-glycosylated protein. The effect of *N*-glycosylation on the activity of *Kluyveromyces cicerisporus* exoinulinase (KcINU) was studied. Endo F1 or PNGaseF treatment suggested that the heterologous KcINU in *Pichia pastoris* was an *N*-glycosylation protein. Zymogram analysis and TTC staining indicated the glycosylation deficiency made enzyme activity decreased. Bioinformatics analysis of the glycosylation KcINU1 revealed eight potential *N*-glycosylation sites of 203, 268, 363, 371, 386, 400, 468 and 527. LTQ Orbitrap mass analysis of the glycosylation KcINU showed five *N*-glycosylation modifications at sites 203, 363, 371, 400, and 468. To make clear the influence of each site *N*-glycosylation on the function, single mutations to Gln of these sites were constructed by site-directed mutagenesis respectively. The mutants of N363Q, N371Q and N400Q reduced enzyme activity, while the mutant of N203Q made the enzyme lose activity completely, however the mutant of N268Q raised the specific activity. The different sites glycosylation affect different roles like substrate binding or structural stability to regulate the KcINU activity. Similar results were found on our other glycoside hydrolases. Taken together, our research indicates that the *N*-glycosylation has important influence on glycoside hydrolases activity.

Glyco(proteo)mics

Keynote Lectures

171. Development and applications of advanced LC-MS/MS based glyco(proteo)mics

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The essence of glycomics resides not so much in enumerating as many distinct glycan entities, but more so the biologically relevant glycosylation features of a cell, tissue or an individual, primarily in terms of the expressed terminal glycotopes, and the elongation and branching pattern of the underlying cores. Precision is required not only in delineating each of the isomeric glycotopes that matter most in functional cellular communications, but also an ability to do so at high sensitivity for large-scale studies to allow distinguishing true pathophysiological changes from profound inter-individual variations. Towards this end, we have integrated and further refined existing nanoLC-MS/MS-based glycomic workflows for concerted non-sulfated and sulfated glycans analysis. Our approach is largely driven by target-selective multistage, multimode fragmentation to zero in on resolving and quantifying isomeric constituents of variably fucosylated, sialylated and/or sulfated glycotopes at high sensitivity. We have adapted methods first introduced in shotgun proteomics and glycoproteomics to glycomics, taking into account the very nature of glycosylation. Emphasis is placed on intelligent automated data acquisition and large-scale comparative data analysis pipeline in order to achieve high throughput and to extend the practical utilities of glycomics to a wider non-expert research community, without compromising precision. Glycotopes thus identified as of functional relevance then form the basis for an equally targeted glycoproteomics to identify its site-specific occurrence on common protein carriers.

172. Isomer specific glycan and glycopeptide sequencing as tools for unraveling disease glycosylation signatures

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The non-template driven biosynthesis of glycoconjugates poses particular challenges for their sequencing despite our knowledge on the biosynthetic pathways, in particular in mammalian organisms. Reliable tools for the robust, sensitive and accurate sequencing of glycoconjugates from minimum amounts of clinical specimens are thus a prerequisite for studying the functional role glycosylation plays in health and disease. Robust sample preparation workflows and multidimensional, automatable analysis technologies are important for successful sequencing of highly complex glycomes associated with clinical specimens such as biopsies or FFPE histopathological slides. Combining the power of porous graphitized carbon (PGC)

nano-LC separation with ESI-MS and MS/MS detection enables the simultaneous acquisition of multiple data points for qualitative and quantitative *N*- and *O*-glycan analysis, which has been automated using manually annotated MS/MS glycan spectra libraries as well as a combination of commercially available software with in-house developed software tools. This approach is applicable on large datasets and has been successfully applied in unravelling the tissue *N*- and *O*-glycome associated with disorders such as inflammatory bowel disease (IBD), hepatocellular carcinoma or skin neoplasia. Despite recent advances in glycomics, direct association of specific glyco-epitopes with the individual glycoproteins represents one final frontier in glycoproteomics. Concomitant identification of peptide identity and glycan-composition has become routine, however differentiation of specific isobaric glycosylation features directly on the glycopeptides still remains challenging. We synthesised *N*-glycopeptides with defined glyco-epitopes such as α-2,3- and α-2,6-linked NeuAc residues that were key components for the evaluation of Ion Mobility (IM)-MS to differentiate isobaric *N*-glycopeptides, resulting in novel approaches for peptide and glycan sequencing on the glycopeptide level.

Lectures

173. Glycoarrays constructed with engineered glycophage nanoparticles

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A deep understanding of protein-carbohydrate interactions have been relatively lacking, mainly because biosynthesis of glycans is not template-driven unlike other biopolymers, and the information content of glycans is enormous. Nevertheless, printed carbohydrate microarrays (glycoarrays) have emerged in the last decade as powerful, high-throughput tools for screening glycan-protein interactions and have been applied in areas such as disease detection, drug discovery and host-pathogen interaction studies. However, glycoarray applications are still limited by the expensive and complex methods available to synthesize glycans or by the challenges in isolating glycans from natural sources. We have recently demonstrated that engineered *Escherichia coli* can secrete filamentous phage particles with covalently linked *N*-glycans and further engineered these so called "glycophages" so that tailor-made *N*-glycans and O-antigens are displayed for use in glycan microarray fabrication. The advantages of glycophage-based arrays presented here, include the low cost and scalability of phage/glycan production, which are biosynthetic processes involving the cultivation of recombinant *E. coli* cells, and the ease with which glycophages can be recovered from the culture supernatant without laborious purification steps. Thus, the optimization steps towards the streamlined process will be presented for preparation and purification of diverse glycan libraries with large quantities of glycophages. We anticipate that the glycophage array technology will be an attractive complement to the existing glycoarrays. Acknowledgments: Marie Curie FP7 Career Integration Grant under REA grant agreement # 322096 and UNESCO-L'Oréal Young Women in Science Award (to E.Ç.).

174. New approaches for the analysis of glycans of glycoproteins

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Analysis of glycoproteins is still a major undertaking. Many protocols have been developed and are being used to elucidate the structure of the glycans by MS or NMR spectroscopy. We have recently developed new analytical protocols to elucidate the structure of glycans and other post-translational modifications. If glycans or glycopeptides have been prepared, it is usually difficult to obtain pure fractions of the individual compounds. We have developed a new technique, called 3DCC, by which NMR spectra of pure compounds can be generated even though the material has not been separated completely. This new technique combines the power of extracted ion chromatograms obtained from mass spectrometry and NMR spectra from the individual fractions each containing mixtures of compounds. Using the cross correlation technique one can now mathematically calculate the NMR spectra of the pure components. Combined with the sensitivity of NMR which allows recording of spectra down to about 6 pmol material, *i.e.* about 12 nanograms, glycans can be analyzed quickly using the information from the mass spectra and NMR combined. We could identify by this technique 4 mass isobaric nonasaccharides and other problematic glycans. Secondly, we have recently shown that the use of ESI/TOF mass spectrometry combined with pre-separation of the compounds in an LC step gives full information on the diversity of the glycans present in a given glycoprotein. Utilization of a back calculation step that allows computation of the individual peaks obtained in the mass spectrum validates the interpretation of the raw data. Glycoproteins with single glycosylation sites and up to 45 glycans have been elucidated in one single simple step.

175. Ultrasensitive impedimetric glycan and lectin biosensors as diagnostic tools

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Current state-of-the-art biochip technology applied in glycomics is based on fluorescent detection with a need to use fluorescent labels, what can negatively influence a biorecognition event. Moreover, fluorescent glycan/lectin biochips do not offer sensitivity of analysis needed for early stage analysis of disease biomarkers. Thus, alternative transducing schemes working in a label-free mode of action are sought with a focus on achieving low limits of detection. The most promising in this aspect is electrochemical impedance spectroscopy allowing to detect analyte down to single molecule level (*i.e.* aM level), when immobilization architecture is controlled at nanoscale. Application of these devices in analysis of serum samples from people having some diseases, in assay of various cancer biomarkers and cancer cell lines will be provided. Acknowledgement: The financial support from the Slovak research and development agency APVV 0282–11 is acknowledged. The research leading to these results has received funding from the European Research Council under the European Union's Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreement No. 311532 and from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement No. 317420.

176. Characterization of the first algae-made human anti-hepatitis B antibodies using a glycoproteomic approach

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Monoclonal antibodies (mAbs) represent actually the major class of biopharmaceuticals. They are produced recombinantly using living cells as biofactories. Among the different expression systems currently available, microalgae represent an emerging organism which displays several biotechnological advantages. Indeed, microalgae are cheap, classified as Generally Recognized As Safe (GRAS) organisms and can be grown easily in bioreactors with high growth rates similarly to CHO cells. Moreover, microalgae exhibit a phototrophic lifestyle involving low production costs as protein expression is fuelled by photosynthesis. However, questions remain to be solved before any industrial production of microalgae-made biopharmaceuticals. Among them, protein heterogeneity as well as protein post-translational modifications need to be evaluated. Especially, *N*-glycosylation acquired by the secreted recombinant proteins is of major concern since most of the biopharmaceuticals including mAbs are *N*-glycosylated and it is well recognized that glycosylation represent one of their critical quality attribute. In this study, we assess the quality of the first recombinant antibodies produced in the diatom, *Phaeodactylum tricornutum*. We are focusing on the characterisation of the *N*-glycosylation macro- and microheterogeneity as well as C- and N-terminal ends of the recombinant algae-made mAb.

Posters

177. Glycoproteomics of the S-layer of *C. difficile*

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Progress made in mass spectrometric (MS) glycoproteomic strategies allows us to expand our knowledge of protein glycosylation of important prokaryotic human pathogens. The precise role of glycosylation in bacteria is unknown due to problems in obtaining purified glycoproteins and an incomplete knowledge of the biosynthetic pathways involved. Understanding the mechanisms of bacterial glycosylation and its role in pathogenesis will underpin practical advances in the design of new glycoconjugate vaccines, antimicrobials and diagnostics. *C. difficile* is one of the main organisms responsible for morbidity in hospitalised patients. Its cell wall is surrounded by a 2D paracrystalline array, the S-layer, composed of two distinctive proteins, the high molecular weight (HMW) and the low molecular weight (LMW) S-layer proteins (SLPs). So far neither genetic nor phenotypic evidence of S-layer glycosylation had been found in *C. difficile*. Indeed, a rigorous MS study has shown no glycosylation in 12 strains.

However, SLPs can vary in sequence between strains and 12 different slpA cassettes have been described. Unusually, in some hypervirulent strains, one of the slpA cassettes carries a novel insert containing 19 ORFs which are similar to glycogenes in other bacterial species. Combining MS and genetics, we have now firmly demonstrated that SLP in strain Ox247 is glycosylated. A variety of MS approaches has been used to establish the putative structure of the Ox247 glycan structure and to show that glycosylation occurs only on the LMW SLP. Following the analysis of several mutants, and applying the recently developed ETD technique, firm evidence has been obtained for *O*-glycosylation of the DILA AQNLTTGAVILNK peptide in the LMW SLP. A glycan chain of up to twelve sugar units, $Hex_3DeoxyHex_8Pentose_1$, has been found attached to the Threonine at position 38 of the sequence determined. Future work will establish whether S-layer glycosylation plays a role in pathogenesis of *C. difficile*.

178. Comparative glycoproteomic study of serum proteins in chronic hepatitis C and hepatitis C induced liver cirrhosis patients

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Liver cirrhosis with hepatitis C viral infection (HCV-LC) has a very high risk to develop hepatocellular carcinoma. Besides routine diagnosis of liver cirrhosis by biochemical test and other imaging techniques, assessment of structural liver damage involves liver biopsy; which shows several disadvantages. Our aim was to monitor the changes in the expression of serum proteins and their glycosylation pattern between chronic hepatitis C (HCV-CH) and HCV-LC patients with respect to healthy controls by glycoproteomics study. Albumin depleted HCV-CH and HCV-LC patients sera by 2D gel electrophoresis gave several protein spots which were identified by LC-MS with respect to control. The change in the level of two prominent protein spots viz; serum haptoglobin (Hp) and alpha 1-antitrypsin (AAT) was evaluated by western blot and ELISA using specific mAbs. The change in glycosylation pattern of serum proteins were assayed using lectins of different carbohydrate specificity. 2D gel electrophoresis of albumin depleted sera of both patient groups revealed four differentially expressed protein spots as compared to healthy controls; of them two identified proteins were further analyzed by ELISA and western blot using mAbs of AAT and Hp to confirm the observed changes of protein expression in 2D gel. Increased level of Hp and AAT was observed in HCV-LC patients whereas those proteins was observed to be present in less in HCV-CH patient groups with respect to control. Lectin-blot showed decreased level of sialylation and increased level of fucosylation of serum glycoproteins of HCV-LC patients groups. This result was supported by Maackia amurensis agglutinin and Lens culinaris agglutinin respectively by ELISA, whereas high glycan branching was observed in HCV-CH patients as determined by Datura stramonium agglutinin. Thus the glycoproteomics approach as demonstrated is useful tool for identification and progression of HCV-CH and HCV-LC.

179. Rapid and quantitative *O*-glycomics of various biological samples by microwave-assisted β-elimination in the presence of pyrazolone analogues

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O-Linked glycosylations of serine/threonine residues (*e.g.* *O*-GalNAc, *O*-Man, *O*-Fuc, *O*-Xyl, *O*-GlcNAc, *O*-Gal, and *O*-Glc types) is a posttranslational modification of proteins and is essential for protein recognition and lipid functions on cell surfaces and within cells. The characterization of differently structured *O*-linked glycans (*O*-glycans) is particularly challenging because there is no known endoglycosidase for such groups. Therefore, chemical digestion approaches have been widely used; however, it is sometimes difficult to suppress unwanted side reactions. Recently, we reported a novel *O*-glycomics procedure using β-elimination in the presence of pyrazolone analogues (BEP). In the present study, we describe a microwave-assisted BEP procedure for rapid and quantitative *O*-glycomic analysis. Following optimization of the reaction conditions, the microwave-assisted BEP reaction substantially improved the recovery of total *O*-glycans from model glycoproteins (PSM) and the reaction time was reduced from 16 to 2 h. Combined with sequential solid-phase extractions, this microwave-assisted BEP procedure enabled *O*-glycomic analyses of various biological samples including serum, tissue, and formalin-fixed paraffin-embedded (FFPE) samples.

180. Improving antibody characterization: rapid PNGase F for accurate *N*-glycan analysis

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A growing number of antibodies and antibody fusions are currently used as therapeutic agents. A conserved *N*-glycan at Asn297 of the Fc region of IgG is critical for functional activity. Moreover, some antibodies have additional *N*-glycans that, together with the conserved site, affect recognition, half-life, and immune reactions. Characterization of the *N*-glycans of therapeutic antibodies requires their complete release with PNGase F, a reaction that often takes several hours. This is the first step in a process followed by glycan labeling and LC-MS. To optimize this workflow, we developed Rapid PNGase F for complete and unbiased release of *N*-glycans in only five minutes, immediately followed by an optimized reductive labeling with 2-aminobenzamide or procainamide. Finally, SPE-HILIC was used for cleanup before LC-MS. Overall, sample preparation time was shortened dramatically. Several therapeutic monoclonal antibodies were used for validation and results were in accordance to published data: sensitivity and accuracy are not compromised by a convenient glycan preparation workflow. The optimized direct labeling technique is also compatible with other endoglycosidases such as Endo S or PNGase Ar. Antibodies were treated with Endo S or a sample of plant-derived antibodies was treated with PNGase Ar. Endo S (an endoglycosidase) releases a reducing sugar, while both PNGase F and Ar (amidases) release *N*-glycosylamines, which rapidly convert to reducing sugars under the reactions' pH. The optimized direct labeling method was successfully used with these samples with excellent reproducibility. Rapid PNGase F allows fast, unbiased, *N*-glycan preparation. Improved, reductive direct labeling can be used after Rapid PNGase F, Endo S, or PNGase Ar.

181. Increasing the fidelity and utilities of mass spectrometry-based protein site-specific glycosylation analysis and glycoproteomics

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Current glycoproteomics is capable of high throughput analysis by relying on data processing and analysis software such as Byonic to automate glycopeptide identification by search and scoring algorithm. This can now return hundreds or even thousands of positive matches along with an estimated low FDR. A major problem then is manual validation, which remains tedious but essential, given the nature of glycosylation. Misassignment often arises due to uncertainties in correctly identifying the overlapping or weak monoisotopic precursors, failure to produce the peptide backbone ion, few peptide cleavage ions, and unanticipated extra modifications on either the glycan or peptide moiety. We have previously shown that, on an LTQ-Orbitrap hybrid or Orbitrap Fusion Tribrid instrument, the quintessential HCD MS2 data, which provides the sugar oxonium ions to filter true glycopeptides and peptide ions for glycopeptide identification, should be complemented by additional CID and/or ETD. The different modes are also useful in handling problematic cases such as sulfoglycopeptides. We present here our continuing effort to explore the best workflow and new computational tools for correctly identifying glycopeptides based on both single glycoprotein and whole glycoproteome analysis. One promising newcomer is Pinnacle, which utilizes and combines evidence from the isotopic fidelity of the parent ion along with high resolution HCD/CID/ETD product ions to build a comprehensive glycopeptide lists, and score them using a multivariate scoring system. We believe and propose that such multifaceted approach is the way forward for productive and precision glycoproteomics.

182. The specificity of α-1,4-*N*-acetylglucosaminyltransferase (α4GNT) and β-1,4-*N*-acetylgalactosaminyltransferase (β4GalNT) on the different *O*-glycan core chains using a mucin type reporter protein

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Recombinant fusion protein productions as biopharmaceuticals are strongly growing within the pharmaceutical industry. Gastric cancer-associated glycosylation phenotypes were glyco-engineered CHO cells by use of glycosyltransferases responsible for the *in vivo* synthesis of glycans. The transferases that are of interest to us are α4GNT and β4GalNT. The α4GNT produces *O*-glycans that have the terminal α-1,4-linked *N*-acetylglucosamine, which was preciously shown to have antimicrobial activity against *H. Pylori*. β4GalNT in the human stomach makes the LacdiNAc epitope and it has specificity to core 2 O glycan; and previous data has

indicated that it may be involved in the suppressing the binding of *H. pylori*. We are investigating the specificity of transiently transfected α4GNT and β4GalNT respectively on *O*-glycan core 1 and core 2 biosynthesis pathways respectively in CHO cells. Liquid chromatography-mass spectrometry (LC-MS) and Western blotting were used to map the *O*-glycome of a mucin-type fusion protein transiently co-transfected with [PSGL-1/mIgG2b, and α4GNT] and [PSGL-1/mIgG2b, β6GlcNAcT and β4GalNT] respectively using Lipofectamine. The LC-MS data for α4GlcNAc/PSGL1 showed only one peak corresponding to GlcNAcα1, 4Galβ1,3GalNAcol (m/z 587) and that of β1,4GalNAc/core 2/PSGL1 showed only one peak corresponding to GalNAcβ1, 4GlcNAcβ1, 6(NeuAcα2,3Galβ1,3)GalNAcol (m/z 1081). Post results analysis, the specificity of these enzymes on different core structures was studied. CHO-K1 cells were transiently co-transfected with α4GNT and PSGL1/mIgG2b (against core 2, core 3 and core 1 Extended) and β4GalNT3/4 and PSGL1/mIgG2b (against core 1, core 3 and core 1 Extended) respectively. The mass spectrometer data showed that both the glycosyltransferases were specific to all the different glycan core structures that were being studied, except for β4GalNT3/4 and PSGL1/mIgG2b on core 1.

183. Comprehensive *N*-, *O*- and glycosphingolipid-glycomics of meat and/or roe of popular seafoods

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Food glycans are of interest from nutritional and health points of view. As an example, exogeneous non-human type glycans such as *N*-glycolylneuraminic acid (Neu5Gc) could accumulate in human tissue and elicits an immune reaction. The major sources of exogenous Neu5Gc are considered to be foods, as it has been demonstrated that Neu5Gc is found in widely varying amounts in foods. The sources are mainly red meats such as lamb, pork, and beef, while Neu5Gc is also found in fish with lesser amounts. In this study, we aimed to clarify the gross glycomic profiles of popular seafoods especially focusing on the sialylation status. The *N*-, *O*- and GSL-glycomes of meat and/or roe of 8 popular seafoods (tuna, cod, salmon, trout, herring, shrimp, crab and sea urchin) were analyzed based on the recently established quantitative analytical techniques for *N*-, *O*- and GSL glycans. We could successfully detect >130 *N*-glycans, >20 *O*-glycans as well as >180 GSL-glycans in total. Sialylated glycans were hardly detectable in shrimp and crab, while we detected unique uronic acid containing glycans specifically in GSLs of these animals. In fish, sialylated glycans were commonly observed, and they were more abundant in roe than in meat. Neu5Gc was found in all fish (both in meat and roe), though the percentages of Neu5Gc in total sialylated *N*-glycans was determined to be less than 1.0 %. By contrast, high relative levels of Neu5Gc-containing *O*-glycans were observed in salmon roe. Thus, it was found that the relative abundance of Neu5Gc could be quite different among different classes of glycoconjugates. Comparison of other glycomic profiles, including occurrence of *O*-acetylated sialic acid, among different classes of glycoconjugates will be also discussed.

184. Characterisation of glycosylation of paramyxovirus surface glycoproteins by mass spectrometry

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The family *Paramyxoviridae* (paramyxovirus) contains a number of significant human and animal pathogens. Represented within this family are human respiratory syncytial virus (hRSV) and Newcastle disease virus (NDV). The former causes severe respiratory tract disease in infants, children and immunocompromised individuals. At present, a safe and effective vaccine is not available for hRSV. NDV is the causative agent of Newcastle disease, afflicting a wide range of avian species. The desire to study NDV is due not only to the significant economic impact it has on the poultry industry worldwide but also its potential use as an oncolytic agent and vaccine vector for human and animal use. Of great importance to paramyxoviruses are the variable attachment glycoproteins, haemagglutinin (H), haemagglutinin-neuraminidase (HN) and major surface glycoprotein (G) along with the fusion (F) glycoprotein. Glycoproteins H, HN and G are involved in viral attachment to host cells, while F is responsible for viral entry by means of fusion with host cell membranes.

Research has shown that the glycosylation sites present on these proteins can modulate protein function. As yet, site-specific glycan heterogeneity of hRSV and NDV attachment and fusion proteins has not been defined at a chemical level. Revealing the glycosylation profile of these proteins may help elucidate mechanisms of viral attachment, replication and immune evasion within paramyxoviruses. Liquid chromatography-mass spectrometry utilising different fragmentation techniques was implemented to structurally characterise site-specific glycan heterogeneity of the digested glycoproteins. Initial research has revealed sialylated *N*-linked and mucin-like *O*-linked glycosylation of recombinant RSV G. Analysis of NDV revealed high-mannose glycosylation of F and HN and additionally, sialylated and sulphated *N*-linked glycans and a novel sialylated *O*-linked glycan of NDV HN.

185. A pH-responsive soluble polymer-based homogeneous system for fast and highly efficient glycoprotein enrichment and identification by mass spectrometry

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Liquid phase homogeneous reactions using soluble polymer supports have found numerous applications in homogeneous catalysis and organic synthesis because of their advantages of no interface mass transfer limitation and a high conversion rate. However, their application in analytical separation is limited by the inefficient/inconvenient recovery of the target molecules from the extremely complex biological samples. Here, we report a stimuli-responsive polymer system for facile and efficient enrichment of trace amounts of biomolecules from complex biological samples. The soluble polymer supports provide a homogeneous reaction system with fast mass transfer and facilitate interactions between the supports and the target molecules. More importantly, the stimuli-responsive polymers exhibit reversible self-assembly and phase separation under pH variations, which leads to facial sample recovery with a high yield of the target biomolecules. The stimuli-responsive polymer is successfully applied to the enrichment of low abundant *N*-glycoproteins/glycopeptides, which play crucial roles in various key biological processes in mammals and are closely correlated with the occurrence, progression and metastasis of cancer. Highly efficient enrichment of *N*-glycoproteins/*N*-glycopeptides with >95 % conversion rate is achieved within 1 h, which is eight times faster than using solid/insoluble enrichment materials. Mass spectrometry analysis achieves low femtomolar identification sensitivity and obtained 1317 *N*-glycopeptides corresponding to 458 *N*-glycoproteins in mouse brain, which is nearly twice the amount obtained after enrichment using commercial solid/insoluble materials. These results demonstrate the capability of this "smart" polymer system to combine stimuli-responsive and target-enrichment moieties to achieve improved identification of key biological and disease related biomolecules.

186. A novel C18-PGC-LC-ESI-MS/MS approach for site-specific protein glycosylation analysis applied to human IgG3

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Site-specific protein glycosylation analysis is often challenging due to the complexity caused by the micro- and macroheterogeneity of protein glycosylation. To obtain qualitative site-specific glycosylation information unspecific protease treatment can be applied, resulting in small peptide moieties carrying the glycan. Detecting these glycopeptides by conventional 1D-LC-ESI-MS/MS can be difficult due to insufficient or irreversible retention on the stationary phase, requiring multiple analyses with different LC-setups. However, since biological sample amounts are usually limited, methods for acquiring comprehensive information in a single run are desired. We developed a platform for site-specific *N*- and *O*-glycosylation analysis of glycoproteins. The purified protein was subjected to unspecific pronase digestion, resulting in glycopeptides with a small peptide portions. The digests were then analyzed by an integrated C18-porous graphitized carbon (PGC)-LC-ESI-MS/MS platform. This allowed retention of hydrophobic glycopeptides on the C18 column and the more hydrophilic ones of the flow-through on the PGC column. In combination with high-resolution QTOF mass spectrometry this method allowed identification of the glycosylation sites and glycoforms from one injection. This method was evaluated using standard glycoproteins and successfully applied for the analysis of various plasma glycoproteins

including IgG3. We obtained a detailed site-specific characterization of human IgG3, without prior enrichment, revealing novel information on the *N*-glycosylation site Asn322. This site showed a different glycosylation profile than the well characterized and conserved site Asn227. In addition, we readily obtained information on IgG3 hinge region *O*-glycosylation. Our platform is a versatile tool applicable to various glycoproteins for detailed qualitative site-specific glycosylation analysis that can be further integrated into workflows for comprehensive glycoprotein analysis.

187. Sweet secrets of a therapeutic worm: the *N*-glycome of *Trichuris suis*

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The porcine whipworm, *Trichuris suis*, has attracted attention due to reports of administration of the eggs as a potential therapy for inflammatory diseases in human patients. However, the nature of the immunomodulatory factors is unknown, but *in vitro* suggest that glycans on the parasite excretory/secretory proteins may have a role. In this study, we have examined the *N*-glycans of *T. suis* using an off-line LC-MS approach in combination with chemical and enzymatic treatments. Other than the typical paucimannosidic and oligomannosidic glycans present, a number of glycans carry LacdiNAc modified by fucose and/or phosphorylcholine; thereby, α-1,3-fucosylated PC-modified HexdiNAc is a novel combination of glycostructural elements. The overall *N*-glycome of this clade I nematode differs in terms of core fucosylation from clade V nematodes such as *Caenorhabditis elegans*, *Pristionchus pacificus* and *Haemonchus contortus*; whereas *T. suis* maximally difucosylates its reducing-terminal GlcNAc, clade V nematodes have up to three core fucose residues. Knowledge of the glycome of *T. suis* will enable more targeted studies on glycan receptors in the host as well aid design of the correct glycosylation of recombinant forms of candidate proteins for future studies on immunomodulation.

188. Quantitative glycomes analysis of *N*-glycan patterns in bladder cancer cells

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Bladder cancer is one of the most common types of human cancer, and its incidence has steadily increased during the past decade. Diagnosis of bladder cancer at an early (nonmuscle-invasive) stage is the best way to reduce the mortality rate. Tumor malignancy in general is closely associated with the types of glycans expressed. Glycosylation status, particularly global glycomes, in bladder cancer are not well known. We integrated lectin microarrays and mass spectrometry (MS) methods to quantitatively analyze and compare glycan expression levels in normal bladder mucosa HCV29 and HUCV1 cells, superficial bladder cancer KK47, and invasive bladder cancer YTS1, J82, T24 cells. Glycopattern alterations were analyzed using lectin microarrays and confirmed by lectin staining and lectin blotting. *N*-glycans were derivatized by amidation of sialylated glycans with acetohydrazide and reductive amination with the stable isotope tags [^{12}C6]- and [^{13}C6]-aniline, and were quantitatively analyzed by MALDI-TOF/TOF-MS. Our findings indicate that sLex, terminal GalNAc and Gal, and high mannose-type *N*-glycans were more highly expressed in bladder cancer cells than in normal bladder mucosa cells, that patterns of fucosylated complex-type *N*-glycans differed significantly between the cell lines, and that the glycome changes were directly related to bladder cancer progression.

189. Quantitative glycosphingolipid-glycomics of human whole serum based on egcase-assisted glycan cleavage, glycoblotting-assisted sample preparation, and MALDI-TOF MS analysis

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Glycolipids are lipid molecules linked to carbohydrate units and classified as either glycosphingolipids (GSLs) containing ceramide, or glycoglycerolpids containing glycerol or diacylglycerol. In mammals, GSLs are predominantly component and form the plasma membrane lipid raft clustered with sphingolipids, sterols, and specific proteins to contribute membrane physical properties and specific recognition sites for various biological events. The structural classification of GSLs is often defined by their glycan structure (*i.e.* ganglio-,

globo-, and (neo)lacto-series). These bioactive GSL molecules affect consequently on the pathophysiology and pathogenesis of various diseases, which include various GSL lysosomal storage diseases various types of cancer, infections atherosclerosis, obesity and central nervous system diseases. Thus, altered expression of GSLs under various diseases may have roles in disease related biomarker discovery. We recently established a procedure for a quantitative and qualitative cellular glycomics of GSLs based on *Rhodococcal* endoglycoceramidase-assisted glycan cleavage, glycoblotting-assisted purification and MALDI-TOF MS analysis. The aim of this study is to apply this method suitable for the quantitative analysis of GSL glycans in serum and clarify the human serum GSL glycome. Herein we report two sample preparation protocols, one with, and the other without GSLs extraction by chloroform/methanol. Both protocols permitted absolute quantitation of ~42 GSL-glycans using as low as 20 μL serum.

190. Comparative glycosylation analysis of Alpha-1 antitrypsin derived from human plasma and recombinant cell culture

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Alpha-1 antitrypsin (A1AT) is a serine protease inhibitor and an acute phase protein. It has been used in augmentation therapy for A1AT deficiency. Here we present a comparative glycosylation analysis of A1AT preparations derived from human plasma (pdA1AT) and recombinant cell culture (rA1AT). In the latter case, A1AT samples were purified from conditioned media of mid log, stationary, and death phases of recombinant Chinese hamster ovary (CHO) cells cultured in batch mode. Analysis at released glycan level was done by HILIC-UPLC with online mass spectrometer, which reveals significant difference in glycosylation of pdA1AT and rA1AT, in terms of fucosylation, sialylation, and branching. Site-specific analysis was also performed at intact glycopeptide level using tandem mass spectrometry with different fragmentation modes. Intact protein mass spectrometry sheds light on glycoform and proteoform distribution during the cell culture. Taken together, the comprehensive glycosylation analysis provides insights into the dynamics of A1AT glycosylation in cell culture and suggests areas for improving the quality of the recombinant product.

Glycobiology of Autoimmune and Inflammatory Diseases

Keynote Lectures

191. A keratin sulfate and its derivatives attenuate inflammation and the progression of emphysema in the murine lung by targeting to Langerin, C-type lectin

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Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide and is predicted to be the third by 2020. The risk factors are cigarette smoking and air pollutants, which make the disease a major public health problem worldwide in terms of associated morbidity and mortality. COPD patients are more likely to develop a greater degree of inflammation in their airways due to bacterial or viral infections and air pollutants than normal subjects. The phenotype of some patients with frequent exacerbations results in a rapid deterioration of lung function and poor outcomes, which are accompanied by extensive pathological changes of the lung inflammation, particularly in the small airways. However, there is no cure for COPD and the efforts of pharmaceutical companies in developing therapeutic agents for COPD have so far resulted in limited success. In this study we report that L4, a keratin sulfate (KS) disaccharide ([SO_3^--6] Galβ1-4[SO_3^--6] GlcNAc) attenuated inflammation and lung destruction in experimental emphysema model mice. Since KS is one of the major glycosaminoglycans in the small airway, but was diminished from the surface of normal human bronchial epithelial (NHBE) cells by elastase treatment *in vitro*, we

attempted to administer L4 in elastase- and cigarette smoke-induced emphysema models in Fut8 heterozygous KO and wild mice. L4 inhibited an early phase of inflammatory cell influx and prevented alveolar destruction. The levels of matrix metalloproteinases, pro-cytokines, and chemokines in bronchoalveolar lavage fluid were also suppressed. Langerin, a C-type lectin on immature dendritic cells was found to be a receptor of L4. L4 and its multivalent derivative, Tri-L4 represent potential glycan-based therapeutics for the treatment of COPD by targeting langerin.

192. Helminth glycans and Th2 responses

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Parasitic helminths carry a complex array of glycans which appear to play a central role in their interaction with the human host. The cellular immune responses to helminth infections are dominated by Th2. Helminth derived molecules have been identified which are able to modify dendritic cell function and thereby skew T cell responses towards Th2. Through generation of mutants it has been possible to establish that glycan interaction with C type lectins on dendritic cells is essential for driving Th2 responses. Unlike what is seen with allergens, the strong Th2 responses induced by helminth antigens and the resultant IgE antibodies, do not lead to allergic diseases. One of the reasons appears to be the poor biological activity of allergen specific IgE antibodies during helminth infections. Some such IgE antibodies are directed towards glycan structures rather than the protein backbone. These studies indicate that studying the structural details of the glycans and their receptors that form the interface with the human immune system might provide novel possibilities to treat a number of inflammatory diseases.

Lectures

193. Immunoglobulin glycosylation analysis in pregnancy and rheumatoid arthritis

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Immunoglobulins (Igs) are involved in many aspects of our body's immune homeostasis. Glycosylation of the Igs changes with both physiological events (*e.g.* pregnancy) and diseases (*e.g.* rheumatoid arthritis (RA) and cancer). The immunoglobulin G (IgG) constant region (fragment crystallizable; Fc) shows increased levels of agalactosylated *N*-glycans in many inflammatory conditions. Approximately 20 % of the IgGs additionally carries an *N*-glycan on the variable region (fragment antigen binding; Fab). We recently developed a method that allows to specifically analyze Fc and Fab glycosylation. When applied to a pregnancy cohort, we found increased levels of galactosylation, sialylation and bisection of Fab glycans as compared to Fc. In addition, pregnancy-associated changes were observed for most glycosylation traits. Next, we studied immunoglobulin A (IgA) glycosylation changes with pregnancy and RA and compared those with the changes found for IgG. For this purpose, we developed a high-throughput method for the simultaneous analysis of IgA *N*- and *O*-glycosylation on the level of glycopeptides. The method revealed clear site-specific differences for sialylation, fucosylation and levels of bisecting *N*-acetylglucosamine. Pregnancy-associated changes occurred on both *N*-glycosylation sites. Interestingly, *O*-glycosylation was less prone to change during pregnancy, although small yet significant differences were observed for traits involving galactosylation. IgA glycosylation analysis in RA revealed *N*-glycan changes similar to those observed on IgG. Interestingly, the *O*-glycans showed higher levels of galactosylation in RA, while the abundance of the other monosaccharides did not differ. Together, these data indicate both specific and general alterations in immunoglobulin glycosylation associated with pregnancy and RA. The potential function of the glycans on immunoglobulins in pregnancy and inflammation should and will be part of future mechanistic studies.

194. Therapeutic potential of lectin to target sialic acid modified receptors on osteoarthritic chondrocytes

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Articular cartilage covers joint surfaces to enable the painless, low-friction movement of synovial joints. Nearly half of the all people are likely to suffer from arthritis in their life time. Current treatments consist mostly of anti-inflammatory and immunosuppressant medications. Arthritis causes degradation of articular cartilage at synovial joints. The cartilage is synthesized by chondrocytes and consists of a collagen-proteoglycan matrix containing numerous glycosylated proteins critical for cartilage structure and functions. A shift in expression of glycoproteins containing α-2,6-linked sialic acids to those containing α-2,3-linked sialic acids has been shown to modify cellular anchoring and signal transduction events. Most notably, an increase in α-2, 3-sialylation of chondrocyte glycoproteins has been associated with the onset diseases including rheumatoid arthritis (RA) and osteoarthritis (OA). However, the pathophysiology of α-2,3-sialylation in cartilage has not been elucidated. The results here presented demonstrate that cartilage explants from patients with OA express significantly higher levels of the α-2,3-sialylated transmembrane mucin receptor podoplanin (PDPN). The *Maackia amurensis* seed lectin (MASL) can be used to target α-2,3-sialylated receptors including PDPN on these cells. Indeed, we show that nanomolar concentrations of MASL can preserve chondrocyte function and prevent cartilage breakdown initiated by ROS, inflammatory cytokines, and metalloproteinases. We reveal novel findings whereby specific lectin may serve as a treatment for cartilage degenerative related diseases. These findings shed mechanistic light on how a specific lectin may serve as a treatment for OA and RA, a so far incurable conditions.

195. Glycan targets for biomarkers of rheumatoid arthritis

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The growing need of biomarkers for disease diagnosis is exemplified by the rising demand from patients and providers worldwide, with the global biomarker markets estimated to grow to $30.6 Billion (CAGR of 16 %) in 2020. The significance of glycosylation changes in the onset of disease is widely recognised and changes in glycan structure can be indicative of disease progression, making them suitable targets for biomarkers. Glycosylation changes have been demonstrated for total serum immunoglobulin (IgG) in patients suffering with rheumatoid arthritis (RA). Changes in the glycosylation patterns of IgG subclasses result in different affinities to Fcγ receptors (FcγR) which in turn can trigger varying immune responses as was successfully demonstrated on Fc glycosylation of IgG1 anti-citrullinated protein antibodies. Studies on the released glycans from total IgG1 have not been demonstrated to date to the best of our knowledge. High-throughput high-performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC) based analyses have been previously demonstrated by our group for serum *N*-glycans in glycoprofiling studies in diseases such as cancer and galactosemia. A novel high-throughput robotic workflow will be presented for IgG and IgG1 glycoprofiling including glycoprotein purification, enzymatic glycan release, fluorescent labelling and UPLC analysis of released *N*-glycans. Results will be presented on glycosylation variability in the total IgG and IgG1 glycans of plasma samples from patients' pre- and post-treatment for RA.

196. Regulation of T cells immune response by *N*-glycosylation in inflammatory bowel disease

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Inflammatory Bowel Disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract that comprises Ulcerative Colitis (UC) and Crohn's disease (CD). The incidence of IBD is increasing worldwide and the disease remains incurable. Although progress has been made in understanding this chronic and disabling disorder, the precise etiopathogenesis of IBD is far from being fully elucidated. Therefore there is an urgent unmet need in the clinic to identify and characterize novel underlying molecular mechanisms of IBD in order to improve the development of novel biomarkers that may help the determination of prognosis and also improve the patients' stratification for appropriate treatment. Glycosylation has been shown to control the immune system and particularly the T cell activity and signaling has been demonstrated to be regulated by

GnT-V-mediated glycosylation. In this study we aimed to address whether the (dys)regulation of the interplay between GnT-V-mediated *N*-glycan branching and T cell activity and function is a major contributory factor and a yet uncovered mechanism in IBD. Our results showed that intestinal T cells from UC patients display a decreased expression of GnT-V-mediated glycosylation, associated with T cell hyperactivity and hyperimmune response. This dysregulation of branched *N*-glycans in the T Cell Receptor was found to accompany disease severity. Patients with severe UC showed the most pronounced defect on *N*-glycan branching in mucosal T cells. Moreover, we further showed that intestinal T lymphocytes from UC patients (with active disease) exhibit a deficiency in MGAT5 gene transcription comparing with normal controls, which underlies the observed dysregulation of T cells glycosylation. Overall, the disclosure of this new molecular mechanism in IBD opens new perspectives to further explore the potential applicability of this mechanism in predicting disease course and/or susceptibility.

Posters

197. Glycosilation profile of immunoglobulin G in moderate kidney dysfunction

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Glycans constitute the most abundant and diverse form of the post-translational modifications. Here we explore the associations between IgG glycans and renal function in 3274 individuals from TwinsUK. We analyzed the correlation between renal function measured as eGFR and 76 *N*-glycan traits using linear regressions adjusting for covariates and multiple testing in the larger population. We replicated our results in 31 monozygotic twin pairs discordant for renal function. Results from both analyses were then meta-analyzed. 14 glycan traits were associated with renal function in the discovery sample ($P<6.5\times10^{-4}$) and remained significant after validation. Those glycan traits belong to three main glycosylation features: galactosylation, sialylation and level of bisecting *N*-acetylglucosamine (GlcNAc) of the IgG glycans. This is the first study to show the role of IgG glycosylation in assessing kidney function. These results provide novel insight into the pathophysiology of CKD and potential diagnostic and therapeutic targets

198. *N*-glycans in the control of T cells immune response in inflammatory bowel disease

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Inflammatory Bowel Disease (IBD) is a chronic immune-mediated disorder of the gastrointestinal tract that includes Ulcerative Colitis (UC) and Crohn's disease (CD). IBD remains an incurable disorder with an increased risk to evolve to colorectal cancer. Several reports support that T cell activity and signaling is tightly regulated by glycosylation. Our group recently found for the first time that IBD patients display a deficiency in a key regulatory mechanism of T cells that is through *N*-glycosylation modifications. In order to further explore this underlying molecular mechanism we went to assess the role of specific *N*-glycans structures (GnT-V-mediated) in the regulation of T cells immune response. For doing so, we used *ex vivo* T cells purified from fresh colonic biopsies obtained from a well-characterized cohort of IBD patients (with different clinicopathological features) and healthy controls. Our *ex vivo* results revealed that *N*-glycans has a functional impact in the regulation of T cells immune response. Patients with active disease showed a decreased T cells branched *N*-glycosylation concomitantly with an increased T cell proliferation, increased production of

Th1 pro-inflammatory cytokines and increased T cell signalling. These results are being validated in mouse models of IBD. Overall, we demonstrated that T cells functions and activity are controlled by GnT-V-mediated *N*-glycosylation, opening new insights in the understanding of IBD pathogenesis.

199. Changes in the IgG glycome associated with inflammatory bowel disease

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Glycobiology is an underexplored research area in inflammatory bowel disease (IBD), and glycans are relevant to many aetiological mechanisms described in IBD. Alterations in *N*-glycans attached to the IgG Fc fragment can affect molecular structure and immunological function. Recent genome-wide association studies reveal pleiotropy between IBD and IgG glycosylation. We conducted two studies to better understand changes in the IgG glycome associated with IBD. In the first study we analyzed IgG glycans in 507 patients with ulcerative colitis (UC), 287 patients with Crohn's disease (CD) and 320 controls. Statistically significant differences in IgG glycome composition between patients with UC, or CD, compared to controls, were observed. Both UC and CD were associated with significantly decreased IgG galactosylation (digalactosylation, UC Odds ratio [OR]=0.71, 95 % confidence interval [CI] 0.5–0.9, p=0.01, CD OR=0.41, CI 0.3–0.6, $p=1.4\times10^{-9}$) and significant decrease in the proportion of sialylated structures in CD (OR=0.46, CI 0.3–0.6, $p=8.4\times10^{-8}$). Logistic regression models incorporating measured IgG glycan traits were able to distinguish UC and CD from controls (UC $p=2.13\times10^{-6}$, CD $p=2.20\times10^{-16}$), with receiver operator characteristic curves demonstrating better performance of the CD model (Area under curve [AUC]=0.77) over the UC model (AUC=0.72) (p=0.026). The observed differences indicate significantly increased inflammatory potential of IgG in IBD. In the second study we analyzed IgG glycans in 87 twin pairs discordant for IBD, 16 twin pairs with IBD and 103 healthy controls. The results indicate that healthy twins are more similar to healthy controls than to their diseased twins.

Glycobiology of Infectious Disease

Keynote Lectures

200. Glycan binding specificity of highly pathogenic influenza viruses

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The aim of this work is to figure out expected connection between avian influenza virus pathogeneicity on the one hand and glycan specificity of hemagglutinin (HA) and (or) neuraminidase (NA) - on the other hand. Highly pathogenic influenza viruses of H5 and H7 subtypes emerge after introduction of low pathogenic avian influenza viruses from wild birds into poultry. Viruses were compared for their HA binding to synthetic sialoglycans and for kinetics of their NA-mediated desialylation of the same sialoglycans. Substrate specificity of NA did not correlate with pathogeneicity of the virus. In contrast, all highly pathogenic viruses differed by the ability to discriminate non-fucosylated and fucosylated sialyloligosaccharides, namely, pairs 3'SLN vs. SiaLeX and 3'SiaLeC vs. SiaLeA.

201. Immune evasion mediated by pathogen-glycans and related immunotherapy strategy

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Many human major infectious disease pathogens (*e.g. Mycobacterium tuberculosis* (*M.tb*), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV)) have rich glycans (sugar chains and glycoconjugates) on the surfaces of microbial organisms. These pathogens-surface glycans have been implicated as immunosuppressive epitopes and might contribute to chronic or latent infections and immune invasion. Mannose-capped lipoarabinomanan (ManLAM) is a lipoglycan serving as a major cell wall component in both BCG and virulent *M. tb*. Here, we demonstrated a novel ManLAM mediated immune evasion mechanism by inhibitory effects on the polarizations of M1 macrophages and Th1 cells via CD44. Our data suggest that BM2, an aptamer specifically against ManLAM with low and no toxicity, holds a potential application as BCG immunoadjuvant and provides a new strategy for TB vaccine design. The development of an efficient vaccine and broadly cross-neutralizing antibody of Hepatitis C Virus (HCV) remains a priority. The highly conserved and heavily glycosylated viral envelope glycoprotein E2 and E1E2 complex are candidate vaccine antigens. Here we demonstrated that *N*-glycans of HCV blocked the exposure of the specific epitopes and mediated immune evasion. Our data suggest that *N*-glycosylation mutant of HCV envelope proteins and its associated neutralizing monoclonal antibody provide potential applications for the treatment of Hepatitis C Virus infection and vaccine design with enhanced immunogenicity.

Lectures

202. Characterisation and localisation of glycans in newly excysted juvenile *Fasciola hepatica*

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Fasciola hepatica is a parasite responsible for a zoonotic disease called fasciolosis. In recent years, the prevalence of fasciolosis has increased considerably and the availability of a prophylactic vaccine would be a considerable progress in prevention of fasciolosis. The *F. hepatica* tegument (Teg) is known to be covered by a glycocalyx that may contain immunogenic antigens. The Newly Excysted Juvenile (NEJ) is the first migratory larvae in contact with the definitive host, hence our work was focused on determing the glycan profile of the NEJTeg and assessing similarities and differences to immunogenic glycans described in other trematodes. After *in vitro* excystation, NEJs were fixed and stained with 17 fluorescently labelled lectins. Our results showed that ConA, PSA, PNA and jacalin lectins were able to recognise glycans on the NEJ surface, the oral and ventral suckers suggesting that mannose, fucose and the mucin-type core-1 structure are the most dominant terminal structures. In order to confirm our findings and perform a deeper glycan characterisation, NEJTeg *N*-glycans were enzymatically released, purified and fluorescently labelled. We found that α6-core fucosylated and non-fucosylated oligomannose and high mannose *N*-glycans are the most dominant *N*-glycans in NEJTeg, confirming our lectin binding experiments. Because of the differences in the glycosylation pattern between the PNG-F and PNG-A released *N*-glycans, it could be also speculated the presence of core α3-Fuc in a set of *N*-glycans. *O*-glycans released by β-elimination were not detected by MALDI-TOF-MS that is not suitable to detect anything smaller than a trisaccharide, indicating that NEJTeg *O*-glycans consist only of the mucin-type core-1 structure. To our knowledge, this is the first report of the structural characterisation of *F. hepatica* NEJ glycans, providing a valuable basis to understand how NEJTeg glycans could play a role in host-parasite interaction at the migratory and immunological level

203. Antibody responses to schistosome glycan antigens and their role in immunity

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During infections with schistosomes, important worm parasites infecting 200–300 million people, humoral and cellular immune responses against numerous glycosylated antigens expressed by larvae, worms and eggs are raised. Antigenic glycan motifs on proteins and lipids are major targets of the antibody (Ab) response, raising the question which role these anti-glycan Abs play in relation to immunity. Using a mass spectrometric glycomics approach we have investigated the expression profiles and structural identity of a large number of glycans produced by the schistosome during its life cycle. Striking shifts and switches in the expression of antigenic glycan motifs such as LeX, multifucosylated LDN, and unique *O*-glycans during maturation of the worm were identified that appear to be linked to establishment in the host. Subsequently, we have generated a microarray of the hundreds of isolated *N*-, *O*-, and lipid-glycans covering the entire glycome of *S. mansoni* and the spatial expression of glycan antigens during parasite development was investigated by microscopy using monoclonal Abs characterised by glycan array analysis. Moreover, we have used the array to determine serum IgG and IgM titers to each glycan in human and animal cohorts. Studies of human *S. mansoni* infections were performed in relation to age, infection intensity, treatment and resistance to reinfection indicating that only a subset of anti-glycan Abs may be correlated to effective immunity. In addition, we performed longitudinal analyses of anti-glycan Abs in *S. japonicum* infected macaques that self-cure infection, and in baboons protected from challenge infection by vaccination with irradiated *S. mansoni* cercariae. Integrated analyses of these studies suggest that multifucosylated glycan motifs expressed on schistosome larvae may be targets of an effective immune response. Initial explorations of synthetic conjugates of such glycans for immunisations in a mouse model for schistosomiasis will be discussed.

204. *O*-linked glycosylation of the mucin domain of the *Herpes simplex* virus type 1 specific glycoprotein gC-1 is temporally regulated in a seed-and-spread manner

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The human herpes simplex virus type 1 glycoprotein gC-1 is involved in receptor interactions and in immune evasion and is also heavily *O*-glycosylated. Since the virus does not encode any glycosyltransferases itself it is completely dependent on the cellular machinery for glycosylation. The human genome encodes 20 different glycosyltransferases that are able to initiate *O*-glycosylation by adding *N*-acetylglactosamine (GalNAc) to a serine or a threonine, namely GalNAc-transferase T1 through T20. The gC-1 has a distinct mucin-like region and in this work we used LC-MS/MS to characterize the *O*-glycans this domain. We also did an expression profile of the GalNAc-transferases by RT-qPCR and immunohistochemistry, all in diploid human fibroblasts that is an authentic target cell for the virus. Finally we applied an *in silico* approach to compare our experimental data with an open access algorithm based on *in vitro* studies on synthetic peptides (ISOGlyP – http://isoglyp.utep.edu/). We found that *O*-glycosylation occurs in a site specific and ordered manner that could be related to specific GalNAc-transferases. We conclude that *O*-glycosylation of the mucin-like region of herpes simplex virus type 1 glycoprotein gC-1 starts with the addition of GalNAc residues at specific positions of the amino acid sequence and then spread along the peptide essentially before any elongation of the glycans occur. Expression pattern of GalNAc-transferases together with *in silico* analysis suggested that GalNAc-T2 is the principle enzyme responsible for initiating *O*-glycosylation of this peptide whereas a number of different GalNAc-transferases can function as follow up enzymes that add subsequent GalNAc units before elongation of the glycans. This work shows that we have an excellent model system for studying details of early *O*-glycosylation in primary unmodified cells that are a natural target for herpes simplex virus type 1.

205. A strategy for *O*-glycoproteomics of enveloped viruses—the *O*-glycoproteome of *Herpes simplex* virus type 1

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Glycosylation of viral envelope proteins is important for infectivity and interaction with host immunity, however, our current knowledge of the functions of glycosylation is largely limited to *N*-glycosylation because it is difficult to predict and identify site-specific *O*-glycosylation.

Here, we present a novel proteome-wide discovery strategy for *O*-glycosylation sites on viral envelope proteins using herpes simplex virus type 1 (HSV-1) as a model. We identified 74 *O*-linked glycosylation sites on 8 out of the 12 HSV-1 envelope proteins. Two of the identified glycosites found in glycoprotein B were previously implicated in virus attachment to immune cells. We show that HSV-1 infection distorts the secretory pathway and that infected cells accumulate glycoproteins with truncated *O*-glycans, nonetheless retaining the ability to elongate most of the surface glycans. With the use of precise gene editing, we further demonstrate that elongated *O*-glycans are essential for HSV-1 in human HaCaT keratinocytes, where HSV-1 produced markedly lower viral titers in HaCaT with abrogated *O*-glycans compared to the isogenic counterpart with normal *O*-glycans. The roles of *O*-linked glycosylation for viral entry, formation, secretion, and immune recognition are poorly understood, and the *O*-glycoproteomics strategy presented here now opens for unbiased discovery on all enveloped viruses.

206. New lectin from insect pathogen *Photorhabdus luminiscence*: structural and functional studies

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Photorhabdus luminescence is Gram-negative bacilli exhibiting dual nature of a lifecycle—one being pathogenic towards *Galleria mellonella* insect larvae, whereas the other representing a symbiosis with entomopathogenic nematodes. A hypothetical protein from *P. luminescence*, PLL, was identified and characterized as a L-fucose binding lectin. The lectin gene was amplified from the genome of *P. luminescence* and cloned with polyhistidine tag. The lectin was found to be a homotetramer of 160 kDa with monomers interconnected with disulphide bridges. The crystal structures of native and recombinant PLL revealed the seven-bladed β-propeller fold with possibly two different binding sites per monomer. Structure-functional analysis suggests the first one to be L-fucose specific, the other to be specific towards a saccharide with a higher level of hydroxylation. Lectin exhibits binding ability to haemocytes, which may be important in insect-nematode-bacterium interactions.

207. Overlapping substrate specificity of nucleotide sugar transporters in the fungal pathogen, *Aspergillus fumigatus*

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Aspergillus fumigatus is an opportunistic fungal pathogen that is the primary cause of the systemic fungal infection, invasive aspergillosis. Current therapies for *Aspergillus* infections are inadequate and there is growing drug resistance. The fungal cell wall is a network of complex carbohydrates and it is the primary target for the development of antifungals due to its absence on mammalian cells. Presentation of sugars on the cell surface are mediated by nucleotide sugar transporters (NSTs), proteins that transport activated sugars from the cytosol into the ER/Golgi lumen to be added to glycosidic chains. Four *A. fumigatus* NSTs (*Af*NSTs) were cloned and characterized, and two *A. fumigatus* NST knockout mutants were created. Assays using Golgi-enriched fractions from yeast overexpressing *Afnst* genes revealed that both *Af*NST1 and *Af*NST5 transported UDP-[^{14}C]-Gal. Saturation transfer difference (STD)-NMR analyses revealed that several UDP-containing sugars were able to bind to the *Af*NSTs, but the combination of nucleotide sugars was not identical. Epitope maps generated from STD-NMR data revealed key contact points of the nucleotide sugars to the *Af*NSTs. This is the first demonstration of multi-substrate NSTs in fungi and the first identification of UDP-Gal transporters in filamentous fungi. The *A. fumigatus* knockout (KO) mutants of *Af*NST1 and *Af*NST5 showed significant differences in growth rate and morphology compared to wild type on media supplemented with the polysaccharide-binding dye, Congo Red. Flow cytometry analyses revealed different levels of galactose and Neu5Ac on mutant spores compared to wild type. In conclusion, although both *Af*NST1 and *Af*NST5are UDP-Gal transporters, substrate differences may explain the observed differences in morphology under conditions of cell wall stress.

Compensatory pathways demonstrate redundancy in fungal carbohydrate metabolism that has implications for the development of antifungal agents targeting cell wall glycans.

Posters

208. Effect of Galectin-3 on the extracellular vesicles from *Cryptococcus neoformans*

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Cryptococcus neoformans is an encapsulated fungal pathogen that causes cryptococcosis and is a major pathogen for immunosuppressed individuals. The fungal capsule is comprised mainly of the polysaccharide glucuronoxylomannan (GXM), which is also released into the extracellular environment as exoGXM. Some of this exoGXM is packaged in extracellular vesicles (EV) that traverse the cell wall for environmental release, where the polysaccharide is likely used for capsule growth or delivered into host tissues. Galectin-3, an endogenous glycan-binding protein, plays essentials roles during microbial infection by modulating innate and adaptive immunity. We predicted galectin-3 would play a role in *C. neoformans* infection. In this work, we identified increased levels of galectin-3 over time in murine tissues (brain, lungs, serum and spleen) infected by *C. neoformans*. We observed that galectin-3 inhibits fungal growth and has a direct lytic effect on the extracellular vesicles from *C. neoformans*. Taken together, our results suggest that galectin-3 may play a role modulating fungal growth and EV virulence factor release during *C. neoformans* infection.

209. Serum *N*-glycome as potential biomarker of disease status and therapeutic efficacy in Chagas' disease

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Chagas' Disease or American trypanosomiasis is a chronic illness endemic in central and south america affecting nearly 20 million persons, caused by *Trypanosoma cruzi*. The transmission is mainly vectorial by members of the *Reduviidae* family, transfusions and transplants, from mother to child, and orally by contaminated beverages. The disease characterizes by an acute phase with high parasitemia and unspecific symptoms that can last for about 4–6 weeks, followed by a long asymptomatic indeterminate phase that can last up to decades before just 20–30 % of patients develop terminal complications, mainly chronic cardiomyopathy and mega syndromes. Many studies have shown that changes in the glycosylation pattern are associated with specific diseases. Here, we present for the first time, an analysis of the serum *N*-glycome in Chagas' disease patients from different clinical cohorts and healthy controls from endemic and non-endemic areas. For this, blood samples were collected from Chagas' disease patients in the acute phase (oral and vectorial transmission from Venezuela) before and after treatment with Benznidazol, in the chronic phase (from Venezuela and Bolivia). The released and 2-AB labeled glycans were resolved by HILIC-UPLC in 40 Glycan peaks (GP). The relative abundances of the GPs adjusted for sex, age and BMI were compared between the study groups. We found a statistically significant difference between groups when considering fucosylated and disialylated glycans. Furthermore, performing a linear discriminant analysis, an excellent separation and prediction ability were observed. One of the most interesting results is the clear

discrimination in the *N*-glycan profiles of the two subgroups of acute patients (oral and vectorial infection). In addition, a normalization of the profile upon chemotherapy was noticed. These alterations in the glycosylation signature could provide new aspects to the pathophysiology of the disease besides serve as new markers in the future.

210. Molecular biomarkers of the susceptible population with influenza viruses: siaylated glycans in human salivary

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Recent studies have elucidated that expression of certain glycoproteins in human saliva are increased or decreased according to age, meanwhile, human saliva may inhibit viral infection and prevent viral transmission. We find that seven lectins (*e.g*., MAL-II and SNA) show significant age differences in both females and males, and seven lectins (*e.g*., WFA and STL) show significant sex differences in children, adults and elderly people. Interestingly, we observe that healthy elderly individuals have the strongest resistance to influenza A virus (IVA) mainly by presenting more terminal α-2-3/6-linked sialic acid residues in their saliva, which bind with the influenza viral hemagglutinations. However, it is often noted that hospitalizations and deaths after an influenza infection mainly occur in the elderly population living with chronic diseases, such as diabetes and cancer. We observed that the expression level of the terminal α-2-3-linked sialic acids of elderly individuals with type 2 diabetes mellitus and liver disease (hepatitis B, hepatic cirrhosis, hepatocellular carcinoma) were down-regulated significantly, and the terminal α-2-6-linked sialic acids were up-regulated slightly or had no significant alteration. But, in the saliva of patients with gastric cancer, neither sialic acid was significantly altered. These findings may reveal that elderly individuals with chronic diseases, such as diabetes and liver disease, might be more susceptible to the avian influenza virus due to the decreased expression of terminal α-2-3-linked sialic acids in their saliva. Our findings imply that the expression level alterations of terminal α-2-3/6-linked sialic acids is a risk factor that could be a biomarker to distinguish those patients who are at a greater risk for infection with IAV, and may provide pivotal information to recommend strongly routine vaccination for them with influenza vaccines.

211. A synthetic glycan-based inhibitor of BambL, a fucose-specific bacterial lectin from *Burkholderia ambifaria*

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Pathogen-associated lectins have so far been considered as an attracting target for the development of anti-adhesive drugs, able to bind in a selective way to the Carbohydrate Recognition Domain of lectins. Such drugs must competitively interfere with the recognition processes between carbohydrates, expressed on the host cell surface, and pathogen lectins, fighting infections. In view of the development of potent inhibitors, it is of great importance to design glycan-based ligands able to selectively bind to specific pathogen-associated lectins with the aim of fighting bacterial infections without interfering with human lectins involved in physiological processes. In this framework, we focused our attention on *Burkholderia ambifaria*, a bacterium member of the *Burkholderia cepacia complex*, a closely related group of Gram-negative bacteria responsible for "cepacia syndrome" in immunocompromised patients. *B. ambifaria* produces BambL, a fucose-binding lectin that displays fine specificity to human fucosylated epitopes. In particular, we report here on the synthesis of the first example of a synthetic fucose-mimetic able to selectively bind the bacterial lectin BambL. The selectivity of the fucose-mimetic was evaluated by assessing the binding affinities vs two different fucose binding lectins: LecB from pathogenic bacterium *Pseudomonas aeruginosa* and DC-SIGN from human. In particular, Hemagglutination Inhibition Assay and Isothermal Titration Calorimetry tests demonstrated that the synthetic mimetic showed a selective binding profile. Indeed, it binds to the pathogen-associated lectin BambL with an affinity comparable to that of the natural ligand while no binding was observed by LecB. Moreover, Surface Plasmon Resonance competition assays showed that this fucose-mimetic does not bind to the human lectin DC-SIGN, thus supporting the selective binding profile towards BambL. The synthesis of multivalent systems containing the fucose-mimetic will be also discussed.

212. Theoretical study of anti-dengue virus inhibitors using the first-principles calculation and MD simulation

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It is well known that Dengue virus (DENV) belongs to *Flaviviridae* family, and that DENV causes a wide range of illness in humans: dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Every year large number of countries report over 100 million (100,000,000) DF with 500,000 DHF cases, which are severe life-threatening infection. These days, infected area of DENV is expanding due to escalation of habitat of *Aedes* mosquito. However, no vaccines or anti-DENV drugs are clinically available at present. Our group has recently developed new small molecular inhibitors for the initial infection of DENV as the analogue of natural glycan. In this study, we theoretically analyzed how the novel inhibitors bind to functional sites on the DENV envelop protein (E-protein) by using the fragment molecular orbital (FMO) method, which is based on pure *ab initio* (the first-principles) calculation without empirical parameters & unphysical caps. Each interaction-energy between the inhibitor and the amino acid residue of the E-protein could be evaluated by the inter-fragment interaction energy (IFIE) analysis. As several polar amino acid residues are present at the edge of the pocket, the glucose moiety was chemically modified with hydrophilic groups. Introduction of both sulfated and carboxylated groups on glucose enhanced not only binding affinity to the E-protein but also inhibition of dengue virus entry. Octyl-2-*O*-sulfo β-D-glucuronic acid may serve as a molecular probe to study the dengue virus entry process. We also adapted integrated procedure coupled the molecular dynamics (MD) simulation with the first-principles calculation. The procedure enables us to correctly evaluate the binding affinities between inhibitors and the target DENV E-protein with structural flexibility and chemical accuracy. We could find that the structural flexibility of the E-protein has changed by binding of the inhibitors to the hinge region.

213. An arabinogalactan from *Isatis indigotica* and its adjuvant effects on H1N1 influenza and hepatitis B antigens

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Isatis indigotica Fort is one of most important medical herbs in China. Its root which is named "Ban-Lan-Gen" is used by drinking for the treatment of influenza, epidemic hepatitis and epidemic encephalitis B for hundreds of years in China. In this study, an arabinogalactan (IIP-2) was firstly isolated from the root of *Isatis indigotica*, and its chemical structure and adjuvant activities was investigated with H1N1 influenza vaccine and recombinant hepatitis B surface protein (HBsAg). The adjuvant effects were evaluated by determining the titers and isotypes of serum antigen-specific antibody of mice using ELISA method. Furthermore, the effects of IIP-2 on the proliferation and TNF-α secretion of MH-S macrophages were also studied *in vitro*. The results revealed that IIP-2 was an arabinogalactan with the molecular mass weight of 66400 Da. It is composed of arabinose and galactose at the ratio of 1.0:1.5. IIP-2 was composed of a β-(1→3,6)-galacan backbone with side chains of→1)-Galp, (1→6)-Galp, α-(1→5)-Araf and→1)-Araf. Our data demonstrated that IIP-2 could increase the antigen-specific antibody levels in mice immunized with H1N1 influenza vaccine or HBsAg antigen, the main isotypes of specific antibody were IgM, IgG1, IgG2a and IgG2b. We also found that IIP-2 could improve the proliferation and TNF-α secretion of macrophages. These results suggested that the arabinogalactan from *Isatis indigotica* enhanced the humoral immunity and this activity might be related to the stimulation of macrophage. Thus, this arabinogalactan could be an efficacious adjuvant for use in vaccines.

Glycoimmunology

Keynote Lectures

214. Programming B cell glycosylation via vaccination

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N-linked glycosylation of antibodies, at asparagine-297 of the IgG heavy chain, plays a critical role in

modulating antibody effector activity including antibody dependent cellular cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP), antibody dependent complement activation (ADCA), etc. While it is clear that antibody glycosylation is profoundly altered in distinct inflammatory diseases, little is known about the specific signals that alter antibody glycosylation during an immune response. However, defining the specific "rules" by which antibody glycosylation is regulated offers a unique opportunity to design next generation vaccines able to direct antibody effector activity for more profound control of various infectious or malignant disease targets. Using cohorts of vaccinated or infected patient populations, we observed that vaccination can reproducibly induce identical antibody glycan profiles across global patient populations, clearly suggesting that antibody glycosylation can be programmed. Moreover, antibody glycosylation is regulated in an antigen specific manner and can be actively manipulated *in vivo* through the use of distinct adjuvants, via both epigenetic imprinting of glycosyl transferases and transcriptional regulation of gene expressionin antigen-specific B cells. Taken together, these data suggest that antibody glycosylation is a highly tunable process, by which B cells selectively regulate glycosylation machinery to direct antibody function. Thus defining the "rules" by which this process is regulated offers a unique opportunity to harness the killing activity of antibodies against various infectious disease and cancer targets.

215. Impact of glycosylation and immune complex size on immunoglbulin G activity

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IgG antibodies are the primary mediators of protective humoral immunity against pathogens and have been used therapeutically for over a century. Besides these protective activities, IgG autoantibodies are the principal mediators of autoimmune diseases such as immune thrombocytopenia (ITP), autoimmune hemolytic anemia (AHA), and systemic lupus erythematosus (SLE). Furthermore, antibodies in the form of intravenous immunoglobulins are used as an anti-inflammatory drug to ameliorate autoimmune diseases such as ITP, chronic inflammatory demyelinating polyneuropathy and skin blistering diseases. The presentation will discuss which molecular and cellular factors influence IgG activity and how we can use this knowledge to enhance the therapeutic and block the self-destructive activity of IgG molecules. A special focus will be on the role of IgG glycosylation and how this can influence the pro- and anti-inflammatory activities of IgG molecules. Moreover, the size of an antibody-antigen complex (immune complex) can strongly influence the impact of the sugar domain or specific sugar residues in this domain on antibody activity, suggesting that both, IgG glycosylation and the size of the immune complex generated *in vivo* need to be considered to predict antibody activity *in vivo*.

Lectures

216. Development of differentially glycosylated IgG antibodies is dependent on co-stimulatory signals

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IgG is an important molecule in the adaptive immune response. The IgG Fc part has one conserved *N*-glycosylation site that influences the function of IgG. De-glycosylated IgG antibodies (Abs) are not able to bind to Fcγ receptors. In addition, the pattern of the IgG Fc glycan can alter the course of an Ab response. Galactosylated and sialylated IgG Abs have shown anti-inflammatory effects and might support the induction of tolerance, whereas IgG Abs without these moieties are linked to inflammatory responses. Here, we analyzed the regulation of IgG Fc glycosylation in the context of different immunogenic milieus. Different mouse strains were immunized with Ovalbumin (OVA) and different co-stimuli. The expression levels of the β-1,4-galactosyltransferase and the α-2,6-sialyltransferase in the OVA-specific plasma cells (PCs) were analyzed by FACS. The *N*-glycosylation of OVA-specific IgG was examined by HPLC after enzymatic glycan release and subsequent 2-AB labeling. Under inflammatory conditions an OVA-specific IgG Ab response was induced leading to germinal center reactions and OVA-specific PC development with low expression levels of the galactosyl- and the sialyltransferases that produced low galactosylated and sialylated OVA-specific IgG Abs, respectively. In INF-γ KO - and IL-17 KO mice this effect was reversed indicating an influence of Th1 and Th17 cells on the Fc glycosylation pattern. Under tolerogenic conditions OVA-specific PCs had higher expression levels of the glycosyltransferases. The IgG Fc glycosylation was comparable to that of total serum IgG in naive WT mice. Our results indicate a strong influence of co-stimulatory molecules on the glycosyltransferase expression levels in PCs and the resulting glycosylation of IgG Abs; thereby altering the effect of the

Ab immune response. Our results might lead to a broader understanding how the IgG Ab glycosylation pattern is determined and might help to improve treatments of autoimmune diseases or vaccinations.

217. Surface glycosylation signature of regulatory T cells (T-reg) correlates with and contributes to their suppressive function

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Background: Regulatory T-cells (T-regs) are a subset of $CD4^+$ T-cells, which express FoxP3 and act to suppress other effector immune cells. Surface protein glycosylation status profoundly influences T-cell biology and functional characteristics. Aims: The main goal of this work was to determine the relationship between the surface glycosylation profile and T-reg phenotypic and functional characteristics. Cell suspensions of lymphoid organs were obtained from C57BL/6-FoxP3-EGFP mice. Surface glycosylation and marker expression was characterized by multi-colour flow cytometric analysis using a panel of 17 lectins with gating on T-reg (GFP^+) and T-conv (GFP^-). T-reg suppressive function was evaluated by culturing purified splenic $CD4^+CD62L^{hi}GSL\text{-}I^{hi/lo}$ T-reg and $CD4^+CD62L^{hi/lo}$ T-conv in various ratios for 5 days followed by analysis of T-conv proliferation. Suppressive potency was also assessed after T-reg glycosidase-treatment with PNGase F and Neuraminidase (which alter cell surface *N*-glycosylation and sialylation, respectively). Freshly-isolated splenic T-regdemonstrated significantly higher surface binding of 5/17 lectins compared to T-conv. In particular, GSL-I binding, which specifically recognizes terminal α-Gal and α-GalNAc motifs, was higher on T-reg from multiple anatomical sites. Among the total T-reg population, higher lectin binding correlated with higher expression levels of multiple known mediators of T-reg suppressive function including CD73, PD-1, PD-L1, ICOS and GITR. Moreover, purified $GSL\text{-}I^{hi}$ T-regs exhibited increased suppressive potency against memory but not naïve T-conv when compared to $GSL\text{-}I^{neg/lo}$ T-reg. Manipulation of T-reg surface *N*-glycosylation and sialylation resulted in significant loss of suppressive potency against memory T-conv. Mouse naïve T-reg have a distinctive surface glycosylation signature that correlates with and functionally contributes to their suppressive potency.

218. Recognition of LPS O-antigen by C-type lectin receptor Dectin-2 shapes TLR4 response

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Lipopolysaccharide (LPS) is an immunogenic component found in outer cell envelope of gram-negative bacteria. It consists of outer polysaccharide chain (O-antigen), core oligosaccharides, and lipid A. While lipid A is well-known to bind to Toll-like receptor-4 (TLR4), the role of O-antigen remains unclear. O-antigens can vary greatly between bacterial strains, and their composition was shown to be important for sepsis severity in mice, highlighting the importance of O-antigen in the immunogenicity of LPS. Here we report, for the first time, a novel receptor crosstalk between TLR4 and mammalian c-type lectin impacts on the immune response to LPS. We compared immunoreactivity of LPS obtained from *Hafnia alvei* PCM1223 which has α-linked mannan in the O-antigen (Man-LPS), and LPS from *Salmonella enterica* O66 which has α-linked galactan (Gal-LPS). We stimulated murine bone-marrow derived dendritic cells (BMDCs) and human blood monocytes with the LPS. We found Man-LPS induced higher levels of IL-10 and TNF-α when compared to Gal-LPS. Next we tested whether Man-LPS was recognized by mannose-specific lectins expressed on mouse BMDCs and human monocytes. Using a reporter assay in which polysaccharide-lectin binding is detectable by β-galactosidase production, we found Dectin-2 bound to Man-LPS, but not Gal-LPS. Mannosidase treatment of LPS significantly inhibited the binding of Dectin-2 to the LPS, suggesting the binding is mediated by O-antigen portion. Moreover, mutations in the carbohydrate-binding domain of Dectin-2 abolished the binding, confirming lectin-glycan interaction. To test whether Dectin-2 enhanced the cytokine production by Man-LPS, we stimulated Dectin-2 knock-out BMDCs with the LPS. In the Dectin-2 knock-out cells, Man and Gal-LPS induced comparable levels of IL-10 and TNF-α suggesting Dectin-2 positively regulates TLR4 signaling. These data demonstrate a novel regulatory mechanism of TLR4 by Dectin-2, augmenting immune response to the mannan-bearing LPS.

219. Phosphoglucomutase 3 (PGM3) mutations impair *N*-glycosylation in hyper IGE syndromes

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Hyper-IgE syndromes (HIES) have been recognized as a group of primary immunodeficiencies characterized by eczema, recurrent skin and lung infections, and elevated serum IgE. Recently, in addition to STAT3, TYK2 and DOCK8, hypomorphic mutations in phosphoglucomutase 3 (encoded by PGM3, which is a crucial enzyme involved in the protein glycosylation pathway) have been identified in HIES patients. PGM3 catalyzes a key step in the synthesis of UDP-GlcNAc, which is the key substrate for the formation of complex *N*-glycan subtypes. Mutations in PGM3 (p.Leu83Ser; p.Glu340del; p.Asp502Tyr) ultimately lead to reduced complex *N*-glycan subtypes (tri and tetra-antennary structures) in affected patients, which was indeed the case as shown by mass spectrometry. This defect in glycosylation did not alter the chemotaxis and surface expression of GPI anchored proteins on granulocytes, nor did it affect the glycosylation of highly elevated IgE. Eosinophilia, impaired T cell proliferation and reversed CD4/CD8 also were identified in patients' cells. Hypomorphic PGM3 mutations characterize multisystem disorders including developmental delay, which might be associated with defective glycosylation, but the exact pathogenic mechanism remains unclear. To investigate the disease mechanism, we successfully generated a genetic rescue model by stably expressing a non-mutant form of PGM3 in EBV-transformed B-lymphocytes from patients and controls using retroviral IRES-EGFP vectors. These cell lines will be used in lectin binding assays, to analyze the glycosylated IgE molecule. Additionally, $Rag2^{-/-}$ mice will be injected with serum from PGM3 deficient and atopic patients to investigate the difference of IgE clearance in those two groups of patients. Additionally, we will analyze the binding ability of CD21 and CD23 to IgE, as well as the interaction of galectins and IgE.

Posters

220. Glycoconjugate vaccine design: combination of conjugation parameters for the development of an improved vaccine against *Salmonella typhi*

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Typhoid fever remains a major public health concern in low-income countries affecting millions of people each year. Antibodies directed against the Vi antigen, which forms a polysaccharide capsule around *Salmonella typhi*, mediate protection and Vi is currently licensed as a vaccine against typhoid fever. Being a T-independent antigen, the Vi polysaccharide is poorly immunogenic in infants and only licensed for children over 2 years of age. Recent years have seen major efforts to develop glycoconjugate vaccines against *Salmonella typhi*. In the design of glycoconjugate vaccines, several variables, such as saccharide chain length, carrier protein, saccharide to protein ratio and conjugation chemistry, can impinge on the immune responses they elicit. Previous studies have often compared the immunogenicity of vaccines differing for multiple parameters at the same time or for just one parameter, making difficult to assign the relative importance of individual variables to the immunogenicity of the vaccine. We used a matrix approach to examine the influence of different combinations of conjugation variables on the immunogenicity of candidate Vi conjugate vaccines. All synthesized vaccines were fully characterized and tested in mice. Results confirmed that conjugation variables were interconnected in determining vaccine efficacy. A few of the candidate conjugate vaccines have been selected for a fuller investigation of the immunological mechanism of these vaccines

221. Vaccine potential of synthetic *Clostridium difficile* glycans

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Clostridium difficile infections are a major global health burden for which a vaccine is not available. We investigate the vaccine potential of pathogen-derived synthetic glycan antigens. Oligosaccharides of the *C. difficile* surface polysaccharides PS-I, PS-II and lipoteichoic acid PS-III were synthesized. Their antigenicity was demonstrated by glycan microarray-assisted screenings for antibodies in serum and stool specimens of *C. difficile* patients. Anti-glycan antibody levels correlated with infection status and disease severity. Auspicious glycan antigens were conjugated to the immunogenic carrier protein CRM_{197}. These glycoconjugate vaccine candidates generated robust, T cell-dependent, high-affinity glycan-specific antibody responses in immunized mice. Glycan-specific monoclonal antibodies were employed to study carbohydrate-antibody interactions that enable the rational design of improved synthetic vaccine antigens. In preliminary murine challenge studies, both active and passive immunization approaches mediated protection against *C. difficile* infection. Our studies highlight the potential of synthetic glycan-based vaccines and therapeutic antibodies to fight *C. difficile*.

222. Synthesis towards bacterial mimetics of HIV-1 GP120 glycans

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GP120, present on the envelope of the human immunodefiency virus (HIV), is an important target for HIV-1 vaccine development, since the tight clustering of oligomannose structures results in the formation of specific epitopes, recognized by several monoclonal antibodies (*e.g.* 2G12). Recently, lipooligosaccharide (LOS), isolated from the bacterium *Rhizobium radiobacter* Rv3 showed a strong resemblance to higher oligomannose structures of gp120. Immunization in mice with heat killed bacteria elicited serum antibodies which bound to monomeric gp120. For further exploitation of this potential antigen mimicry by Rv3 LOS analogues, several building blocks *en route* to larger oligosaccharide conjugates were synthesized. Acknowledgments: Support by Austrian Science Fund (FWF grant P26919-N28).

223. Lung cancer cell-secreted IL-6 glycoforms differentially modulate the polarization of tumor-associated macrophage

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Interleukin-6 (IL-6) is critical in modulating both tumor growth and anti-tumor immunity. Here, we describe the glycosylation pattern of lung cancer cell-secreted IL-6 and examine the function of the IL-6 glycoforms in polarization of tumor-associated macrophages (TAMs). IL-6 with different molecular weights were detected in various lung cancer cells. Because in NetNGlyc 1.0 Server, one possible *N*-glycosylation site has been predicted at N73 on IL-6, we used tunicamycin treatment and site-directed mutagenesis on N73 to demonstrate that lung cancer cell-secreted IL-6 is modified by *N*-glycosylation. To explore more detailed attached glycans, we measured the expression of glycosyltransferases by qPCR and found that the expression of fucosyltransferase 8 (FUT8), responsible for core fucosylation on *N*-glycosylated proteins, was higher in lung cancer cells than normal bronchial cells. Core fucosylation on IL-6 was reduced by silencing FUT8. We generated AS2-IL6, AS2-IL6-shFUT8, and AS2-IL6-N73Q cells for producing full-glycosylated IL-6 (G-IL6), core fucose-depleted IL-6 (DeCF-IL6), and Asn73-*N*-glycan-depleted IL-6 (N73Q-IL6). To examine the effect of the IL-6 glycoforms on differentiation of TAMs, THP-1 monocytic leukemic cells were incubated with phorbol-12-myristate 13-acetate for transforming to macrophages, followed by co-culture with the AS2 cell derivatives to mimic the education of TAMs. The co-cultured TAMs of each AS2 derivative showed distinct morphology. Intriguingly, the G-IL6-producing cells promoted the expression of M2-TAM markers CD204, CCR1, arginase-1, and M2-specific cytokines that benefit anti-tumor activity, while DeCF-IL6 and N73Q-IL6 favored the formation of M1 macrophages. Together, we report the presence of specific IL-6 glycoforms secreted from lung cancer cells. Moreover, the glycosylation on IL-6 changes its

activity on the polarization of TAMs, suggesting its critical role in orchestrating the tumor microenvironment of lung cancer.

224. The *N*-glycosylation of neutrophil cathepsin G and its role in the host innate immunity against *Pseudomonas aeruginosa*

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Cathepsin G, a serine protease found in the azurophilic granule of neutrophils, is known for its anti-microbial activity during host defence against bacterial infection via proteolysis dependent and independent mechanisms. However, the structure-function relationship of its attached *N*-glycans remains largely unknown. Herein, we conducted a detailed site-specific quantitative characterisation of the *N*-glycosylation micro- and macro-heterogeneity of neutrophil-derived human cathepsin G using complementary LC-MS/MS technologies on multiple glycoanalyte levels (glycan, glycopeptide and intact glycoprotein) and performed functional studies of its glycoforms with the aim to map their anti-microbial activities towards *Pseudomonas aeruginosa*. Under-reported truncated chitobiose core-type (GlcNAc1-2Fuc0-1) and paucimannosidic (Man1-3GlcNAc$_2$Fuc0-1) *N*-glycans were abundantly displayed on cathepsin G, which yielded a higher level of understanding of the glycoprotein structure. Purified glycoforms of intact cathepsin G and isolated glycan moieties displayed bacteriostatic activities towards virulent-specific *P. aeruginosa* strains, but not laboratory-strains of the bacteria under biologically relevant concentrations and conditions. Taken together, we here present new glyco-focused insight of the structure of cathepsin G and provide evidence supporting the functionally important roles of novel types of protein glycosylation on this important anti-microbial protein in human neutrophils during innate immunity.

225. Synthesis of a novel glycopolymer carrying multivalent plant antigenic *N*-glycans

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Plant complex type *N*-glycans that bear β1-2 xylosyl and α1-3 fucosyl residues on the tri-mannosyl core show strong antigenicity against mammals. In previous report, however, we found that the free plant antigenic *N*-glycan, Man$_3$Fuc$_1$Xyl$_1$GlcNAc$_2$ (M3FX), significantly suppressed the production of IL-4 from Japanese cedar pollen allergen (Cry j1)-specific Th2 cells, suggesting that the M3FX can be used as a lead compound to develop the anti-allergic drug. Based on this finding, in this study, we have developed a novel glycopolymer bearing multivalent M3FXs to modulate the cellular immune system. Three types of Asn-glycopeptides (M3FX, Man$_8$GlcNAc$_2$, and NeuAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$) were prepared from *Ginkgo biloba* seeds, *Vigna* beans, and egg yolk. Asn-glycopeptides were coupled to poly-γ-L-glutamic acid (γ-PGA) by 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium Choloride *n*-Hydrate. The resulting glycopolymers were purified by a combination of gel-permeation and RP-HPLC. The incorporation of *N*-glycans into γ-PGA (mol%) was estimated by amino acid composition analysis. The incorporation rates of M3FX-Asn, Man$_8$GlcNAc$_2$-Asn, and NeuAc$_2$Gal$_2$GlcNAc$_2$Man$_3$GlcNAc$_2$-Asn to γ-PGA were 15.4, 8.6, and 11.1 %, indicating that nearly 800 molecules of *N*-glycans were incorporated into γ-PGA. Immunological activities of neoglycopolymers will be discussed.

226. Anti-cancer activity of a low-molecular weight oligosaccharide prepared by enzymatic degradation of ginseng marc-derived polysaccharide against murine skin melanoma cells via activation of RAW264.7 murine macrophage cells

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Panax ginseng C.A. Meyer has been traditionally used to prevent or treat various medical disorders for its cardioprotection activity, vasorelaxation, stimulation of the central nervous system, and host resistance against infection. In spite of its biologically beneficial properties, high molecular weight polysaccharide becomes an obstacle for application as a health beneficial therapeutic agent. In this study, we prepared lower-molecular weight oligosaccharide (GOS, MW. 2.2 kDa) by enzymatic degradation of polysaccharide (MW. 439–605 kDa) and evaluated for its immunostimulating activities in RAW 264.7 murine macrophage cells. Treatment with GOS

(100~500 μg/ml) dose dependently enhanced the production of TNF-α, IL-6, and NO in RAW 264.7 cells. Western blot analysis showed that GOS dose-dependently induced phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38, and Nuclear factor κB (NFκB), which are upstream signaling molecules for cytokine production. Next, whether GOS-stimulated macrophages are capable of killing murine B16F10 melanoma cells was examined. While GOS was not cytotoxic to the RAW 264.7 macrophage cells at the concentration tested (up to 1000 μg/ml), when B16F10 melanoma cells were co-cultured with the GOS-activated macrophages, the cell viability of melanoma cells was decreased in a dose-dependent manner. Taken together, these results suggest that ginseng marc-derived GOS may have anti-cancer activity against melanoma cells through potentiating macrophage function.

227. Natural anti-glycan antibodies of mammals and birds

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Natural anti-glycan antibodies (nAbs) are important part of innate immune system, but at the same time they are least of all studied. Their role and origin are still poorly understood. Blood-group related antigens, tumor-associated and xeno-antigens are well-known counter-part of human nAbs. Anti-glycan nAbs pattern of healthy donors have been investigated with the help of printed glycan array (PGA). It was shown that about a half of typical mammalian glycans bound human serum IgG and IgM; most of the specificities have not been described yet. Discrete PGA data about anti-glycan nAbs of other mammals as well as birds were summarized (partly, our experimental data, partly available from http://www.functionalglycomics.org). Data on nAbs from mice, rats, primates and hens were compared with data on human nAbs. Baboons pattern of nAbs was similar (but not identical) to human one. Anti-glycan nAbs repertoire of mice, rats and hens was dramatically shrunk, top-binding glycans were different. Common epitopes recognized by nAbs such as chitooligosaccharides, L-rhamnose, L-fucose, and nAbs to core structures can be mentioned. Notably all examined species have own features in nAbs repertoires, which reflect their individual immunological "history" as humans have. The information about the natural anti-glycan antibodies repertoire is necessary because laboratory mammals and birds are widely used as immunological models. The reported study was supported by RFBR research project No 13-04-00549 A and Russian Science Foundation (project No. 14-14-00579).

228. Differential effects of super- /SUB- molecular recognition factors on *Dolichos biflorus* agglutinin (DBA)—glycan interactions and their accommodating sites

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Dolichos biflorus agglutinin (DBA) is one of the well-known applied lectins that are widely used in routine serology to differentiate human $\mathbf{A}_1$ and $\mathbf{A}_2$ erythrocytes. However, the knowledge of super-molecular (polyvalent), subsite and sub-molecular (functional group/ epimer) recognition factors in DBA-glycan interactions and their recognition sites are very limited. In this study, most recognition factors of DBA-glycan binding were characterized by different conditions of enzyme-linked lectinosorbent assays (ELLSA) and inhibition assays. The super recognition factor of polyvalent GalNAcα1→related glycotopes expressed by mass relative potency (mass RP) was up to 3.8×10^3, 7.3×10^5 and 1.2×10^6 times more active than GalNAc, Gal and GlcNAc respectively, while molar ratio of sub-molecular recognition factors (epimer and functional group) in Glc, Gal, GlcNAc and GalNAc, was 0.4, 1.0, 1.8 and 2.4×10^2 respectively. Since the polyvalency of glycotope (GalNAcα1→) affects its steric structure, conformation and reactivity, the polyvalent glycotope is defined as super glycotope of DBA. The intensities of recognition factors of DBA can be expressed by decreasing order of high density of polyvalent GalNAcα1→(super glycotopes) >>monomeric GalNAcα related glycotopes >>their β-anomer> $-NHCH_3CO$ at carbon-2 of GalNAc>configuration of -OH at carbon-4 of GalNAc. The combining site of DBA should consist of a small cavity shape as major site most complementary to monomeric GalNAcα→, a broad and shallow groove type as subsite to accommodate from mono- to tetra-saccharides (GalNAcβ, Galβ, LFuc, GalNAcβ1→3Galα1→4Galβ1→4Glc). This study provides an essential knowledge of DBA recognition at molecular, super/sub-molecular levels, which

should expand and upgrade the classic concept of the combining (recognition) site proposed previously.

Glycolipids, Membrane Domains and Cell Signaling

Keynote Lecture

229. Glycosphingolipids in health and diseases: the role of plasma membrane composition and organization

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Intracellular metabolic processes determine the basic glycosphingolipid plasma membrane (PM) composition. In addition to this, the glycosidases and the glycosyltransferases, associated with the PM are capable under specific signals to modify the oligosaccharide chains introducing minor changes of the glycosphingolipid composition instrumental for the PM organization changes and properties. Glycohydrolases, in particular, are under detailed studies. They show trans activity that results strictly related to the extracellular pH, being in general optimal under mild acidic conditions. There are mild acidic conditions in membrane domains known as lipid rafts, where glycosphingolipids are highly enriched together with cholesterol, receptor proteins, proteins involved in cell signaling, and antiports or general ion exchange proteins. Several proton pumps are present on the cell surface and inserted into lipid rafts together with the glycosidases. Thus their activity and the following catabolic process at the cell surface that under particular conditions can lead to apoptosis can be increased by activating the proton pump and decreasing the local pH of their extracellular environment. In addition to this, it has been demonstrated that a cross talk among the glycosphingolipid hydrolases exists. A portion of the cell surface glycohydrolase expression derives by fusion of lysosomes to the PM, but some is associated to non–lysosomal enzymes like for the β-glucocerebrosidase GBA2 and the α-sialidase NEU3. Both the enzymes are associated to any kind of cells, but their activity can be very variable being maximum in neuronal cells were glycosphingolipids and particularly gangliosides are abundant. Both seem to be associated to neuronal differentiation and degeneration. NEU3 is also specifically associated to some tumors were the enzyme is used to reduce the content of ganglioside GM3 producing high amount of antiapoptotic lactosylceramide.

Lectures

230. Roles of β-1,3-galactosyltransferase V, SSEA3, SSEA4 and Globo H in breast cancer

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Glycosphingolipids (GSLs) are highly expressed in cancer and therefore can be specific targets for anti-cancer development. Recent studies show that members of the globo series pathway, especially SSEA3 (Gb5), SSEA4 and globo H, are specific GSLs observed in many malignant tissues, particularly breast cancer. Their expression correlates with progression, metastasis, and poor survival in patients. The enzyme β1,3-galactosyltransferase V (B3GalT5) catalyzes the addition of galactose to Gb4 (GalNAcβ3Galα4Galβ4GlcβCer) to form SSEA3, which serves as the precursor of both SSEA4 (α-2,3-sialyl SSEA3) and globo H (α-1,2-fucosyl SSEA3). B3GalT5 is therefore a key enzyme in controlling the expression of these three cancer-related molecules. However, the functional roles of B3GalT5, SSEA3, SSEA4 and globo H in breast cancer remain unclear. Here, the malignancy of breast cancer cells MCF-7 and MDA-MB-231 related to B3GalT5 was studied. We found that the mammosphere formation and cell survival were decreased in B3GalT5 knockdown cells. In addition, knockdown of B3GalT5 also suppressed cell migration and adhesion, and induced cell apoptosis mediated by caspase-3 and resulted in reduced expression of focal adherent kinase (FAK) protein. Preliminary data also showed that FAK interacted with the globo-series glycans to facilitate the signaling of cancer progression.

231. Properties and functions of anti-LacCer monoclonal antibodies are highly depend on the organization of LacCer-enriched domains

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Glycosphingolipids (GSLs) are composed of hydrophobic ceramide and hydrophilic sugar moieties. Based on the physicochemical characteristics of GSLs, they form lipid rafts on plasma membranes of cells. We introduce here that two different anti-lactosylceramide (LacCer) antibodies T5A7 and Huly-m13 showed the different binding behaviors and specificities. T5A7 bound to human and mouse neutrophils, while Huly-m13 only bound to human neutrophils. SPR analysis revealed that T5A7 and Huly-m13 bound to a lipid monolayer composed of LacCer, DOPC, cholesterol, and sphingomyelin (1:10:10:1), while T5A7 but not Huly-m13 bound to the 0.1:10:10:1 lipid monolayer, suggesting that the binding affinities of Huly-m13 to LacCer depend on the amount of LacCer in the domains. STED microscope observation revealed that Huly-m13 and T5A7 bound to distinct LacCer domains/clusters. The binding avidity of Huly-m13 to LacCer-coated wells was similar to its avidity to DOPC/LacCer, POPC/LacCer and DPPC/LacCer mixture-coated wells, indicating that the binding of Huly-m13 to LacCer coated onto wells was not affected by acyl-chain structures of PC. Moreover, lactose inhibited the binding of Huly-m13 to LacCer/DOPC liposome-coated and LacCer/DOPC mixture-coated wells, indicating that Huly-m13 binds to LacCer clusters in LacCer-enriched domains. In contrast, the binding avidity of T5A7 to LacCer-coated wells was much weaker than to DOPC/LacCer-, POPC/LacCer- and DPPC/LacCer mixture-coated wells, indicating that the binding of T5A7 to LacCer is affected by the PC structures. Lactose inhibition of T5A7 binding to DOPC/LacCer mixture-coated wells was lower than its inhibition of Huly-m13, suggesting that T5A7 recognizes the PC-enhanced three-dimensional structure of LacCer clusters. It seems that Huly-m13 may bind to the core region of lactose clusters in LacCer-enriched domains, while T5A7 binds to dispersed LacCer clusters in the phase boundary regions of the microdomains.

Posters

232. LXRα-silencing induces apoptosis by glycosphingolipid-derived ceramide in different tumor cell type

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Cell surface ceramide is a key signaling molecule necessary to regulate important cellular processes, like differentiation, proliferation and apoptosis. Its production derives from several sphingolipids, including gangliosides by activity of the plasma membrane glycosidases, like sialidase NEU3, β-HEX, β-GAL and β-GLU. We found that several human cancer cell lines (*i.e.* multiple-myeloma, melanoma and breast cancer) shared the same sensitivity to LXRα signaling abrogation. We focused our attention on one of LXRα target genes, SREBP-1c, a transcription factor regulating *de novo* lipogenesis, which is the main pathway used by cancer cells for the synthesis of new structural lipids. Both SREBP-1c and its target gene FASN turned out to be down regulated in shLXRα-sensitive but not in shLXRα-insensitive cells. Finally, we have found in the shLXRα-sensitive cells an increase of apoptotic cell death due to an increase of PM ceramide up to 3 times with respect to the shLXRα-insensitive cells. We also observe a reduction in ganglioside GM3, suggesting that ceramide is because of the activity of PM sialidase NEU3, β-GAL and β-GLU. These data suggest that in shLXRα-sensitive cells the tumor-promoting role of LXRα might be due to an effect on the SREBP-1c-driven *de novo* lipogenesis. FASN inactivation could be responsable for a reorganization of cell membrane given a decrease in the synthesis of FA, therefore an alteration of lipid metabolism: increase in glycohydrolase activity and in PM GM3, LacCer, GlcCer and pro-apoptotic ceramide. This pathway may represent a novel potential drug-target in multiple-myeloma and other tumors.

233. Environmental oxygen levels greatly influence on the constituent ceramide species of glycosphingolipids from human colon cancer cells

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Since normally structural analyses of glycosphingolipids (GSLs) have focused on the diversity of glycan sequences. However, the constituent ceramides of GSLs also show diversity, because they are composed of different lengths of fatty acids (FAs) and long-chain bases (LCB), either or both of which are occasionally hydroxylated with the presence or absence of unsaturation. Moreover, the expression efficiencies of glycan sequence of GSLs on the cell surface serving such as cancer antigens and for microbe recognitions are crucially influenced by their constituent ceramide species. Dihydroceramide desaturase (Δ4-desaturase), sphingolipid Δ4-desaturase/C-4-hydroxylase synthesizing phytosphingosine, and fatty acid 2-hydroxylase synthesizing hydroxyl FAs are involved in the constituent ceramide synthesis for GSLs, and all of them are commonly oxygen-requiring enzymes. Therefore, environmental oxygen levels are expected to greatly influence on the constituent ceramide species of GSLs in the cells. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometrywith high-energy collision-induced dissociation, we analyzed the alteration of the constituent ceramide species of GSLs from human colon cancer cell line Caco2 and LS174T, according to the change of oxygen levels from normoxic condition (20 %) to hypoxic condition (0.5–1 %). We further examined the reoxygenation (hypoxic condition to normoxic condition) effect on the constituent ceramides species using Caco2 cells. These results suggest that individual constituent ceramide species of GSLs greatly alter according to the environmental oxygen levels.

234. Interaction of disialyl gangliosides GD2/GD3 with growth factor receptors sustains the stem cell phenotype of breast cancer

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Our previous studies demonstrated a causal relationship between GD2/GD3 gangliosides and maintenance of breast cancer stem cells (BCSCs) properties, based on reversal of stem cells properties following knockdown of GD2/GD3 synthases. Recently, we analyzed growth factor receptors (GFRs) associated with GD2/GD3 and their related signaling pathways to clarify how interactions with GD2/GD3 maintain stem cell properties and signaling in BCSCs. Immunofluorescence staining indicated that most GD3 is co-localized with epidermal growth factor receptor (EGFR). In contrast, GD2 and cMet showed only partial colocalization in the ER-Golgi intermediate compartment. Interactions between GD2/GD3 and GFRs were also detected by coimmunoprecipitation of BCSC lysates with anti-GD2 and anti-GD3 antibodies. We observed that there was strong interaction between GD3 and EGFR but weak GD2/cMet association. On the other hand, β1-integrin, which modulates functions of various GFRs, was strongly associated with GD2. Immunogold labeling and transmission electron microscopy confirmed the localization of GD2/GD3and GFRs in membrane micro domains. GD3 was clustered with EGFR in electron-dense membrane patches, whereas GD2 and cMet showed little colocalization on BCSC membrane sheets. EGFR expression and ERK signaling were increased in BCSC populations showing high GD2/GD3 expression. Co-immunoprecipitation with a chemical cross-linker in living cells was used to identify protein complexes associated with GD2/GD3. A nano-LC-MS/MS showed there was a total of 143 GD2-associated and 320 GD3-associated proteins. Our findings provide an important basis for further studies of functional molecular interactions that maintain stem cell properties in BCSCs.

235. Highly efficient preparation of sphingoid bases from glucocerebrosides and their biological activities

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Glycolipids are basic structural and functional components of the plasma membrane of essentially all eukaryotic cells. Cerebrosides are a class of glycosphingolipids, consisting of ceramide and a single sugar residue (glucose or galactose) at C-1. The principal cerebroside in plants is glucocerebroside (GlcCers). GlcCers have been found to be involved in many cellular processes such as cell proliferation, oncogenic transformation, differentiation, and tumor metastasis. The core part

cerebrosides are sphingoid bases and these emerged as bioeffector molecules, controlling various aspects of cell growth. Many of the naturally occurring and synthetic sphingoid bases are cytotoxic for cancer cells and pathogenic microorganisms or have other potentially useful bioactivities; hence, they offer a promise as pharmaceutical leads. Even though sphingoid bases have of greater chemotherapeutic importance, their supply has been limited due to their low abundance and difficulty in purification. On the other way, GlcCers are abundant in natural resources and there are many reports to prepare sphingoid bases from GlcCers using classical acid or base hydrolysis, however these methods are not practically efficient because they produce many byproducts with a quantitative yield. There are few enzymes to hydrolyze glycosidic linkage between sugar and ceramide in gangliosides, however they will not work in case of GlcCers. Herein, we are presenting a novel chemoenzymatic method for the preparation of sphingoid bases from naturally abundant GlcCers. Our method is practically efficient, economical and applicable to any kind of cerebrosides. And also we have developed a novel glutaraldehyde resin for chemoselective capture of free sphingoid bases from biological samples and natural extracts. We are constructing chemical library of structurally distinct natural sphingoid bases and testing these bases for inhibitors of multidrug resistance in cancer therapy.

Glycoproteins and Glycolipids in Disease

Keynote Lectures

236. Glycosylation in muscular dystrophy

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Muscular dystrophies are genetic diseases that cause progressive muscle weakness and wasting. In congenital muscular dystrophies, muscle weakness is apparent at birth or shortly thereafter. The aberrant protein *O*-mannosylation of α-DG is the cause of some forms of congenital muscular dystrophy, and these muscular dystrophies are termed α-dystroglycanopathies. Recent studies revealed that at least 17 genes disrupt the glycosylation of dystroglycan and cause disease, and that structures of *O*-mannosylglycans are highly complicate including the presence of the LeX epitope, HNK-1 epitope, sialyl lactosamine structure, and Xyl-GlcA polysaccharide. In addition, a novel phosphorylated trisaccharide structure has been proposed. α-DG hypoglycosylation greatly reduces its binding affinity for extracellular matrix components such as laminin, thereby disrupting the dystroglycan-extracellular matrix linkage and leading to membrane fragility. Originally, *O*-mannosyl glycan was only known to be present on a limited number of glycoproteins, especially α-DG. It is unclear whether defects in additional *O*-mannosylated proteins beyond α-DG contribute to the observed disease complexity. Once a clear relationship was established between *O*-mannosyl glycan and the pathological mechanisms of some congenital muscular dystrophies in humans including us, research on the biochemistry and pathology of *O*-mannosyl glycans has been expanding. However, details of molecular pathology of dystroglycanopathies are still unclear. I will focus on the relation between aberrant glycosylation of α-DG and congenital muscular dystrophies. Possible regulatory mechanism of protein *O*-mannosylation will also be discussed.

237. High-throughput glycomics in patient stratification for personalized medicine

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Glycosylation is an essential posttranslational modification generated by a complex biosynthetic pathway comprising hundreds of glycosyltransferases, glycosidases, transcriptional factors, ion channels and other proteins. This process results in the creation of branched oligosaccharide chains, called glycans, which become integral part of proteins and significantly contribute to their structure and function. Since glycans are created without the genetic template, alternative glycosylation creates an additional layer of protein complexity by combining genetic variability with past and present environmental factors. Individual variability in glycome composition is very large, but glycosylation of an individual protein seems to be under strong genetic influence, with the heritability of the IgG glycome being up to 80 %. Structural details of the attached glycans are of great physiological significance and many pathological conditions are associated with various types of glycan changes. Since the onset of genome wide association studies (GWAS), thousands of genetic loci have been associated with different diseases and traits. However, it is becoming increasingly evident that GWAS studies are only a beginning of the understanding of complex human diseases. Hypotheses generated in these studies have to be put in the context of complex biology of life and a more elaborate approach that combines different 'omics phenotypes is needed to understand disease mechanisms and perform patient stratification that transcends genomics. Glycomics, as by far the most complex epiproteomic modification, has an immense potential in

this respect, which is only beginning to be investigated. During the last 2 years researchers in Genos analysed IgG and/or total plasma glycomes in over 30,000 samples from population and case/control cohorts. Through the analysis of these large datasets we are shedding some initial light on the importance of individual variation in protein glycosylation for the development and progression of different diseases.

Lectures

238. Glycosylation of plasma proteins in HNF1A—maturity onset diabetes of the young

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Recent improvements in analytical approaches enabled us to perform the first study that combined genome-wide association approach and high-throughput glycomics. The study showed that Hepatocyte Nuclear Factor (HNF) 1α and its downstream target HNF4α regulate the expression of key fucosyltransferase and fucose biosynthesis genes and that HNF1α is both necessary and sufficient to drive the expression of these genes in hepatic cells. This revealed a new role for HNF1α as a master transcriptional regulator in the fucosylation process. Since loss-of-function HNF1A mutations are causal for the HNF1A subtype of maturity-onset diabetes of the young (MODY), we performed a pilot study on patients with different subtypes of diabetes and identified potential, glycosylation based, discriminative marker for this often misdiagnosed disease. The current diagnostic approach is based on sequencing of the whole gene and difficult subsequent diagnosis assignment, therefore any improvement in this process would represent a great value for both patient and healthcare system. Accurate diagnosis allows prompt screening of relatives to identify other cases in family members and offers adequate treatment. We are currently trying to assess performance in a "real-life" clinical setting among unselected patients with a diagnosis of young adult-onset diabetes and produce a rational diagnostic protocol. We hypothesize that by measuring plasma glycan profile we will identify patients with the highest risk of having HNF1A-MODY, who can then undergo diagnostic sequencing. We also hypothesize that, since glycosylation is shown to be altered by pathogenic HNF1A variants, it could be used as plasma marker in assessing pathogenicity of variants that are either novel, or have not been conclusively categorised as disease-causing. The study involves patients with increased risk of diagnostic misclassification, recruited through the Croatian Registry of Diabetes and the Young Diabetes in Oxford study.

239. Hyper-*O*-GlcNAcylation is a major mechanism of glucose toxicity & diabetes complications

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O-GlcNAc is an abundant and highly dynamic protein modification directly derived from glucose via the hexosamine biosynthetic pathway. *O*-GlcNAcylation is a nutrient sensor that regulates signaling, transcription and myriad cellular processes. *O*-GlcNAc cycling has a complex interplay with phosphorylation and other post-translational modifications, and is required for life in both plants and animals. Prolonged elevation of *O*-GlcNAcylation, as occurs in diabetes in most tissues, is a major underlying molecular event causing glucose toxicity and diabetic complications. For example, elevated *O*-GlcNAcylation contributes to diabetic cardiomyopathy by dysregulation of the contractile machinery in cardiomyocytes, by directly altering the activity of cardiac CAMKII, and by dysregulation of cardiomyocyte mitochondrial functions. Increased *O*-GlcNAcylation, contributes to diabetic nephropathy by altering the expression of extracellular matrix proteins, such as proteoglycans and matrix metalloproteases (MMPs), and by altering the localization and expression of key slit diaphragm proteins, such as podocin and nephrin. Hyper-*O*-GlcNAcylation results in altered slit diaphragm structure. *O*-GlcNAcylation also plays a direct role in appetite control by the brain. An inducible, αCAMKII promoter targeted knockout of *O*-GlcNAc transferase in murine brain, leads to a morbidly obese mouse within about 3-weeks. The massive weight gain is due to a clear satiety defect mostly affecting TRH neurons in the PVN of the hippocampus. Thus, chronic elevation of this sugar modification, as occurs in diabetes, appears to be a major molecular mechanism explaining glucose toxicity. Supported by NIH P01HL107153, R01DK61671 and N01-HV-00240. Dr. Hart receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU.

240. Lectin-based techniques for analysis of glycosylation changes using microarray and surface plasmon resonance platforms

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Glycosylation is post- and co-translational modification affecting proteins and the changes in glycopattern are associated with many biological functions and diseases including cancer. We are focused to the development and application of lectin-based bioanalytical techniques using surface plasmon resonance (SPR) and microarray platforms enabling evaluation of differences in protein glycosylation. These methods can serve for analyses of glycosylation profile of both the complex protein samples (*e.g.* lysates) and the isolated proteins in biological, immunochemical, biomedical and biomarker research. Microarray as high-throughput method enables analysis of large set of samples while SPR, optical label-free technique serves for label-free real-time detection and study of molecular interactions on the modified surface. The aim of our studies is determination of glyco-alterations connected with physiological and pathological changes (*e.g.* colorectal and prostate cancer). Acknowledgements: This work is supported by the Marie Curie Initial Training Network PROSENSE (grant No. 317420, 2012–2016), CMST COST Action CM1101, APVV SK-SRB-2013-0028 and VEGA 2/0162/14.

241. Hippocampal abnormalities in a mouse model for an inherited human Golgi-GDP-fucose transporter deficiency

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Neuronal development, learning and memory are influenced by glycoproteins in which the monosaccharide fucose plays an important role. Genetic defects in the human Golgi-GDP-fucose transporter SLC35C1, essential for the supply of the sugar, evoke a general loss of fucose residues linked to glycoproteins leading to 'Congenital Disorder of Glycosylation-IIc' also termed 'SLC35C1-CDG'. Patients suffer from persistent leukocytosis, severe infections, mental and growth retardation. In a patient-like knockout mouse model for SLC35C1-CDG we found a diminished dentate gyrus zone and an increased amount of synapses in the hippocampus. Consistently, several behavioral abnormalities with distinctive features in fear conditioning were detected. Moreover, a variety of proteins belonging to the TGF-β superfamily, which play an important role in hippocampus formation show a significantly deregulated expression. Lectin affinity chromatography combined with mass spectrometry analyzes revealed a severe hypofucosylation of cell adhesion molecules, ion channels, transporters and synaptic vesicle-associated proteins, assuming a crucial role of glycoconjugate fucosylation in hippocampal morphology and function.

Posters

242. Is protein glycosylation modulated in SRD5A3-deficient model cell lines?

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Congenital Disorders of Glycosylation are group of metabolic diseases caused by impaired protein glycosylation. One of the variants, SRD5A3-CDG, is caused by nonsense mutations in the gene encoding polyprenol reductase. SRD5A3 plays a crucial role in the dolichol cycle by catalyzing the conversion of polyprenol to dolichol. Phosphorylated derivatives of dolichol are essential for protein glycosylation in eukaryotes. Decreased content of dolichol-phosphate affects the assembly of the dolichol linked $Glc_3Man_9GlcNAc_2$ glycan and its transfer to proteins. For better understanding of the biological consequences of decreased dolichol levels, we established a cellular model of SRD5A3 deficiency. Flp-In technology was used to obtain different HEK293T and HeLa cell lines expressing miRNA for silencing endogenous *SRD5A3* and simultaneously complementing with mutated variants of *SRD5A3*. Mutations selected for complementation corresponded to those found in patients with SRD5A3-CDG. TaqMan gene expression analysis was carried out to establish the efficiency of silencing of the endogenous *SRD5A3* mRNA and expression of exogenous transcript *SRD5A3*. Biochemical analysis of model cell lines demonstrated that expression of mutated *SRD5A3* led to aberrations in lipid metabolism. HPLC/UV analysis revealed that all tested cell lines, including control - SRD5A3 knockout show a reduction of dolichol levels and polyprenol accumulation. In parallel to lipid analysis we tried to establish whether the decreased level of dolichol affected glycosylation of cellular proteins in HEK293T/HeLa Flp In cell lines. These cell models will be used in the future to elucidate the physiological consequences of SRD5A3 deficiency. It will help to characterize polyprenol reductase at molecular and biochemical level and to clarify the role of this enzyme in various cellular processes, including regulation of the dolichol cycle, glycosylation of proteins and synthesis of steroid hormones.

243. Effects of psychiatric disorders-related intronic single nucleotide polymorphisms (iSNPS) of the polysialyltransferase ST8SIA2/STX gene

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Polysialic acid (polySia) is a homopolymer of sialic acid with a degree of polymerization of 8–400. PolySia is expressed not only in embryonic brains, but also in distinct regions of adult brains where neurogenesis is ongoing, such as hippocampus and the olfactory system. When present on neural cell adhesion molecule (NCAM), polySia is known to exhibit anti-adhesive effects on cell-cell interactions due to its bulky polyanionic nature, to regulate the neurogenesis. We have recently demonstrated that polySia also functions as a reservoir scaffold for the brain-derived neurotrophic factor (BDNF) and the fibroblast growth factor 2 (FGF2), which are biologically active molecules in neurogenesis and neural functions. PolySia is known to be biosynthesized by two polysialyltransferases ST8SIA2/STX and ST8SIA4/PST. Interestingly, the genome-wide screenings of psychiatric disorders have suggested that there are associations between those disorders and polymorphisms in the ST8SIA2/STX. However, underlying mechanisms for these associations remain unknown. The objective of this study is to understand the effects of iSNPs of ST8SIA2/STX on the expression of mRNA, ST8SIA2STX and their final product polySia. In this study, we focused on intronic SNPs of the ST8SIA2/STX that were identified in autism spectrum disorder and bipolar spectrum disorder patients. The results showed that iSNPs affected the amount of ST8SIA2/STX by changing the stability of ST8SIA2/STX pre-mRNA. These alterations of the enzyme quantity may change the quantity and quality of polySia, which is known to affect the reservoir functions of polySia. Therefore, iSNP of ST8SIA2/STX may impair the neural function via impairment of quality and quantity of polySia. This study was supported in part by the program for leading graduate schools IGER of Nagoya University.

244. Glycomic changes during colon adenocarcinoma re-differentiation

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Modern glycomic and glycoproteomic research is partially focused on two important fields of biomedicine. First are the studies on cancer-related glycosylation changes and their impact on the disease, the second focuses on cell differentiation, to find and describe new differentiation markers. The research model of this study consists of Caco-2 adenocarcinoma cell line which, under specific culture conditions represents the ability to spontaneous differentiation into enterocyte-like cells. The cells were analysed when growing in sparse culture – representing the undifferentiated state, and 12 days post-confluence—representing differentiated phenotype. The transition towards enterocyte-like morphology was analysed in terms of expression of well-known brush-boarder marker enzymes: sucrase-isomaltase and alkaline phosphatase. To study the *N*-glycome, glycans released from cellular proteins were analysed by NP-HPLC after fluorescent labelling with 2-AB. The glycosphingolipids (GSLs) were studied by NP-HPLC according to Neville *et al.* 2004. The *N*-glycan analysis revealed, that the main change that seem to accompany the cell fate transition towards enterocyte-like phenotype is the quantitative remodeling within the relative amounts of oligomannose glycans (M4-M9). The important down-regulation of M7, M9 and especially M8 was observed, together with the up-regulation of M4 and M5. In case of GSLs, differentiated Caco-2 cells showed up-regulation of LacCer, GD3 and GD2 and down-regulation of GM3 and Gb3. The Caco-2 cell line is a good model of both enterocytic differentiation and re-differentiation of cancer cells towards the less aggressive phenotype. Our preliminary studies suggest, that the changes of the specific balance between oligomannose *N*-glycans and remodeling of GSLs composition could be the important attributes of these phenomena. The project was financially supported by National Science Centre in Poland, decision No. DEC-2012/07/D/NZ1/00523

245. Protein fucosylation in diagnosis of maturity onset diabetes of the young

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Maturity onset diabetes of the young (MODY) is a monogenic form of diabetes caused by mutations in one of the 11 different genes, which consequently leads to pancreatic beta-cell dysfunction. HNF1A-MODY is the most common form seen in adults and it is frequently misdiagnosed as type 1 or type 2 diabetes. Identification of this form of diabetes is of great significance, since the hyperglycaemia in HNF1A-MODY is very sensitive to oral treatment with sulfonylurea and may lead to an individual being able to discontinue assumed life-long insulin treatment. Current diagnostic protocols miss cases through low sensitivity and, up to date, the only reliable method for diagnosis confirmation is the sequencing of susceptible gene. Our previous studies have identified HNF1A as a master regulator of plasma protein fucosylation and have also determined that the fucose level is significantly lower in HNF1A-MODY patients than in other types of diabetes and healthy controls. The aim of the current study was to evaluate the diagnostic accuracy of the glycan test in a clinical setting using an unselected population with young-onset diabetes. Patients enrolled had increased risk of diagnostic misclassification and were recruited through the Croatian Registry of Diabetes and Young Diabetes in Oxford Study. Employed computational approach, based on random forest classification of total *N*-glycan profiles, served to determine the best algorithm to distinguish HNF1A-MODY patients from population without HNF1A-MODY. Described approach has the potential to produce a rational diagnostic protocol and could improve the differential diagnosis of diabetes.

246. Glycolipid mimetics: lipophilic carbasugars as inhibitors and chemical chaperones for GM1 gangliosidosis

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The hereditary lack of catalytically active lysosomal D-galactosidases for the degradation of gangliosides (Fabry's disease, GM1 gangliosidosis, Morquio B) causes severe damages of bones as well as neuropathical effects. Recently, chaperone mediated therapy has been proposed to improve enzyme activity and thus reduce symptoms. This approach relies on small molecules that support the folding and the transport of the catalytically compromised enzyme mutants into the lysosome. Interestingly, potent glycosidase inhibitors have been found suitable for this purpose. Based on the carbasugar 4-epi-validamine as the common scaffold, we have synthesized a range of new D-galactosidase inhibitors featuring lipophilic sub-moieties. They show potential as pharmaceutical chaperones for GM1 gangliosidosis and Morquio B. Chemical access as well as biological activities will be discussed.

247. Glycolipid mimetics: fluorine containing isoiminosugars as chemical chaperones for GM1 gangliosidosis and Fabry's disease

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Fabry's disease and GM1 gangliosidosis are hereditary lysosomal storage disorders caused by the lack of catalytically competent D-galactosidases. In Fabry's, an α-galactosidase is concerned whereas GM1 gangliosidosis is characterized by the lack of lysosomal β-galactosidase. For both types, chaperone mediated therapy has been proposed as a potential means to ameliorate the symptoms of the diseases. This approach is based on the action of small molecules that help the correct folding of the catalytically compromised enzyme mutants to guide them to the lysosome. In particular, suitable D-galactosidase inhibitors may provide the desired molecular properties in this context. We have designed a range of derivatives of the isoiminosugar isofagomine for this purpose. In particular, compounds containing a fluorine atom in β-position to the ring nitrogen seemingly improve the inhibitor's activity with the Fabry enzyme. Synthetic details as well as biological data for this new class of inhibitors will be presented.

248. Glycolipid mimetics: lipophilic 4-epi-isofagomine derivatives as chemical chaperones for GM1 gangliosidosis and Morqiuo B

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GM1 gangliosidosis is one out of over fifty defined lysosomal storage diseases. It is triggered by a genetically caused lack of catalytically competent lysosomal β-galactosidase and leads to accumulation of gangliosides in nerve and bone tissues thus causing severe damages. For a potential treatment, chaperone mediated therapy (CMT) has been envisaged, employing small molecules that induce correct folding of the enzyme and its transport into the lysosome. Some iminoalditols have been shown to improve the catalytic activity of the enzyme by around one order of magnitude. Alternatively, selected isoimino sugars may provide similar effects. We have devised a route to lipophilic derivatives of the isoimino sugar 4-epi-isofagomine which enables broad access to potential drug candidates for CMT of GM1 gangliosidosis/Morquio B disease. Details of the synthesis as well as results of biological evaluation will be presented.

249. A powerful approach towards *C*-glycosyl type iminoalditol building blocks for biological applications

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Iminoalditols are well known for their excellent inhibition of glycoside processing enzymes. Several iminoalditols also exhibit anti-microbial as well as anti-cancer properties while some have been reported as immune system stimulating agents and others as pharmacological chaperones (PC). The latter initiated their application for the chaperone mediated therapy (CMT) of lysosomal storage diseases (LSD). We have developed a multicomponent approach towards *C*-glycosidic iminoalditol structures by applying the Staudinger/aza-Wittig reaction sequence employing azidodeoxysugars as substrates to obtain the corresponding cyclic imine species. These intermediates can undergo attack by nucleophiles such as cyanide, to provide the corresponding 1-*C*-cyano iminoalditol derivatives. Follow up chemistry for structural modification, such as fluorescent labelling and subsequent deprotection, led to 1-*C*-modified iminoalditol structures, which have been shown to exhibit excellent biological activities with the respective glycosidases. Such compounds were found to have great potential as PCs for CMT of LSD. Now we are investigating other nucleophiles for trapping the imine intermediate such as, Grignard reagents and amino acid moieties, in order to introduce structural diversity to the iminoalditol scaffold for additional applications such as mechanistic tools for glycoprocessing enzymes. Synthetic details as well as biological evaluation of selected products will be presented. MZ is a recipient of a DOC fellowship of the Austrian Academy of Sciences at the Institute of Organic Chemistry.

Glycosylation in Bacteria

Keynote Lectures

250. Glycomics and glycoproteomics: powerful technologies for microbiology

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Glycosylated proteins are ubiquitous components of eukaryote cellular surfaces, where the glycan moieties are implicated in a wide range of cell-cell recognition events. Once thought to be restricted to eukaryotes, glycosylation is now being increasingly reported in prokaryotes, especially pathogenic bacteria. Structural determination is essential for understanding the roles that glycans play in biological systems. Mass spectrometry (MS), with its ultra-high sensitivity and ability to analyse complex mixtures of glycans, is the most powerful tool currently available for glycan structure analysis. Our laboratory is engaged in numerous world-wide collaborations in which we exploit high sensitivity mass spectrometric methodologies for the structural characterization of glycans. This presentation will review MS strategies incorporating MALDI-TOF-TOF MS/MS and nanoLC-ES-MS/MS for defining the microbial glycosylation. The broad range of our research activities will be illustrated by data from ongoing collaborative projects embracing host pathogen interactions and human disease. Acknowledgements: This

work is supported by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust. We are grateful to our students and postdoctoral scientists without whom this research would not be possible.

251. Application of glycan array analysis in the discovery of novel bacterial—host interactions

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Glycans are important structures in many host—pathogen interactions. Bacterial lectins such as adhesins and toxins exploit host glycans as targets. Host lectins recognize bacterial glycans in innate immune processes. The molecular details of many bacterial—host interactions remain to be discovered. Understanding these processes is key for the development of novel strategies for prevention and therapeutics. We have applied glycan array to discover novel interactions between bacterial and human cells. Using the Institute for Glycomics glycan arrays comprising 400 different structures, we have discovered novel glycan targets for the archetypal cholesterol dependent cytolysin toxins, pneumolysin and streptolysin. Previously it had been believed that cholesterol rich membrane of host cells were the only receptor of these toxins. We report high affinity binding to glycan structures present on red blood cells. For example, pneumolysin binds to sialyl Lewis X with a KD of 1.88×10^{-5} M. Binding to this structure is required for efficient red blood cell lysis by pneumolysin. Using similar approaches we have defined novel classes of bacterial and host lectins.

Lectures

252. Functional and structural characterization of rhamnose biosynthesis enzymes in Group A Streptococcus

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The human pathogen Group A Streptococcus (GAS) ranks in the top 10 of infection-related causes of mortality worldwide. The Group A Carbohydrate (GAC) is the molecular signature and a virulence determinant of GAS. The production of dTDP-L-rhamnose is critical for GAC biosynthesis but also more broadly for the viability or virulence of other medically-important bacteria including *Mycobacterium spp.* and *Enterococcus faecalis*. dTDP-L-rhamnose is synthesized from α-glucose-1-phosphate through a four-step enzymatic process catalyzed by the enzymes RmlA-D. The only structurally characterized RmlA-D enzymes are from the Gram-negative bacterium *Salmonella enterica* and have provided valuable insight into the enzymatic mechanism of action, including requirement for Mg^{2+}-dependent homodimerization for RmlD. In GAS, homologues of all four L-rhamnose biosynthesis enzymes can be identified through bioinformatics. In contrast to *S. enterica*, the GAS RmlD homologue appears to be encoded by the gacA gene, the first gene of the recently identified GAC biosynthesis gene cluster, and is located separately from rmlA-C on the genome. We demonstrate through biochemical and structural analysis that GacA is the 4-dTDP-dehydrorhamnose reductase of GAS but functions in a novel cation-independent monomeric manner. Sequence analysis of 210 RmlD homologues shows that RmlD homologues from all Gram-positive and 45 % of Gram-negative species likely function as monomers due to lack of a dimerization motif. Therefore, GacA defines a new class of monomeric RmlD enzymes. Analysis of a saturated mutant GAS library using Tn-sequencing identified gacA, as well as rmlA-C, as essential genes for GAS. Therefore, we confirmed the enzymatic function of GacA *in vivo* through functional replacement of *S. mutans* rmlD with GAS gacA. The results from this study will help future screens for novel inhibitors of dTDP-L-rhamnose biosynthesis.

253. Glycoprotein export in the oral pathogen *Tannerella forsythia* via a type IX secretion system

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The oral pathogen *Tannerella forsythia* is covered with a unique 2D crystalline surface (S-) layer composed of two glycoproteins. These S-layer proteins (TfsA, TfsB) together with several other proteins are modified with

an *O*-linked oligosaccharide with the structure 4-Me-β-ManpNAcCONH$_2$-(1,3)-[Pse5Am7Gc-(2,4)]-β-ManpNAcA-(1,4)-[4-Me-α-Galp-(1–2)]-α-Fucp-(1,4)-[α-Xylp-(1,3)-]-β-GlcpA-(1,3)-[β-Digp-(1,2)-]-α-Galp. Notably, the S-layer proteins are equipped with a conserved C-terminal domain (CTD) known to act as a translocation signal for certain proteins across the outer membrane of *Bacteroidetes* species via a type IX secretion system (T9SS). The *T. forsythia* genome predicts the presence of the components for a T9SS in conjunction with a suite of CTD proteins. To investigate, if T9SS is functional in *T. forsythia*, two defined T9SS mutants were generated and analyzed with respect to secretion, assembly and glycosylation of the S-layer proteins as well as proteolytic processing of the CTD and biofilm formation. In either mutant, TfsA and TfsB were incapable of translocation, evidenced by the absence of the S-layer in transmission electron microscopy of ultrathin-sectioned bacterial cells. Despite being entrapped within the periplasm, MS analysis revealed that the S-layer proteins were modified with the complete, mature glycan indicating that protein translocation and glycosylation are two independent processes. Further, the T9SS mutants showed a denser biofilm architecture compared with the wild-type. This study demonstrates the functionality of T9SS and the requirement of CTD for the outer membrane passage of extracellular proteins in *T. forsythia*. The T9SS may constitute a valuable target for the design of strategies to combat periodontitis. Support came from the Austria Science Fund FWF, project P24317-B22 and the Doctoral Programme BioToP-W1244.

Posters

254. Enhanced α-2,6-sialyation by bacterial sialyltransferase reaction coupled with dephosphorylation of cytidine-5′-monophosphate

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Bacterial sialyltransferases (STs) have several advantages including easy and economic expression in *E. coli*, compared to eukaryotic ones requiring expensive expression system such as a mammalian cell culture. In this study, we directly compared three bacterial α-2,6-STs from *Photobacterium damselae*, *Photobacterium* sp. JT-ISH-224 and *P. leiognathi* JT-SHIZ-145, which were recombinantly expressed in *Escherichia coli*. In all of the prolonged ST reactions, sialylated glycans increase in early time and then decrease in late time. We found that this pattern is related with inherent sialidase activities of bacterial STs. These sialidase activities are greatly increased by free CMP generated from a donor substrate CMP-*N*-acetylneuraminic acid (CMP-Neu5Ac) during ST reactions. Decrease of sialylated glycans in prolonged ST reaction is prevented through an inhibition of sialidase activity by simple treatment of alkaline phosphatase which dephosphorylates CMP to cytidine. Through supplemental additions of alkaline phosphatase and CMP-Neu5Ac to the reaction using the recombinant α-2,6-ST from *Photobacterium leiognathi* JT-SHIZ-145, the content of bi-sialylated *N*-glycan increases up to ~98 % without decrease in prolonged reaction. This optimized reaction is successfully applied for α-2,6-sialylation of asialo-fetuin, which results in great increase of multi-sialylated *N*-glycans. It suggests that this strategy using a recombinant bacterial α-2,6-ST coupled with alkaline phosphatase treatment have a promise for economic glycan synthesis and glyco-conjugate remodeling.

255. Supercharging reagent for enhanced liquid chromatographic separation and charging of acidic and giant glycopeptides for nanolc-ESI-MS/MS

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Shigella flexneri, coating with plentiful *O*-antigens, is the cause of bacillary dysentery and shigellosis. Type 2a *O*-antigen is the most prevalent isolated from patients and consists of several repeating unit of one β-D-GlcNAc and three α-L-Rha with non-stoichiometric glucosylation. This *O*-antigen is a promising component of vaccine for prophylaxis of shigellosis. Bacterial toxins are widely used as tools for mucosal vaccination. Hence, exotoxins conjugated with *O*-antigens would be good vaccine candidates against *S. flexneri*. To develop such vaccines, analytical tools are highly demanded. With the development of proteomic technique, obtaining site-specific glycosylation on its carrier protein is achievable. However, the molecular weight of *O*-antigens on the covalently linked protein is rather large. One possibility to detect the corresponding large ions in MS analysis is the increase of their charge state. Addition of *m*-nitrobenzyl alcohol (*m*-NBA) into electrospray solutions is known to increase the charge state of

macromolecules. We rationalized that *m*-NBA might also be able to influence the ionization process of giant glycopeptides, resulting in the increase of their charge state and therefore enhance the sensitivity of detection of mass spectrometer. Commercial glycoproteins were first used to examine the effect of the charge state by adding m-NBA into mobile phase. Our data showed that with the help of *m*-NBA, the charge states of sialylated glycopeptides increased and the chromatographic separation of neutral and acid glycopeptides revealed a remarkable improvement. Next, we applied this system for the characterization of an exotoxin with covalently linked *Shigella O*-antigens produced by *E. coli*. It allowed us to identify peptides with glycan chains up to 17 repeating units. Our results indicated that incorporation of *m*-NBA into reverse-phase LC solvents improves sensitivity, charging and chromatographic resolution for acidic and giant glycopeptides.

256. Identifying different classes of *O*-otases in *Acinetobacter* species and their potential in glycoengineering

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Protein glycosylation is the covalent modification of proteins with sugars. In bacteria, different forms of protein glycosylation exist, mainly *N*- and *O*-glycosylation. Bacterial *O*-glycosylation occurs when a lipid-linked glycan is transferred *en bloc* to acceptor proteins in the periplasm via *O*-Oligosaccharyltransferases (*O*-OTases). Alternatively, OTase-independent *O*-glycosylation occurs through the sequential addition of sugars to proteins by cytoplasmic glycosyltransferases. Bacteria belonging to the genus *Acinetobacter*, which persist in hospitals and cause a multitude of drug-resistant infections, possess an OTase-dependent general *O*-glycosylation system that is essential for virulence. Unlike other characterized *O*-glycosylation systems where a given organism encodes a single *O*-OTase, we have identified two functional *O*-OTases in most sequenced *Acinetobacter* strains. One of the two OTases (TfpO) is specific for type IV pilin glycosylation, while the other OTase (PglL) glycosylates multiple proteins. PglLs and TfpOs are phylogenetically distinct and are homologous to the *Neisseria* PglL and the *Pseudomonas* PilO/TfpO respectively. Moreover, we have identified the *O*-OTase PglLComP in *A. baylyi* that is homologous to PglL, but glycosylates only its cognate pilin ComP, suggesting that PglLComP represents an evolutionary link between PglLs and TfpOs. Identifying novel OTases with different specificities is particularly important for the biotech industry, where OTases can be exploited in the production of glycoconjugate vaccines. Glycoconjugates are currently produced by chemical conjugation, which has many drawbacks including high cost and batch-to-batch variations. OTase-dependent methods for glycoconjugate synthesis present a viable alternative as they produce homogenous glycoconjugates and are more cost effective. Our work is focused on determining the glycan specificity of *Acinetobacter O*-OTases to realize their full potential in the glycoconjugate vaccine industry.

Intestinal Glycoscience

Keynote Lecture

257. A milk-oriented microbiota in breast fed infants: a glycan-driven microbial enrichment

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Human milk contains numerous components that shape the microbial content of the developing infant gastrointestinal tract. A prominent feature of milk is an array of complex glycans and glycoconjugates that serve a passive immune function by sequestering and deflecting pathogens while simultaneously enriching a protective, milk-oriented microbiota (MOM) often dominated by bifidobacteria. Recent research suggests the timing of establishment, and proper function of, a MOM is critical for infant development. An infant's MOM is initially generated through environmental transfer to the gut and subsequently shaped by diet (milk) and host genetics. Once established, MOMs dominated by bifidobacteria exhibit low residual milk glycans and higher levels of short chain fatty acids in the feces. The mechanistic basis for milk glycan consumption by bifidobacteria has been the subject of active research. Different infant-borne bifidobacteria contain specific glycosidases and transport systems

required to utilize free glycans or glycoconjugates. Consumption of milk glycans enhances specific bifidobacterial interaction with the infant host through both direct and indirect routes. Bifidobacterial growth on free milk glycans beneficially modulates intestinal function. In addition, metabolites generated during growth on milk glycans dampen inflammation and strengthen gut barrier function. In aggregate, these studies suggest a co-evolutionary relationship between mammalian milk glycans, infant-borne bifidobacteria and the infant host resulting in a programmed enrichment of a protective bifidobacterial-dominant MOM during a critical stage of infant development. Importantly, disruption of this programmed enrichment can lead to a "poorly functioning" MOM that may pose a risk for negative health outcomes. Further analysis of this naturally evolved system will shed light on effective pre- and probiotic tools that support and ensure a protective MOM for all at risk infants.

Lectures

258. Region-specific *N*-glycan mapping in inflammatory bowel disease and control tissue using PGC-LC-ESI-MS/MS

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Chronic inflammatory bowel diseases (IBD) are affecting a large part of the society but onset and progression are comparably poorly understood. The colon mucosa is heavily glycosylated and lectins as well as glycan receptors were suggested to be involved in the aetiology of IBD. However, there is just little known about IBD related glycosylation. It was found that mucin glycosylation changes within the length of a healthy colon, which is of particular importance to sample collection for studies that aim the comparison of inter- and intra-individual differences. In our study for the first time we systematically analysed *N*-glycan profiles of colon biopsies from IBD and control patients, taken from seven distinct positions between ileum and rectum of the colon. The bowel section *N*-glycome was analysed using porous graphitized carbon (PGC)-LC-MS/MS analysis, and the glycan compounds were detected in negative ion-mode. Considering the tremendous data complexity of each single LC-MS/MS analysis, qualitative glycan identification is very time and effort consuming. This step was considerably facilitated using an in-house developed semi-automated glycan spectra library approach. In total, we detected 144 *N*-glycan structures, including structural and linkage isomers. At a final stage statistical analysis was applied to determine region-specific *N*-glycan features in IBD colon tissue. Distinct *N*-glycan differences were observed between the ileum, which is the last part of the small intestine and the other sample locations, belonging to the large intestine. This includes a gradient in antenna fucosylation, in particular lewis y/b structures as well bisecting *N*-acetylglucosamine and hybrid glycan structures. However, within all sample locations of the large intestine there were hardly any significant difference in glycan expression detected. This study gave novel insights into bowel *N*-glycosylation and will further facilitate disease-specific glycosylation analysis of IBD.

Posters

259. Structural diversity of human gastric mucin glycans

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The main components of the mucus layer that lines all mucosal surfaces are heavily glycosylated mucins. In the stomach, these mucins can both protect the epithelial surface from the acidic gastric juice and bind to the causing pathogen of ulcer and gastric cancer, *Helicobacter pylori*. Mucin glycosylation differs between individuals and can change during disease. Here we have examined the mucin glycosylation in depth of 16 individuals with and without gastric disease. We found an enormous diversity in

glycosylation, both between individuals and within the glycosylation of mucins from a single individual: mucin glycan chain length ranged from 2 to 13 and over 200 structures were found, each individual carrying 39–103 structures. Each mucin sample had 1–14 glycan structures that were unique for that individual. As for *O*-glycans, they are mostly with core 1 or 2. Sialylation was present but the sulfation was low. Blood group antigens, Lewis epitopes, LacdiNAc (GalNAcβ1-4GlcNAc), and terminal α-1,4-GlcNAc were also observed, which lead to structural diversity of gastric *O*-glycans.

Neuroglycoscience

Keynote Lecture

260. Structural and functional changes of polysialic acid related with genetic alterations of ST8SIA2/STX in psychiatric diseases

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Polysialic acid (polySia) is a unique and functionally important glycotope especially in vertebrate brains and involved in learning, memory, circadian rhythm, and social behaviors. PolySia and ST8SIA2/STX, one of two polysialyltransferase genes, are also known to associate with psychiatric disorders and cancers. Due to its bulky and hydration properties, polySia exhibits repulsive fields displaying an anti-adhesive effect on cell-cell interactions. Recently, we have demonstrated that polySia also exhibits attractive fields displaying a reservoir scaffold for various neurological active molecules: the brain-derived neurotrophic factor (BDNF), catecholamine neurotransmitters, and the fibroblast growth factor 2 (FGF2). The reservoir functions for BDNF and FGF2 are also exemplified in glycosaminoglycans (GAGs); however, polySia and GAGs exhibit different binding properties with respect to strength, stoichiometry, and the repertoire of binding counterparts. Importantly, binding properties of polySia to BDNF and FGF2 highly depend on the degree of polymerization. Accumulating data led us to hypothesize that the quantity and quality of polySia on cell surface are highly regulated for normal brain functions. We have thus focused on impairments of polySia properties in relation with the risk of diseases, such as schizophrenia, autism, and mood disorder, in which the SNPs of ST8SIA2/STX are partially involved. A variety of SNPs actually affected the quality and quantity of polySia and its functions as attractive fields. All these data suggest that highly regulated polySia expression is important for normal brain functioning.

Lectures

261. Sialyltransferase ST3GAL IV deletion protects against epilepsy, but induces anxiety

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Temporal lobe epilepsy (TLE) often becomes refractory, and the epilepsy patients are at a greater risk for developing anxiety, depression, psychosis, and learning disorders. Therefore, it is necessary to identify molecules that were previously unknown to contribute to epilepsy and its associated disorders. Previously, the sialyltransferase ST3Gal IV is upregulated within the neural circuits through which amygdala-kindling stimulation propagates epileptic seizures. Presently, kindling stimulation failed to evoke epileptic seizures in ST3Gal IV-deficient mice. Furthermore, approximately 80 % of these mice failed to show tonic-clonic seizures with stimulation, whereas all littermate wild-type mice showed tonic-clonic seizures. This indicates that the loss of ST3Gal IV does not cause TLE in mice. Meanwhile, ST3Gal IV-deficient mice exhibited decreased acclimation in the open field test, increased immobility in the forced swim test, enhanced freezing during delay auditory fear conditioning, and sleep disturbances. Thus, the loss of ST3Gal IV modulates anxiety-related behaviors. These findings indicate that ST3Gal IV is an effective target for treating epilepsy, and is a key molecule in the mechanisms underlying anxiety. ST3Gal IV-KO mice showed decreased growth hormone (GH) and Igf1 mRNA expression in the brain, while the kindling stimulation increased GH levels in neuronal cells during development of epilepsy and following acquisition of seizures. These suggest that regulation via growth hormone has a robust impact in epileptogenesis, which is regulated by ST3Gal IV. It is known that GH is principal

hormones involved in lipid metabolism. These propose that ST3Gal IV regulates lipid metabolism. Presently, we also introduce that oil-rich diets differentially modulate anxiety and depression in normal and anxious mice.

262. Bisecting GlcNAc on BACE1 is a novel therapeutic target for Alzheimer's disease

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Alzheimer's disease (AD) is the most common form of dementia and a serious issue all around the world. So far, however, an effective medicine has not been developed. In this study, we found that GnT-III, a glycosyltransferase for the biosynthesis of bisecting GlcNAc, plays a pathological role in AD. GnT-III knockout mice show a drastic decrease in production of amyloid-β (Aβ), a hallmark and the causative peptide of AD, resulting in improvement of cognitive function. We found that BACE1, an essential protease for Aβ generation, is a novel target for bisecting GlcNAc and is highly modified with bisecting GlcNAc in AD patients compared with non-AD individuals, suggesting that bisecting GlcNAc promotes AD by regulating BACE1 function. Indeed, the reduction of Aβ in GnT-III-knockout mice is caused by a shift in intracellular distribution of BACE1 from early endosomes to lysosomes without loss of its catalytic activity. Furthermore, in contrast to the severe nerve defects and semilethality in BACE1-knockout mice, GnT-III-knockout mice essentially show no severe phenotypes. These results suggest that a GnT-III inhibitor could be a promising drug target for AD. To search for GnT-III inhibitors, we newly established a high-throughput GnT-III assay method. Using our new system, we tested 140, 000 synthetic compounds and found several chemicals that inhibited GnT-III activity with IC50 less than 20 μM. This study will provide a new strategy for large-scale screening of glycosylation inhibitors as glycan-targeted therapeutics.

Posters

263. New insights into the role of alterations in protein glycosylation in Alzheimer's disease pathology

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Human neurodegenerative diseases, such as Alzheimer's disease (AD) which is the primary cause of dementia, Creutzfeldt-Jakob disease and Parkinson's disease, are devastating illnesses that predominantly affect elderly people. The hallmark of these diseases are pathogenic oligomers and fibrils of misfolded amyloidogenic proteins (*e.g.* Aβ and hyper-phosphorylated tau in AD), which cause progressive loss of neurons in the brain and nervous system. Interestingly, several reports indicated vast deviations from normal protein glycosylation in the brain of AD patients, while others demonstrated alterations in glycosylation of specific proteins related to AD pathology in the disease state, such as tau and APP, the Aβ precursor. Yet, a causal link between alterations in protein glycosylation and AD-related neurodegeneration remains to be demonstrated. This is the goal of our work. Using an *in silico* approach we found that many glycosylation-related enzymes exhibit different expression profile in brains of human AD patients as compared with healthy subjects. Utilizing a more direct approach, we experimentally intervene in global protein glycosylation and study the effect of enhancing or reducing expression of the glycosylation-related genes in transgenic *Drosophila* over-expressing human tau, which serve as an established AD model. We were able to identify leading glycosylation enzymes, both augementing and ameliorating tauopathy symptoms using the fly as a model. We will next verify these effects of alterations in global protein glycosylation on

AD pathology using human cultured cells over-expressing Aβ or tau and AD model mice.

264. Distribution of parvalbumin and calbindin-D28k interneuron in the brain of ST3GAL2, ST3GAL3 and ST3GAL2/3 double null mice

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Based on previous research of Schnaar *et al*., it is known that *St3gal2* and *St3gal3* genes are responsible for nearly all of the terminal sialylation of brain gangliosides in the mouse. *St3gal2*-null mice differed significantly from wild type, expressing half the normal amount of GD1a and GT1b. *St3gal2/3*-double null mice were >95 % depleted in gangliosides GD1a and GT1b. *St3gal2/3*-double null mice were small, weak, and short lived. They were half the weight of wild type mice at weaning and displayed early hindlimb dysreflexia. GABA-ergic neurons, have important role in performing feedback inhibition. This study describes the distribution of parvalbumin and calbindin-D28k interneurons in the brain of *St3Gal2*, *St3Gal3* and *St3al2/3*-double null mice. Two brain regions, hippocampus and cerebellum were analysed. In hippocampus we analysed three different region, CA1, CA3 and dentate gyrus (DG). Cerebellum was analysed for molecular and granular layer and Purkinje cells. There is no difference in number of all neurons in CA1 and DG region of hippocampus, but all knock out mice have less neurons than wild type in CA3 region (p=0,029). In cerebellum there is difference in number of neurons in molecular layer (p=0,021), but not in the granular layer. Calbindin-D28k positive neurons only appear in DG region of hippocampus with significantly the least number in *St3Gal2* mice (p=0, 033). Purkinje cells of cerebellum are calbindin-D28k positive and there is the least neurons in *St3Gal2* mice (p=0,036). In conclusion, *St3gal2* and *St3gal3* genes affect calbindin-D28k distribution in hippocampus and cerebellum, but they do not affect parvalbumin distribution. *St3Gal2* knock out mice have the greatest decrease in number of calbindin-D28k positive neuron so we assume that GD1a ganglioside plays a role in maintanance and function of calbindin positive interneurons, but additional analysis are needed.

265. The sweet side of neurons - xCGE-LIF based characterization of neuron and synaptic *N*-glycan composition

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Glycosylation has been described as important posttranslational modification of proteins involved in neuronal development, learning and memory formation and synaptic plasticity in general. The majority of neuronal cell surface proteins are modified by *N*-glycans, though a comprehensive characterization of the *N*-glycosylation changes accompanying stimulation of neurons has not been performed yet. To fill this lack of knowledge a cell culture based neuronal model system was established and its *N*-glycome was investigated by multiplexed capillary gel electrophoresis with laser-induced fluorescence detection (xCGE-LIF). Accordingly, neurons were isolated from E18 rat cortex and allowed to differentiate within 18 days. After stimulation by the neurotransmitter L-glutamate, synaptosomes and synaptic junctions were isolated by density gradient centrifugation and subjected to *N*-glycan analysis. *N*-glycans were released by PNGase F, labeled with fluorescence dye APTS and analyzed by xCGE-LIF. After migration time normalization to an internal standard, a peak assignment with *N*-glycan structures using an in-house database was performed. All annotations were carefully validated by exoglycosidase digestions. Mainly high-mannose type *N*-glycans could be detected, as well as a great variety of complex type *N*-glycans bearing the Lewis X epitope. Ongoing experiments will help to evaluate the importance of glycosylation for synaptic plasticity.

266. Possibility that ST3GAL IV deficient mouse is a schizophrenia model

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We previously screened molecules that are involved in epilepsy using a temporal lobe epilepsy (TLE) mouse model and a sialyltransferase ST3Gal IV was nominated as an epilepsy-responsive molecule. To determine whether ST3Gal IV is a determinant of seizure occurrence, we established the ST3Gal IV-knockout (KO) mouse model. Then, we demonstrated that ST3Gal IV-KO mice failed to show seizures with repeated kindling stimulation, however, exhibited decreased exploration/acclimation in the open field, enhanced cognitive fear in delay auditory fear conditioning, and increased immobility in the forced swim test. It suggested that the disappearance of epilepsy induces other neuropsychiatric disorders as side effects. Epilepsy patients are at a greater risk for developing anxiety, depression, and schizophrenia. So to investigate the possibility of schizophrenia occurrence, ST3Gal IV-KO mice received startle response test as an end phenotype and we examined the pre-pulse inhibition test in the KO mice. While there was little difference of startle response between ST3Gal IV-KO mice and wild-type mice, the KO mice exhibited decrease of pre-pulse inhibition (PPI) statistically. It suggested the possibility that ST3Gal IV-KO mice contract schizophrenia. Next, we investigated pathological changes in the brain of the KO mice. As a result, the KO mice showed abnormal shape of microtubule-associated protein 2 (MAP2)-positive dendrite in the polymorphic layer of the dentate gyrus. We have observed aberrations of MAP2-positive dendrite in the granular layer of the dentate gyrus following acquisition of epileptic seizures. Hence, ST3Gal IV might modulate structures of MAP2-positive dendrites in the dentate gyrus. Now we investigate whether the KO mice recover from decrease of PPI by therapeutic drug.

267. Probing the brain glycome by tissue glyco-capture and nano-LC/MS and MS/MS

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Tissue glyco-capture (TGC), a highly sensitive MS compatible method for extraction of glycans from tissue, was combined with structure-specific nano-LC/MS for sensitive and detailed profiling of the mouse brain glycome. Hundreds of glycan structures were directly detected by accurate mass MS and structurally elucidated by MS/MS, revealing the presence of novel glycan motifs such as antennary fucosylation, sulfation, and glucuronidation that are potentially associated with cellular signaling and adhesion. Microgram- level sensitivity enabled glycomic analysis of specific regions of the brain, as demonstrated on not only brain sections (with a one dimensional spatial resolution of 20 μm) but also isolated brain structures (*e.g.*, the hippocampus). Reproducibility was extraordinarily high (R>0.98) for both method and instrumental replicates. The pairing of TGC with structure-specific nano-LC/MS was found to be an exceptionally powerful platform for qualitative and quantitative exploration of the brain glycome. Furthermore, we have explored the developmental changes of glycosylation in frontal cortex region of human brain tissue during development from neonate (39 days) to adult (49 years). Indeed, we could monitor the glycosylation in human brain during the stage of development. The results of this study would be useful as the informative data for the age-dependent neurological diseases such as Alzheimer's disease.

268. GALNT17/Wbscr17 knockout mice show decreased growh and hyperprolactinemia

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We have been investigating roles of mucin-type *O*-linked sugar chains with the linkage structure, GalNAcα1->Ser(Thr). Their biosynthesis is initiated by a group of enzymes, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAc-Ts). GALNT genes coding GalNAc-Ts consist of a large gene family with 20 members in human. We have previously identified GalNAc-T17, which is catalytically inactive and is expressed predominantly in the brain. GALNT17 is estimated as one of causative genes for Williams-Beuren syndrome, which is a rare neurodevelopmental disorder. Therefore, GALNT17 is alternatively designated Williams-Beuren syndrome chromosome region 17 (WBSCR17). However, the function of GalNAc-T17 in

the brain is still unclear. To investigate the physiological roles of GalNAc-T17, we generated Galnt17 knockout mice. The homozygous mutant mice exhibited decreased body weight, reduced food intake, and female reproductive abnormality. Further analysis revealed that the Gh-IGF1 system was downregulated, while the secretion of Prolactin was upregulated in the mutant mice. Considering the expression of Galnt17 in the hypothalamic arcuate nucleus, which includes neuroendocrineneurons controlling the anterior pituitary gland, Galnt17 might participate in the hypothalamus-pituitary axis, and regulate secretions of the pituitary hormones.

269. Dissecting the role of glycohydrolases in the central nervous system: GBA2 in the neuronal differentiation

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Mammalian neurodevelopment is characterized by qualitative and quantitative changes in plasma membrane glycosphingolipids due to a fine regulation of their metabolic pathways. While the biosynthetic pathway is largely studied scant is the information available on the catabolic one. For this reason, I studied the activity of the main glycohydrolases expressed in the central nervous system during neuronal differentiation. To this purpose the activities of the principal glycohydrolases involved in glycosphingolipid catabolism have been evaluated in different experimental models such as brains and cerebella of mouse at different ages and neuronal cell cultures (immortalized mouse neuronal cell lines GN11 and GT1-7, primary cultures of mouse cerebellar granule cells and human neuroblastoma cells SH-SY5Y). The results obtained indicate that the process of neuronal differentiation is associated with a marked increase in the activities of all the glycohydrolases evaluated; in particular, the activity of the non-lysosomal β-glucosylceramidase GBA2 undergoes the most relevant increase representing the prevalent form of β-glucocerebrosidase in mature neurons. In order to evaluate the possible role of GBA2 in the neuronal differentiation, SH-SY5Y cells have been stably trasfected for GBA2 overexpression. Cells overexpressing GBA2 acquired a neuronal phenotype and showed a significant increase in ceramide levels. These results are in line with literature data that demonstrate the involvement of ceramide in the neuronal differentiation. Therefore, it is possible to hypothesize that the hydrolysis of glucosylceramide to ceramide, catalyzed by GBA2 at the plasma membrane level, has a functional role in the neuronal differentiation process. Collectively these findings suggest that GBA2 may represent a possible neuronal marker and demonstrate for the first time its direct involvement in the neuronal differentiation.

270. Immunoglobulin G and total plasma protein *N*-glycosylation in Parkinson's disease

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Parkinson's disease (PD) is a progressive neurodegenerative condition involving both motor and non-motor features. It is accompanied by dysfunction or loss of dopamine producing neurons in the substantia nigra pars compacta of the midbrain and the presence of Lewy bodies, cytoplasmic aggregations of the protein α-synuclein in brain neurons. Clinical symptoms of disease arise from this pathophysiology. Parkinson's disease is occurring in about 1 % in individuals aged 60 years or older, in 4–5 % of those aged 85 years or older and it is estimated to afflict 0.3 % of the general population worldwide. The glycans covalently added on proteins, play important roles in almost every biological process, considering that all membrane and secreted proteins as well as numerous intracellular proteins are being modified by oligosaccharides. IgG *N*-glycosylation was analysed in 98 PD patients and in 95 patients as healthy controls and total plasma protein *N*-glycosylation was analysed in 107 PD patients and in 59 patients as healthy controls. Mono-galactosylated and di-galactosylated IgG *N*-glycans are decreased in PD patients compared to the control group which follows the same trend as in some other inflammatory and autoimmune diseases as well as in aging (inflamaging). Galactosylation decreases proinflammatory function of IgG. This suggests the potential role of galactosylated IgG *N*-glycans as general biomarkers for aging, inflammatory and autoimmune diseases. Total protein plasma *N*-glycosylation should be investigated further in male population.

Proteoglycans

Keynote Lecture

271. Function and biosynthesis of dermatan sulfate

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Dermatan sulfate epimerases (DS-epi1 and 2), together with dermatan 4-*O*-sulfotransferase 1 (D4ST1) are the only dermatan sulfate specific enzymes, *i.e.* they ultimately determine if the polysaccharide will be CS or DS. We have previously cloned dermatan sulfate epimerases, both of them converting glucuronic into iduronic acid. DS-epi1 together with D4ST1 generate DS with long blocks of iduronic acid disaccharides while DS-epi2 only generate few iduronic acid disaccharides along the chondroitin chain. We have generated milligram quantities of recombinant DS-epi1 and D4ST1 using a HEK293-EBNA expression system. Recombinant DS-epi1 chondroitin oligosaccharide was incubated in deuterated water. The tetrasaccharide was the shortest attacked substrate. The product formation did increase from a tetrasaccharide to reach a maximal product formation with octa- and deca-saccharide. Further, using a hexasaccharide we could show that the non reducing terminal disaccharide was not attacked by the epimerase. Finally using longer oligosaccharides it was possible to show that the DS-epi1 did introduces deuterium into several adjacent disaccharides. By co-incubation of DS-epi1 and D4STI with cold chondroitin (defructosylated K4) recombinant dermatan sulfate was obtained composed of long IdoA and GalNac-4S building blocks accounting for 95 % of the whole chain. Recombinant dermatan sulfate is biologically active as judged by its binding capabilities. Mice with targeted DS-epi1, DS-epi2 and double knock out mice was generated The DS-epi1 targeted mice are smaller and have musculoskeletal and immune deficiency disorders. The DS-epi2 targeted mice have no overt changes. The double knock-out mice have a similar phenotype as the DS-epi1 targeted mice but do not survive delivery for unclear reason.

Lectures

272. Estrogen-induced osteoanabolism is mediated via enhanced production of chondroitin sulfate-E

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Bone remodeling is a physiological process that maintains skeletal integrity by removing old bone and replacing it with new bone mineral matrix. An imbalance between bone resorption and bone formation causes a number of bone diseases. Osteoporosis is an age-related disorder of bone remodeling in which bone resorption outstrips bone matrix deposition. Although anticatabolic agents (*e.g.*, bisphosphonates) are commonly used to prevent osteoporotic bone loss, complementary and alternative treatments that cause osteoblastic cells to stimulate bone formation are actively sought. Sex steroid hormones, particularly estrogens, are bidirectional regulators for bone homeostasis; therefore, estrogen-mediated events are important potential targets for such anabolic therapies. Here, we show that estrogen-induced, osteoanabolic effects were mediated via enhanced production of chondroitin sulfate-E (CS-E), which could act as an osteogenic stimulant in our cell-based system. Conversely, estrogen deficiency caused reduced expression of CS-E-synthesizing enzymes, including GalNAc4S-6ST, and led to decreased CS-E production in cultures of bone marrow cells derived from ovariectomized mice. Moreover, GalNac4S6ST-deficient mice had abnormally low bone mass that resulted from impaired osteoblast differentiation. These results indicated that strategies aimed at boosting CS-E biosynthesis are promising alternative therapies for osteoporosis.

273. The control of hyaluronan synthesis

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Increased amount of hyaluronan (HA) in extracellular matrix (ECM) promotes neo-angiogenesis, cell migration and inflammation. HA synthesis is regulated by several factors including covalent modification of the synthetic enzymes HAS2. We described the role of AMPK in the phosphorylation of HAS2 which blocks the HA synthesis. In human smooth muscle cell (AoSMC) model, we show that the hexosamine

biosynthetic pathway (HBP) may increase the concentration of UDP-*N*-acetyl-glucosamine leading to an increase of HA synthesis. It was reported that proteins can be *O*-glycosylated by specific transferase OGT, and we found that the inhibition of *O*-GlcNAcylation reduced HA production whereas opposite effect was obtained inducing protein *O*-GlcNAcylation altering the enzyme life span. The *O*-GlcNAcylation increases the gene expression of the HAS2, in fact many nuclear proteins can be *O*-glycosylated by OGT. We studied whether the expression of the HAS2 could be controlled by *O*-GlcNAcylation in AoSMCs. We found that the natural antisense transcript (NAT) of HAS2 (HAS2-AS1) was absolutely necessary to induce the transcription of the HAS2 gene. Moreover, we found that *O*-GlcNAcylation modulated HAS2-AS1 promoter activation, but not the HAS2 promoter, whereas HAS2-AS1 NAT, working in cis, regulated HAS2 transcription by altering the chromatin structure around the HAS2 proximal promoter via *O*-GlcNAcylation and acetylation. From ChIP data we were able to indentify the protein involved in this mechanism: p65 from NFkB signaling pathway. This protein can be *O*-GlcNAcylated and in this form triggers the expression of NAT transcript as well as the stabilization of HAS2 promoter. These results indicate that HAS2 transcription can be finely regulated not only by recruiting transcription factors to the promoter of the gene but also by modulating chromatin accessibility by epigenetic modifications which include a long non coding RNA HAS2-AS1 and p65.

Posters

274. Structure and function of D-glycuronyl C5-epimerase

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Heparan sulfates (HS) are glycosaminoglycan ubiquitously expressed on the cell membrane and in the extracellular matrix. These highly sulfated glycans are essential for many physiological processes including embryonic development, cell growth, inflammation, and blood coagulation. D-glucuronyl C5-epimerase (Glce) is a key enzyme in HS synthesis, converting D-glucuronic acid (GlcA) to L-iduronic acid (IdoA) to form diverse structure motifs which are important for protein ligand recognition. We have determined the crystal structures of Glce in apo form (unliganded) and in complex with heparin hexasaccharide (product of Glce following *O*-sulfation), both in a stable dimer conformation. A Glce dimer contains two catalytic sites, each at a positively charged cleft in C-terminal α-helical domains binding one negatively charged hexasaccharide. Mutagenesis studies indicate that three tyrosine residues, Y468, Y528, and Y546 in the active site are found to be pivotal for the enzymatic activity. The complex structure also suggests the mechanism of product inhibition, *i.e.* 2-*O*- and 6-*O*-sulfation of HS keeps the C5 carbon of IdoA away from the active-site tyrosine residues. Further study shows that Glce in rat cortical neurons promotes dendritic morphogenesis. Besides, we demonstrates that Glce is a direct target of miR-34c-5p. In addition, miR-34c-5p and its target Glce regulate the dendritic morphogenesis through PI3K/Akt signaling. These data advance understanding of the key modification regulation in HS biosynthesis and the function of Glce in neuritogenesis.

275. Synthesis of keratan sulfate oligosaccharides having biotin-linker

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Keratan sulfate (KS) has a linear glycan chain composed of a repeating disaccharide unit (−4Gal1-4GlcNAc1-). Primary positions of Gal and GlcNAc are often sulfated. It is known that the KS oligosaccharide inhibits the extension of axons. Damaged neurons in the adult mammalian central nervous system can not regenerate spontaneously. It is known that the inhibitory factors associated with KS oligosaccharide. However, the mechanisms of the interaction between neuron and KS oligosaccharide were not identified. The length of the KS oligosaccharide and the sulfateion pattern might deeply relate to the activity of inhibition. Here, we report the synthesis of different types of KS oligosaccharides attached to biotin via a hydrophilic linker at the reducing terminal for biological use. We synthesized the sulfated KS oligosaccharides by using common disaccharide unit (Gal-GlcN) suitably protected at its amino and hydroxyl groups. The common disaccharide unit is designed as both disaccharide donor and acceptor for convenient glycan elongation. Disaccharide donor and acceptor were equipped with chemoselectively removable NAP (2-naphthylmethyl) and TBDPS (tert-butyldiphenylsilyl) groups at the 6 positions of the Gal and GlcN residues, respectively, for regioselective sulfation. Disaccharide acceptor was stereoselectively coupled with the disaccharide donor. The protected oligosaccharides were sulfated at the primary positions of Gal and / or GlcN. These sulfated oligosaccharides were suitably deprotected and biotinylated at the reducing terminals.

276. Amounts and sulfation patterns of chondroitin sulfate in the tissues of fish

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Chondroitin sulfate (CS) is commercially used in health food supplements and medications. One major source of CS is the cartilage of shark, however, indiscriminate fishing has led to the protection of sharks by international treaties. The other main source of CS is porcine aorta, but the pig is an intermediary host of the influenza virus which can infect humans. In spite of the higher price and the limited supply, the demand for CS is still increasing. It is necessary to find alternative sources for CS. To avoid infectious diseases and save protected animals, we have turned our attention to marine species. The total catch of the fish in 2009 was almost 145 million tons (JAICAF 2010) including farmed fish. Moreover, non-targeted species such as deep-sea fish and under-sized individuals are usually not used. In addition, the inedible parts of fish, estimated to account for almost half of a catch's weight are also thrown away. We have already reported the amounts and sulfation patterns of CS in several tissues of the diamond squid (*Thysanoteusis rombus*). Based on the analytical strategies we isolated CS from the inedible parts of several kinds of fish including pacific bluefin tuna (*Thunnus orientalis*), yellowfin sole *(Pleuronectes asper*) and deep-sea fish such as eelpouts. We show the amounts and the compositional analysis of CS including sulfation patterns of CS derived from the fish.

Structural and Chemical Glycobiology

Keynote Lectures

277. Structure-function studies of glycans using mass spectrometry

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The analysis of carbohydrate and glycoconjugate structures and their interactions with proteins are core to modern glycomics. Mass spectrometry (MS) is one of the most useful analytical tools in glycomics because of high sensitivity and minimal requirements for labelling of probes. We have been interested in developing MS –based methodologies for structure-function studies of glycans with focus on two aspects: (i) application of MALDI-Tof MS for the label free analysis of glycan binding proteins on glycan arrays; (ii) determination of three-dimensional structure of carbohydrates with the aim to develop *de novo* sequencing protocols using MS-based techniques.We have recently reported a method for the sequence determination of oligosaccharides, which includes ion mobility MS for three dimensional structure determination. This sequencing method allows us to determine the identity of each sugar building block at high resolution after in-source fragmentation, thus opening up the way for generic sequencing strategies. This technology has now been expanded to allow us to distinguish between the two anomeric configurations thus completing the full stereochemical structural analysis of glycan oligomers and conjugates.

278. Novel approaches for MS-based *N*-glycoproteome and *N*-glycome analysis

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MS-based glycoproteomics and glycomics are challenging because naturally occurring glycoproteins are often present at low levels, and the levels of the attached glycans are even lower. To sensitively characterize the glycoprotein by MS, we have developed series of new strategies to selective enrich the glycopeptides based on boronic chemistry, hydrazide chemistry, reductive amination reaction and oxime click reaction. For example, we explored alternative reactions that can specific performed between glycoproteins and solid phases including reductive amination and oxime click reaction. With the use of a newly synthesized aminooxy-functionalized magnetic nanoparticle, the oxidized glycan chains on glycopeptides readily react with the aminooxy groups through oxime click reaction, resulting in the highly selective extraction of glycopeptides. Compared to the traditional hydrazide chemistry-based method, which takes 12–16 h coupling time, this new method rendered excellent enrichment performance within 1 h. Furthermore, the enrichment selectivity (extracting glycopeptides from mixtures of non-glycopeptides at a 1:100 molar ratio), and reproducibility (CVs<20 %) were also dramatically improved. To selective enhance the signals of glycans in MS, we proposed a brand new matrix (HYNIC) to realize highly sensitive and selective analysis of glycans (amol) in MALDI-MS. Recently, we developed a general and simple labeling method, termed glycan reductive isotope-coded amino acid labeling (GRIAL), for MS-based quantitative *N*-glycomics. Using isotope-coded amino acid as the label,

the GRIAL exhibited a good CV(<10 %) and a high correlation coefficient (R^2=0.99) within 2 orders of magnitude within the dynamic range. In summary, we have developed novel strategies that enable the highly sensitive and specific MS analysis of the glycoproteome and the glycome.

Lectures

279. Live-cell bioorthogonal Raman imaging of glycans

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Visualization of glycosylation dynamics is essential for dissecting the biological functions of glycans. For the past two decades, the bioorthogonal chemical reporter strategy has emerged as a powerful tool for labeling and fluorescent imaging of glycans in live cells. However, this method relies on chemoselective reactions (*e.g.,* click chemistry) to attach fluorophores onto the chemical reporters for visualization. Developing bioorthongal chemistry remains an ongoing challenge and it is currently an active filed of research. Herein, we present an alternative strategy, which bypasses the requirement of chemical reactions by exploiting Raman spectroscopy and microcopy. Termed bioorthogonal Raman imaging, our strategy metabolically incorporates small Raman reporters, which possess Raman siganals that do not overlap with the naturally existing biomolecules in a cell, into glycans. The Raman reporters are then visualized by surface-enhanced Raman scattering (SERS) microscopy or stimulated Raman scattering (SRS) microscopy. Furthermore, the bioorthaonal Raman imaging strategy has been expanded to image other biomolecules including nucleic acids, proteins, and lipids. In conclusion, live-cell bioorthongal Raman imaging is an attractive new microscopy beyond the fluorescence imaging and label-free imaging.

280. Lubricin and how its carbohydrate protein and protein protein interactions provide its funtionallity

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Purpose: Biolubrication is key for sustaining the mobility of joints. The consequence of faulty biolubrication is pronounced in pathological conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA), where degradation of the joint is exacerbated by defect of the lubricating superficial layer on the cartilage. For OA and RA, molecular investigation of this layer and its molecules in healthy and disease conditions is crucial. We have characterised the heavily glycosylated mucin like lubricin in the synovial fluid using proteomic and glycomic techniques. Lubricin was found to be associated with extracellular matrix (ECM) proteins of joint tissue and this association was verified *in vitro* using recombinant protein constructs of lubricin and its identified binding partners. Immunohistological staining was also used to identify the specific staining of lubricin to cartilage (protein interaction) and to synovial neutrophils (carbohydrate interaction). Our data suggest that part of lubricin becomes linked to the Cartilage Oligomeric Matrix Protein (COMP) via covalent and noncovalent interaction. This association to a cartilage located protein explains how lubricin can provide lubrication even under high stress conditions in a healthy joint by its specific interaction to cartilage. This organization allows the glycosylated mucin domain of lubricin to generate a friction free joint surface. Analysis of oligosaccharides from OA patients suggests that there are pathological changes that could influence the lubrication property. Also, identification of complex oligosaccharides present in healthy and diseased state indicates that its glycosylation may have additional function as an immune regulator. The data suggest that the mechanisms for localization of lubricin to synovial surfaces provide insight into transformation from a healthy state to pathological state, including also changes of the glycosylation that would directly link to the pathology found in OA and RA

281. Structural characterization and membrane binding properties of MGD1, the major galactolipid synthase in *Arabidopsis*

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Galactolipids, such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are a unique lipid class ubiquitously found in photosynthetic organisms, from cyanobacteria to land plants. They are essential to the biogenesis of plastids and photosynthetic machinery. MGDG synthesis is catalyzed in a single step by a MGDG synthase (called MGD), which transfers a galactosyl residue

from UDP-galactose to diacylglycerol (DAG). MGD1 is the major galactolipid synthase in *Arabidopsis* and is essential for the massive expansion of membrane thylakoids. It is a monotopic protein localized in the inner envelope membrane of chloroplasts. Its product MGDG is the substrate for DGDG synthesis which is catalyzed by DGD1, located into the outer envelope membrane. The catalytic domain of MGD1 has been successfully expressed and purified as an active and soluble form into *E. coli*. The crystal structure of the catalytic domain of MGD1 has been obtained free and in complex with UDP. MGD1 displays the canonical GT-B fold consisting into two distinct Rossmann-type domains. Exploitation of the active site gives insight into residues critical for binding UDP-Gal and clues for DAG recognition. Using a Langmuir membrane model, which allows tuning of both lipid composition and packing, we investigated the membrane binding properties of MGD1. MGD1 presents a high affinity to the substrate DAG but surprisingly, also to its product MGDG, which maintains the enzyme bound to the membrane. MGD1 harbors a long and flexible region (~50 amino acids) which was shown to contribute to the anchoring of the enzyme into the lipid bilayer and to the capture of its DAG acceptor.

282. Tau-derived glycopeptides as novel inhibitors of amyloid aggregation

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Harmful amyloid oligomers and fibrils of certain disease-specific proteins are the hallmarks of various neurodegenerative diseases, *e.g.* Aβ and tau in Alzheimer's disease (AD). Inhibiting the misfolding and aggregation of these proteins is an attractive strategy for developing disease modifying therapeutics for these maladies. In this study we rationally designed glycopeptides as novel inhibitors of tau aggregation. In this strategy the "two-component" aggregation inhibitory molecules contain (i) the peptide backbone as an aromatic core that would target and bind the aromatic residues in the amyloidogenic monomer, thus confering target specificity, and (ii) sugar moieties that would provide steric hindrance component thus preventing aggregation of the target protein. Sugars were chosen as steric hindrance motif since sugar moieties on proteins have been shown to promote correct folding, and prevent aggregation, of the glycosylated proteins. Capitalizing on the specificity of peptides to bind target proteins we have synthesized various glycopeptides that target specifically the hexa-peptide core domain of tau (PFH6) and have shown their ability to inhibit its aggregation *in vitro*. In the future we shall assess the ability of the novel glycopeptides to disassemble preformed PHF6 amyloid fibrils. Feeding the various inhibitory glycopeptides to available transgenic *Drosophila* over expressing human tau, which serve as an established model of AD, will allow to examine their ability to reduce tau aggregation in the treated flies and to ameliorate their AD-related symptoms.

283. Recent applications of the inverse-electron-demand Diels-Alder reaction in glycobiology

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Bioorthogonal chemical ligation reactions, such as the Staudinger-Bertozzi ligation and azide-alkyne cycloaddition (click chemistry), have experienced an increasing popularity in recent years. More recently, the inverse-electron-demand Diels-Alder (DAinv) reaction (also known as tetrazine ligation) has found application. Advantages of this reaction are its speed, irreversibility, and independence of toxic metal catalysts. In addition, the DAinv reaction can be orthogonal to the azide-alkyne cycloaddition, enabling dual-labeling strategies. Presented here is the application of the DAinv reaction for the live-cell labeling of cellular carbohydrates. Metabolic glycoengineering relies on the promiscuity of the enzymes involved in glycan biosynthesis and offers the possibility to introduce carbohydrate residues with a non-natural functional group acting as dienophile in a DAinv reaction into cellular glycoconjugates. Employing *N*-acylmannosamine derivatives containing terminal alkenes, we labeled cell-surface sialic acids by reaction with electron deficient 1,2,4,5-tetrazines. Methylcyclopropene tags undergo much faster DAinv reactions and can be labeled within only 15 min. Combining DAinv and click chemistry, we could achieve dual labeling of two different metabolically incorporated sugars in a single step. Metabolic glycoengineering is a powerful tool to incorporate unnatural sugars into the whole glycome. To detect glycosylation of specific proteins, we employed fluorescence lifetime imaging microscopy (FLIM) of EGFP-tagged and metabolically labeled proteins exploiting fluorescence resonance energy

transfer (FRET) between EGFP and the labeled glycan. Application of the DAinv reaction was key to visualize protein-specific glycosylation inside living cells for the first time.

284. Single-entity heparan sulfate glycomimetic clusters for therapeutic applications

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Heparan Sulfate (HS), a highly sulfated glycosaminoglycan, plays a crucial role in a range of essential physiological processes. Functions of HS depend on ionic interactions between negatively charged sulfates and carboxylate groups with a variety of proteins such as cytokines, growth factors, lipases and proteases. HS oligosaccharides can mimic or interfere with HS functions in biological systems but exploitation has been hindered by the complexity of their synthesis. The complex synthesis of HS octa- to dodecasaccharides has been investigated by a number of groups, including ours, but despite many useful modifications and improved glycosylation protocols, multi-step syntheses of HS targets remain cumbersome and costly. Polyvalent displays of small specific HS structures on dendritic cores offer more accessible constructs with potential advantages as therapeutics, but the synthesis of single entity HS polyvalent compounds has not previously been achieved. Here we report the synthesis of a novel targeted library of single entity glycomimetic clusters capped with varied HS saccharides. They have the ability to mimic longer natural HS in their inhibition of the Alzheimer's disease protease BACE-1. We have identified several single entity HS clusters with low nM IC50 potency. None displayed any measurable ability to accelerate antithrombin-III mediated inactivation of Factor Xa and had no anticoagulant activity. Unlike heparin, such synthetic compounds would thus be expected to have no significant side effects related to anticoagulation. These have also been checked for *ex vivo* activity in a mouse brain slice assay which replicates many aspects of the *in vivo* context, crucially including bioavailability. Using an *in vivo* model, we further demonstrated the passage of C14-labeled clusters of HS through the blood–brain barrier. These novel HS clusters offer a novel framework for the manipulation of HS-protein interactions in general.

Posters

285. Synthesis and biological activity of inositol phospholipid as NKT cell modulator

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Entamoeba histolytica membrane has lipopeptidophosphoglycan (EhLPPG), which has glycosylphosphatidylinositol (GPI) anchor type glycan structure and also contains inositol phospholipid moieties, EhPIa and EhPIb. The inositol phospholipids have been shown as natural killer T (NKT) cell stimulators and induced selective IFN-γ but not IL-4 production via CD1d restricted manner. Because of these biological activities and the unique structures of EhLPPG, especially at the inositol phospholipid moieties EhPIa and EhPIb, with having characteristic long-chain fatty acids, we synthesized EhPIa,b and also the molecules with the sugar moiety to clarify the detailed biological activities. In the present study, we developed new synthetic methods including regioselective phosphorylation reaction of myo-inositol and Ni catalyzed sp3-sp3 cross coupling reaction for the synthesis of the long-chain fatty acids. In order to achieve the total synthesis of these unique inositol phospholipids, we also adopted a new protecting group strategy utilizing Allyl and Alloc groups as the permanent protecting groups for the hydroxyl groups in the inositol. These protecting groups can be cleaved at the final step of the total synthesis with Ru or Pd complex highly effectively. Based on these newly developed methods, we succeeded in the first total syntheses of EhPIa, EhPIb and GlcN-EhPIb. With utilizing these structurally-established synthesized compounds, the NKT cell stimulatory activities in murine and human systems were investigated. In murine system, selective IFN-γ induction was obtained in the NKT cell stimulatory activities.

286. Characterization of O-antigen based-GMMA vaccines against nontyphoidal *Salmonella*

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Nontyphoidal *Salmonella* (NTS), which include *S. typhimurium* and *S. enteritidis*, are the leading cause of bacteraemia in many parts of Africa. Currently, there are no licensed vaccines against NTS and efforts are ongoing to identify protective antigens and best strategies for vaccine development. Lipopolysaccharide (LPS) has been implicated as a target of the protective immune response and the serovar-specific O-antigen chains (OAg) of LPS have been employed in subunit vaccines. The Vaccine Institute for Global Health is working on the development of Generalized Modules for Membrane Antigens (GMMA) as an innovative delivery system for OAg of enteric bacteria. Advantages of this approach over traditional glycoconjugate approaches include simplicity and low-cost of production, co-delivery of multiple *Salmonella*-specific proteins, and a potential self-adjuvanting effect due to the presence of innate signaling molecules. By genetically manipulating the bacteria, the level of GMMA shedding can be greatly enhanced. Reactogenicity can be reduced by genetic detoxification of the lipid A moiety of LPS. A key question that may influence the effectiveness of NTS GMMA vaccines is whether OAg derived from GMMA is the same as that found on wild type bacteria. In the present work, OAg purified from *S. typhimurium* and *S. enteritidis* wild-type strains were compared to OAg extracted from different mutated strains, by using analytical methods including colorimetric assays, high performance size exclusion chromatography, high performance anion exchange chromatography with pulsed amperometric detection, gas chromatography, NMR spectroscopy and mass spectrometry. It was verified that mutations introduced can affect the amount and length of the OAg produced, while the overall structure and composition of the sugar chains remains unchanged. Other studies on GMMA size, protein and lipid A content are being performed, in order to fully characterize these new NTS candidates vaccine.

287. Discrimination of epimeric glycans and glycopeptides using IM-MS and its potential for carbohydrate sequencing

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Mass spectrometry is the primary analytical technique used to characterize the complex oligosaccharides that decorate cell surfaces. Monosaccharide building blocks are often simple epimers, which when combined produce diastereomeric glycoconjugates indistinguishable by mass spectrometry. Structure elucidation frequently relies on assumptions that biosynthetic pathways are highly conserved. Here, we show that biosynthetic enzymes can display unexpected promiscuity, with human glycosyltransferase pp-alpha-GanT2 able to utilize both uridine diphosphate *N*-acetylglucosamine and uridine diphosphate *N*-acetylgalactosamine, leading to the synthesis of epimeric glycopeptides in vitro. Ion-mobility mass spectrometry (IM-MS) was used to separate these structures and, significantly, enabled characterization of the attached glycan based on the drift times of the monosaccharide product ions generated following collision-induced dissociation. Finally, ion-mobility mass spectrometry following fragmentation was used to determine the nature of both the reducing and non-reducing glycans of a series of epimeric disaccharides and the branched pentasaccharide Man3 glycan, demonstrating that this technique may prove useful for the sequencing of complex oligosaccharides.

288. The capsular polysaccharide Vi from *Salmonella typhi*: synthesis and molecular dynamic simulations of short analogue fragments

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Vi capsular polysaccharide (Vi antigen) is the virulence factor of *Salmonella enterica serovar typhi*, the causative agent of typhoid fever in humans. It is a linear homopolymer made up of α-(1→4)-linked *N*-acetyl galactosaminuronic acid with a 60–70 % *O*-acetylation at C-3. Vaccination with purified Vi antigen from *Salmonella typhi* can protect against typhoid fever, although many aspects of the mechanism of action have yet to be established. It has been demonstrated that the immunogenicity of Vi is strongly related to the content of *O*-acetyl groups and seems not related to the presence of the carboxyl groups. In fact, the acetate groups dominate the molecular surface of the polysaccharide and confer hydrophobic properties to it, probably shielding the carboxylic groups from interaction with other molecules, even if only partial 3-*O*-acetylation seems necessary to maintain the flexibility of the molecules. Being interested in the study of the role of the negative charge of the Vi biopolymer on the biological activity, we

have planned the preparation of analogues where the carboxylic group has been substituted with a pH-independent ionizable group, *i.e.* the sulfate group. The sulfate group has been selected after preliminary investigations through molecular dynamics simulations on a hexasaccharide Vi antigen fragment, that showed similarities between the conformational behavior of the natural antigen and the sulfate analogue, where the galacturonic residues have been replaced with 6-*O*-sulfogalactoses. Herein we will report our results on the conformational analysis and the stereoselective synthesis of Vi antigen sulfated-analogue fragments.

289. Study of the *O*- and *N*-glycosylation profiles in cancer cell lines by modulation of the *N*-acetylglucosamine-based cellular pathways

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Cancer cells are characterized by metabolic alterations in particular intracellular pathways that should be exploitable in diagnosis and therapy of cancer. A key role in the development and in the maintenance of the proliferative aptitude of cancer cells is known to be associated to a disregulation of *N*- and *O*-glycosylation patterns of proteins that control the normal functioning of the cells. *N*- and *O*-glycosylations are fundamental post- and co-translational modifications that occur on many proteins; these modifications can actively participate in several aspect of the transformation of cells into their tumor phenotype. In *N*- and *O*-glycosylation, a crucial role is associated to the *N*-acetylglucosamine (GlcNAc) and the biochemical pathways that lead to its introduction as modifications in proteins and enzymes, such as proteins that control cell growth, apoptosis, cellular differentiation and proliferation (*O*-GlcNAc), or membrane transporters and receptors (*N*-glycosylation). In this context, our work is focused on the study of the cellular processes that lead to altered levels of *O*-GlcNAcylation in cancer cels, with a particular attention towards the Hexosamine Biosynthetic Pathway (HBP), that is at the basis of *O*-GlcNAc modification. We are focusing our study on the behaviour of this pathway in cancer cell lines by means of its positive or negative modulation, exploiting a chemical biology approach. In particular, we are generating synthetic analogues of HBP intermediates which can act as inhibitors or activators of enzymes of the pathway. Exploiting cell biochemistry methodologies, we are studying the effect of the administration of these compounds on different cancer cell lines, by means of changes in *N*- and *O*-glycosylations exploiting. Moreover, we are determining the ability of the synthesized compound to inhibit or activate the HBP by *in vitro* enzymatic assays or by *in silico* docking of the designed structures to our target enzymes.

290. More than just oligomannose: *N*-glycomics of a *Penicillium* species

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In the filamentous fungi, *N*-glycans are present on a range of secreted and cell wall proteins; but most of them are uncharacterized and their function is poorly understood. In this study, we analysed the *N*-glycans released by peptide:*N*-glycosidase F from proteolysed cell pellet of one *Penicillium* species (*P. dierckxii*, which belongs to the *Eurotiomycetes*). Although the major structures found are in the range of $Hex_{5\text{-}10}HexNAc_2$ as shown with mass spectrometry, a second series of *N*-glycans with a modification of 123 Da could be detected. Using HPLC the *N*-glycans were sub-fractionated in order to facilitate MALDI-TOF MS analysis of isomeric structures or glycans of different types. Furthermore, hydrofluoric acid and exoglycosidase treatments were performed in combination with MS/MS of original and digested glycans in order to propose structures. Thereby oligomannosidic glycans could be determined that were modified with outer chain och1-dependent mannosylation but also with the zwitterionic moiety phosphoethanolamine. These data are the first to show this kind of modification on the *N*-glycans of filamentous fungi. Furthermore, our results indicate that mere mass spectrometric screening is insufficient to reveal the subtly complex nature of *N*-glycosylation of a fungal species.

291. Evolutionary analysis of *O*-GlcNAc transferase using the evolutionary trace method

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O-GlcNAcylation is a one of glycosylation that is catalyzed by *O*-GlcNAc transferase (OGT). Human OGT (hOGT) consists of two domains: a catalytic region and tetratricopeptide repeat (TPR) region containing more than 10 TPR units, which interacts with other proteins. OGT activity has been reported in Metazoa and Viridiplantae, but not in Monera. This study was conducted to investigate the acquisition of OGT function

across taxonomic ranks using evolutionary trace (ET) method, which predicts protein functional sites by identifying of evolutionarily conserved amino acids and mapping them on 3D structures. We predicted OGT functional domains and compared them among representatives of different phylogenetic groups such as *Homo sapiens*, *Arabidopsis thaliana* (AtOGT), *Aureococcus anophagefferens* (AaOGT) and *Prochlorococcus marinus* (PmOGT). Three-dimensional structure of hOGT was used for structure homology modeling of OGTs from other species. OGT activity was confirmed in *H. sapiens* and *A. thaliana*. ET analysis revealed that the amino acid sequence of UDP-GlcNAc binding site was not evolutionarily conserved among the tested species: therefore, these sites in different OGTs compared by steric, electrostatic and hydrophobic properties. These analyses revealed that UDP-GlcNAc binding sites were similar in hOGT and AtOGT and that in AaOGT, contrary to hOGT, the site was negatively charged. These findings and the fact that OGT-like protein was identified in the cyanobacteria *P. marinus* suggest that OGT evolved by divergent evolution from a common descent. Although the evolution of *H. sapiens* and *A. thaliana* took different directions, UDP-GlcNAc binding sites in hOGT and AtOGT were similar, indicating their origin through common descent. At the same time, AaOGT, which was likely not bind UDP-GlcNAc because its UDP-GlcNAc binding site and UDP-GlcNAc were negatively charged, may hayve a different biological function.

292. Conformational impacts of *O*-glycosylation on human Notch1 EGF12 Glycopeptides

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Notch signaling pathway has key roles of cell developing process. Notch protein contains 36 epidermal growth factor (EGF)-like repeats on extra cellular domain. Each EGF domains have conserved three disulfide bonds, these position is C1-C3, C2-C4 and C5-C6. Many EGF domains are glycosylated by *O*-fucose [mature form is Neu5Acα(2→3)Galβ(1→4)GlcNAcβ(1→3)Fucα1→Thr] and *O*-glucose [Xylα(1→3)Xylα(1→3)Glcβ1→Ser]. *O*-Fucosylation of EGF12 is one of the most important modification to Notch signaling activities because glycosylation pattern of this *O*-glycan regulates binding affinity with their ligands. However, the detail functions of *O*-fucosylation as well as *O*-glucosylation are still unclear. Previously, we reported chemical synthesis and NMR structural analysis of mouse EGF12 peptide carrying *O*-fucosyl glycans, and demonstrated that local peptide conformation was altered by glycan elongations. In the present study, our attention is focused on human EGF12 and impact of *O*-glucosylation in addition to *O*-fucosylation. Here, we present chemical synthesis of human Notch1 EGF12 glycopeptides carrying *O*-fucose- or *O*-glucose-type glycans. In disulfide bond formation of *h*EGF12 analogues, we revealed that reaction proceeded efficiently in the presence of calcium ion in redox buffer due to stabilization of their native conformation. NMR study of synthesized EGF12 glycopeptide analogues demonstrated that *O*-fucosylation could stabilize β-sheet conformation of EGF12, and *O*-glucosylation might enhance the peptide solubility by shielding hydrophobic part of EGF12 glycopeptides. These glycosylation effects on peptide conformation might be important for Notch signaling pathway.

293. Structural and functional study of POMGnT1 related glycopeptide

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Muscular dystrophy is caused by genetic disorder to progresses muscle atrophy and muscle weakness. Deficiency of *O*-mannosylated glycan synthesis on α-dystroglycan (α-DG), a cell surface protein of neural and muscular cells, is a cause of muscular dystrophy to reduce binding activity with an extracellular protein, laminin. Recently, it is revealed that phosphated *O*-mannosyl glycan is required for the laminin-binding activity of α-DG. Deficiency of protein *O*-mannose β-1,2-*N*-acetylglucosaminyltransferase (POMGnT1), a causative gene for muscle-eye-brain disease, induce abnormal synthesis of the phosphated *O*-mannosyl glycan. However the controlling mechanism of the glycan synthesis is still unknown because the *N*-acetylglucosamine (GlcNAc) residue catalyzed by POMGnT1 is not involved in the phosphorylated glycan sequence. To elucidate the function of the GlcNAc residue catalyzed by POMGnT1, we constructed a glycopeptide library of the mucin-like domain of human α-DG bearing POMGnT1 modified-type *O*-mannosylated glycans at Thr residue. The glycopeptides displayed microarray allowed us to find a glycopeptide which interact with laminin very weakly. Conformational study of the selected glycopeptides by NMR showed that the introduction of the GlcNAc residue catalyzed by POMGnT1 induce conformational changes of the peptide backbone. This result indicates that the POMGnT1 works as conformational modulator to activate phosphorylated *O*-mannosyl glycan synthesis.

294. Biological significance of endomannosidase activity in the endoplasmic reticulum

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Newly synthesized glycoproteins with $Glc_1Man_9GlcNAc_2$ (G1M9) glycan are folded to form functional structures through calnexin/carleticulin (CNX/CRT) cycle. Even though about 30 % of newly synthesized glycoproteins are introduced into degradation pathway, the mechanisms that release these proteins from CNX/CRT cycle remain unclear. Recently, we established an *in vitro* quantitative analysis system to evaluate *N*-glycan processing in the endoplasmic reticulum (ER) using synthetic fluorescent G1M9 glycan that includes BODIPY on the reducing terminus (G1M9-BODIPY). Using this system with an ER fraction from rat liver in the presence of both inhibitors for glucosidases and mannosidases, we surprisingly found an endomannosidase (EM) activity that hydrolyzed G1M9-BODIPY to form M8A-BODIPY and glucosylmannose (Glc-Man). No one reported the EM activity in the ER but in the Golgi apparatus. Then we investigated the EM activity focusing on the pH dependence and the glycan specificity, indicating that the EM activity in the ER (ER-EM) differs from that in Golgi apparatus. Furthermore, we analyzed the ER-EM activity using a specific substrate for Golgi-EM, Glc-Man-4-methylumbelliferone. The substrate was digested in a fraction that included Golgi apparatus, while not in the ER fraction. In the clear contrast to this, the substrate was digested in the ER fraction in the presence of a hydrophobic molecule, Fmoc-glycine. These results suggest that ER-EM is regulated allosterically with hydrophobic molecules such as unfolded glycoprotein that exposes hydrophobic regions. In addition, glycoprotein with M8A glycan generated by ER-EM is neither re-glucosylated by UGGT (UDP-Glc:glycoprotein glucosyltransferase) nor recognized by CNX/CRT in the ER. Taken together, our findings suggest that ER-EM will play important roles for triaging glycoproteins depending on the hydrophobicity and for releasing unfolded glycoproteins from CNX/CRT cycle.

295. A convergent synthesis of branched inner-core oligosaccharides of Neisserial LOS and their biotin conjugates

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Lipopolysaccharides (LPSs) and lipooligosaccharides (LOSs) are important surface antigens for a wide variety of gram-negative bacteria. LPS and LOS consist of a lipid A and a polysaccharide (PS) or an oligosaccharide (OS). The core OS moiety of LPS and LOS consists of a structurally variable region and a conserved inner-core OS and has immunogenicity. 3-Deoxy-D-manno-oct-2-ulosonic acid (Kdo) is a major component of the inner-core OS. We have reported the syntheses the preparation of 2–4 and 2–8 linked Kdo disaccharides from a common Kdo derivative prepared D-mannose and 4,5-branched trisaccharides by using glycosylation of the 5-OH group of the 2–4 linked Kdo disaccharide. Here, we wish to report the synthesis of branched inner-core OS, penta- and tetrasaccharides of Neisserial LOS containing 4,5-branched Kdo structure by a convergent approach and the preparation of their biotin conjugates. Branched inner-core penta- and tetrasaccharides were successively synthesized by [2+2] or [3+2] approaches. The acceptor, 2–4 linked Kdo disaccharide was prepared by reported procedure. The tri- or disaccharide donors [Gal(β1-4)Glc(β1-4)Hep, Hep(α1-3)Hep] were coupled with above Kdo acceptor at 5-OH position with alpha selectively in moderate yield. Deprotection of isopropylidene group and followed by alkaline hydrolysis afforded corresponding penta-and tetrasaccharides in moderate to good yield. The reducing allyl group was attached with methyl thioglycolate under UV irradiation and resulted methyl esters were converted into hydrazine amide. Coupling of the spacer compounds with Biotin-sulfo-OSu gave the corresponding biotin-OS conjugates.

296. Selectivity of lectin interactions with new glycomimetics based on calixarene scaffold

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Reversible interactions between oligosaccharide chain of glycolipids/glycoproteins and carbohydrate-binding protein receptors (lectins) mediate critical biological recognition on the cell level. The identification of lectin-carbohydrate interactions in human disease raises the possibility of the development of carbohydrate-based therapeutic products, but fundamental barriers to the development of such agents still remain: a) many lectins

exhibit overlapping selectivities for particular carbohydrate structures, b) carbohydrates themselves commonly bind their receptors with only weak monovalent affinity, and c) *O*-glycosidic linkages are accessible to both chemical and enzymatic degradation. To avoid these problems, we focus on the synthesis of a new class of glycomimetics having high stability and displaying glycocluster effect. The synthesis starts from 2-(glycopyranosyl)ethyl azides with D-galacto, D-manno and L-fuco configuration. For their subsequent connection to bis/tetrakis(propargyl) calix[4]arene derivative, we use Huisgen cycloaddition reaction. The affinity and selectivity of new glycomimetics were verified against lectins from pathogenic bacterial species *Pseudomonas aeruginosa*, *Burkholderia cenocepaciae* and others using SPR and ITC.

297. Study of the *O*-GlcNAc transferase reaction mechanism using the hybrid QM/MM simulations

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The inverting glycosyltransferase *O*-GlcNAc transferase (OGT) posttranslationally modifies a variety of proteins. The misregulation of *O*-GlcNAc-ylation is linked to a wide variety of diseases, so knowing its reaction mechanism is very significant. Known OGT structures and experimental biochemical data suggest several possible mechanisms. The first published structure by Lazarus and Walker suggested that the His498 residue acts as a catalytic base (Figure 1A). Later on, Schimpl *et al*. published a new set of crystal structures complemented by other biochemical data where the α-phosphate moiety of the UDP moiety was proposed as a catalytic base (Figure 1B). However, at the same time, Lazarus *et al*. published crystal structures and proposed a specific catalytic mechanism, a so called "proton shunting mechanism". In the present study, all experimentally proposed mechanisms were investigated at the DFT QM/MM level and with sophisticated theoretical approaches such as hybrid QM/MM DFT Carr-Parinello ab initio molecular dynamics combined with a string method for reaction path optimization to investigate which of the three proposed mechanisms might be the most probable.

298. Two independent pathways on mannose trimmings in the ER glycoprotein quality control

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Glycoprotein quality control system in the endoplasmic reticulum (ER) is necessary for proper folding of nascent protein, whereas about 30 % of them have been known to be essentially unfolded. To maintain cellular homeostasis, balance of the secretion and degradation of the glycoproteins should be regulated. $Man_8GlcNAc_2$ (M8) isomers, M8A/B/C, generated from $Man_9GlcNAc_2$ (M9)-protein are believed to be signals for the secretion (M8B) and degradation (M8A/C). Production of these signals might be mediated several mannosidase candidates. ER α-1,2-mannosidase I and ER degradation enhancing α-1,2-mannosidase-like protein (EDEM) are well studied as a origin of these functions, whereas those of precise substrate specificity are poorly understood to date. To understand regio selectivity of the mannosidase candidates, recombinant enzymes will be useful. Although, mannosidase activity of the recombinant EDEM has not been detected *in vitro*, we have performed detection of EDEM-like activity in the ER fraction with synthetic oligosaccharide substrate. In this study, we first explored selective inhibitor for the mannosidase candidates in the ER fraction to simplify the complex mannose-trimming pathways related to the glycoprotein sorting. With the selective inhibitor, then we examined mannose trimmings using synthetic substrate. Comparison of inhibitory activities for M8 production by various glycosidase inhibitors was carried out. As a result, we found 1-deoxymannojirimycin selectively inhibited the M8A/C production. Inversely, we surprisingly found high concentration of 1-deoxynojirimycin specifically inhibited the M8B production. With these complementary selective inhibitors, we examined glycan processing from M9 to M5, respectively. The resulting glycan analyses clearly showed that two independent mannose-trimming pathway will exist in the ER. This indicates that complete role sharing on glycoprotein sorting might be controlled based on regioselective mannose trimmings.

299. Total synthesis of *O*-mannosyl glycans and characterisation using label-free discovery array platform

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Complex sugars (glycans), glycopeptides, glycoproteins and glycolipids are essential to life and most abundant class of biomolecules on Earth. They occur either alone or covalently linked to proteins and lipids (glycoconjugates). This complexity translates to many roles and functions such as: energy stores and to maintain structural integrity, interactions between viruses, bacteria and the surfaces of mammalian cells and molecular recognition for intracellular trafficking. As these biomolecules mediate a host of biological roles, abnormal changes in their composition and amounts can result in a number of pathological conditions; a variety of cancers, acute pancreatitis and muscular dystrophies to name but a few. *O*-mannosylation has an important role in regulating the function of α-dystroglycan (α-DG), as defective glycosylation is synonymous with various phenotypes of congenital muscular dystrophies associated with neuronal migration defects. To understand the structure-function relationship, further detailed biological investigation will require synthetic methodology for the generation of pure samples of homogeneous oligosaccharides with diverse sequences. Herein, we describe the total synthesis of *O*-mannosyl glycans using a combination of chemical and enzymatic methods for their syntheses in solution and on-chip respectively. Utilisation of a label-free, highly sensitive, high-throughput MALDI-TOF MS glycan array platform has been employed to study the interactions and affinities of *O*-mannosyl glycans with a variety of glycan binding partners (GBP) including: lectins and whole cells *e.g.* bacteria. This GBP analyze by either the intact protein mass or, after on-chip proteolytic digestion, the peptide mass fingerprint and/or tandem mass spectrometry of selected peptides. The platform described herein is a valuable tool for studying complex and clinically relevant systems and also applicable to a wide variety of biopharmaceutical and biotechnology applications.

300. Identification of immunodominant epitopes on the O-antigen from *Shigella flexneri* serotype 3a by use of synthetic oligosaccharides

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Shigellosis, or bacillary dysentery, caused by the non capsulated enteroinvasive bacteria *Shigella*, is a major burden especially in the pediatric population living in developing countries. Species and serotype diversity added to geographical distribution and emerging antibiotic resistance strongly support the need for a multivalent vaccine. In particular, strains belonging to the species *flexneri* are the major pathogens responsible for the endemic form of the disease. Protection against re-infection is, to a large extent, achieved by antibodies specific for the O-antigen moiety of the bacterial lipopolysaccharide (LPS) and a number of LPS-based vaccines are being investigated. In recent years, vaccine candidates encompassing synthetic oligosaccharides mimicking the "protective" determinants carried by the LPS O-antigen moiety have been considered as a promising opening to potent, better standardized, *Shigella* LPS-based vaccines, including in our group. This presentation describes our multidisciplinary efforts toward the development of the aforementioned vaccines, with a particular emphasis on *S. flexneri* 3a, one of the most prevalent serotypes. The repeating unit of the *S. flexneri* 3a O-antigen is a branched pentasaccharide, *O*-acetylated at two sites. Here, the two-step strategy developed so far for the selection of the carbohydrate haptens toward the design of potent glycoconjugate immunogens will be summarized. Structural input and antigenicity data will be discussed. Along this line, our most recent understanding of the influence of *O*-acetylation and side-chain glucosylation on O-antigen:protective antibody recognition will be examined. In addition, efforts to develop efficacious synthetic routes to oligosaccharides featuring the identified epitopes will be highlighted.

301. Rational development and biological application of water-soluble fluorescent photochromic diarylethenes which have sugar substituents

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Photochromic molecules are molecules that reversibly transform between two isomers with different absorption spectra while maintaining the same molecular weight when irradiated with appropriate wavelength of light. Diarylethene compounds are known to exhibit excellent photochromic

performance. The diarylethene undergoes cyclization/cycoreversion reactions upon irradiation with light. Extensive studies have been carried out to apply the photochromic molecules as optical memory media that can optically store information. In such use, the media were prepared by dissolving photochromic molecules in an organic solvent, then spreading the resulting solution over a substrate. Recently, bioimaging using fluorescent microscope, which is a method to observe an image by binding fluorochrome molecules to biomolecules, has been actively studied. Bioimaging that employs green fluorescent proteins (GFP) is frequently used, but this method is disadvantageous in that the label molecule is large, and the protein-protein interaction affects the target biomolecule. Diarylethene is expected to achieve bioimaging with high resolution, since they are low molecular weight compounds. However, it is indispensable to provide water-solubility to the compounds for the application to biological samples. Under such situation, a highly water-soluble diarylethene compound obtained by a different means was desired. In this study, highly fluorescent photochromic diarylethene having various sugar substituent groups were rationally designed and synthesized in order to provide non-ionic water-solubility to photochromic diarylethene derivatives. The derivatives underwent reversible photochromism in aqueous solution as well as in methanol upon irradiation with ultraviolet and visible light. The fluorescence properties and water-solubility of these derivatives were examined. Application of these derivatives as fluorescent bioprobes to *Xenopus tailbud* was also performed.

302. It's better to bend than to break

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Herein we describe a molecular interaction between flexible seven membered ring (septanose) monosaccharides that deform upon binding to the rigid bacterial adhesin FimH to accommodate to the shape of the binding pocket. The interaction was firstly evaluated in a competitive binding assay and molecular docking experiments. Subsequent isothermal titration calorimetry (ITC) measurements, molecular dynamics (MD) simulations and NMR chemical shift perturbation (CSP) experiments revealed that the difference in affinity between n-heptyl α-D-mannoside and the corresponding septanose derivative was solely due to an induced conformational change (entropy cost) of the septanose ligand. Combining the different analytical methods considerably improved our understanding of FimH-Ligand interaction and will guide the design of therapeutically relevant FimH antagonists.

303. Sialic acid as a glyco-biomarker of seasonal variation in dairy cow

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Thermal comfort is one of the basic principles of dairy cows welfare, but there is no specific criteria to assess. *N*-glycans composition changes due to the physiology inside the body of organisms, caused by environmental changes. *N*-glycome data of dairy cow serum is scare. Meanwhile, there are two major kinds of sialic acids in cows used for stress diagnosis and discovery. One of them is *N*-acetylneuraminic acid (Neu5Ac) which mainly found in mammals; however the other is *N*-glycolylneuraminic acid (Neu5Gc) cannot synthesize in human but found in human cancers, fetal samples, and a dietary source of red meat consumption of cow. According to Japan meteorological agency database in Sapporo, 2012 dairy cows exposed lethally to heat stress particularly in summer season (August). Our strategy focused on SweetBlotTM, UltraflexIII, Glycomod analysis, and SPSS software to analyze *N*-glycans, then serum IgG purification by column separation, confirmed with SDS/PAGE, and using DMB reagent: (1,2-diamino-4,5-methylenedioxybenzene:2HCl), followed by HPLC to detect the amount of sialic acids in serum *N*-glycans. The results showed that AVG cows weight, milk yield kg/day, days in milk, and lactose%, fat%, protein% in milk were the lowest in summer "August" with $P<0.001$, 36 *N*-glycans listed, and we found: i) novel *N*-glycan structures seasonally; high-mannose was in spring with significance $P\leq0.001$, complex type- with (two NeuAc-group) was in winter with significance $P<0.001$, complex- with (three NeuGc-group) was in summer with significance $P<0.001$, and complex- with (one NeuGc-group) was highest expression in autumn but without significance. ii) bio-medical indicator of

environmental stress. iii) assessment of a novel criterion to detect the thermal comfort of cows. The data clearly indicated that sialylated *N*-glycans; as a biomarker of stress in dairy cows. Currently we analyze the serum IgG *N*-glycome, to enable us to create anti-NeuGc, as a therapy of heat stress in dairy cows.

304. Structural characterization of glycan binding properties of mucus binding protein (MUB) from *L. Reuteri* employing label-free glycoarrays

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Alterations in glycan and microbial composition may increase the susceptibility towards intestinal disorders, such as inflammatory bowel disease (IBD) and cancer. A better understanding of molecular interactions between gut bacteria and host mucins are needed to identify therapeutic targets and prevent glycan related diseases. *Lactobacillus reuteri* (probiotic) possesses a mucus binding (MUB) protein, which has been characterized as a cell surface adhesin. MUB consists of two different types of repeats (Mub1 and Mub2), which are tandemly arranged forming a 15 repeat structure, responsible for the intestinal mucus adherence. It is believed that the binding of MUB protein to mucus involves multiple interactions including specific binding to terminal sialylated glycans, however this interactions are not fully characterized. In order to elucidate the capability of MUB segments to recognize specific glycans, we have used a label-free glycoarray platform in which aminoethyl glycosides are covalently attached to a functionalised self-assembled monolayer (SAM) of alkanethiols on gold. Both glycan functionalization and glycan-protein interactions can be analyzed by MALDI-Tof MS. A range of recombinant Mub repeats (Mub R5, −RI, RV, −R8V, −RV-VI, and RI-II-III) and full-length MUB were tested for carbohydrate-binding properties. Interestingly a strong interaction between a type 1 repeat Mub R-I-II-III and Neu5Ac-α-2,6- linked glycan was observed. This interaction was significantly weaker with the α-2,3-linked analogue and no interaction to non-sialylated glycans was observed. Single repeats, Mub R5, −RI, RV or double repeats, −R8V, −RV-V displayed no affinity towards the tested glycans. The Mub R-I-II-III segment was fully characterized after "on-chip" trypsin digestion followed by MS(/MS) analysis. Here we have shown that MUB is able to target sialylated structures, providing significant insights regarding the specificity of this probiotic adhesin to recognise host glycans.

305. Label-free discovery array platform for the characterisation of glycan-binding bacteria

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Glycans are the most abundant class of biomolecules on Earth, being rarely simple molecules and this complexity translates to their many roles: energy stores, maintain structural integrity, interactions between viruses, bacteria and mammalian cell surfaces and molecular recognition for intracellular trafficking. Many bacteria initiate their attachment to host cells with glycan-lectin binding. Some pathogenic bacterial strains have been associated with alteration of the normal human gut flora, leading to several diseases (*e.g.* ulcerative colitis, sepsis etc.). Probiotic strains have been identified as useful in restoring normalcy of the bacterial floral domain in humans. A systematic study of bacterial strains might enable the identification of glycan motifs that serve as a common platform for bacterial invasion of both pathogenic and probiotic strains. To exploit the glycan-driven functions of bacteria, we must unambiguously elucidate their structure and glycan-binding affinities to be able to define structure-function relationships. Current state-of-the-art methodology includes real time PCR and microarrays, however, use of fluorescence tags for detection is a major drawback. Here, we report the use of label-free glycan-arrays with highly sensitive, high-throughput MALDI-MS. The platform can unequivocally identify bacteria (*eg. E. Coli*, *S. Uberis*, *L. Reuteri*, *Bifidobacterium* etc.), captured from complex sample mixtures. Identification of bacteria bound to the functionalised array is achieved by analysing either the intact bacteria or after on-chip proteolytic digestion. Peptide mass fingerprint and/or tandem MS of selected peptides, can yield highly diagnostic information. Further, principal component analysis can be used for classification of the bacteria without a priori information. The platform described herein is a valuable tool for studying complex and clinically relevant

systems and also for a wide variety of biopharmaceutical and biotechnology applications.

306. Characterization of bioactive glycan motifs on biotherapeutic glycoproteins with advanced MS technologies

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Recent advancements in biotherapeutic protein design have underscored the importance of glycosylation towards optimal drug bioactivity. The presence/absence of certain glycosyl modifications and structural motifs, as well as their abundances in a biopharmaceutical preparation can significantly affect drug immunogenicity and stability. However, the inherent structural diversity of glycosylation significantly hinders analysis. We employed structure-specific LC/MS and LC/MS/MS to rapidly screen and elucidate bioactive glycan motifs on biotherapeutic glycoproteins. Our analytical platform was applied to EPOs, mAbs produced by various cell-based expression systems, and enzyme treatment. Briefly, *N*-glycans were enzymatically released and purified by porous graphitized carbon (PGC) solid phase extraction. Native *N*-glycans were comprehensively analyzed by PGC chip based nanoLC/Q-TOF MS to obtain chromatographic isomer separation profiling and structural characterization. Accurate masses were used to compositionally annotate and profile the glycans, while tandem mass spectrometry using CID was further performed to confirm the composition and elucidate the structure of glycans. We were successful in identifying atypical and/or contaminant glycosylation on various types of biotherapeutic glycoproteins. First generation EPOs had generally less *O*-acetylated, whereas second generation EPOs showed high abundances *O*-acetyl modifications per sialic acids. Numerous NeuGc-sialylated glycans as well as galactose-α-1,3-galactose epitopes were also discovered in mAbs produced by mouse Sp2/0 cells (instead of CHO or HEK cells). Additionally desired glycosylation such as high mannose phosphorylation in α-galactosidase was readily identified. We have created a biotherapeutic-specific database including retention time, accurate mass, glycan composition, and unique fragment ion. The analytical platform and DB enables us to identify bioactive glycan motifs in a quick and efficient manner.

307. Synthesis and biofunctional study of immunomodulative lipopolysaccharide partial structures

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Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria, and known as endotoxin for its potent immunostimulating and inflammatory activities. Lipid A is the active principal of LPS and links to the polysaccharide part via the peculiar acidic sugar, Kdo. Recent studies have suggested several pathological implications of LPS from parasitic bacteria due to their influence on the host immune responses. To address this issue, we have synthesized the LPS partial structures of the parasitic bacteria, *Helicobacter pylori* and *Porphyromonas gingvalis* to evaluate their biological activities. In comparison with *E. coli* lipid A, lipid As of *H. pylori* and *P. gingvalis* have the distinctive several chemical structures. We thus established an efficient synthetic strategy that is widely applicable to the synthesis of various lipid As by using a key disaccharide intermediate with selectively cleavable protecting groups. For the synthesis of Kdo-lipid A, stereoselective Kdo glycosylation was effected by using *N*-phenyltrifluoroacetimidate under microfluidic conditions. All synthetic parasitic bacterial lipid As did not induce strong inflammatory responses. Lipid As having tri or tetra-acyl groups with 1-phosphate showed inhibitory activity against IL-6 and IL-8 induction of *E. coli* LPS, whereas triacyl lipid A with 1-ethanolamine phosphate, and penta-acyl lipid A with 1-phosphate showed weak inducing activity. On the other hand, all synthetic parasitic bacterial lipid As showed IL-18 inducing activity. Since IL-18 has been shown to correlate with chronic inflammation, parasitic bacterial LPS may be implicated in the chronic inflammatory. We have been then investigating immunoregulatory activity of LPS from symbiotic bacteria. The results will also be reported.

308. Insights into the impact of co- and post-translational modification by sugars on peptide folding from the study of a family of "glyco"-foldamers

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Considerable effort has thus been invested in delineating and understanding the impact of carbohydrates on the conformational preferences of glycoproteins and glycopeptides in solution and also in manipulating their interactions with their cognate receptors. These endeavors have revealed themselves not to be straightforward and success in rationalizing or predicting such processes has been possible only in a handful of well-studied cases. Important insights into such questions have been gleaned from the study of glycopeptide- and oligosaccharide-mimetics and especially those that are structurally well defined. However, attempts to correlate secondary structures with the biological activities of glycopeptide mimetics have been relatively sparse despite the importance of such targets in the quest for carbohydrate-based therapeutics. In the present work we sought to examine the effects of appended sugar moieties on the conformational behavior of a family of δ-sugar amino acid (δ-SAA)-derived foldamers. Foldamers are synthetic oligomers able to adopt ordered conformations in solution and their study has helped enlighten our understanding of the origins of the preferred secondary structures and biological activities of polymers prevalent in Nature. Considering the importance of glycoproteins we were struck by the absence of reports describing the impact of glycosylation or the absence thereof, on the preferred secondary structures of foldamers and how such preferences might impact on their biological activity. The study provides evidence that the secondary structures adopted by the various δ-SAA-derived foldamer are dependent on the identity of the sugar moiety appended to their backbones. Moreover, the observed conformational preferences are also mirrored in differences between their interactions with selected biological targets.

309. *N*-glycans of algae—surprises at the green outposts

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With regard to *N*-glycosylation, green plants are extremely conservative. Angiosperms such as mono- and dicotylidons, gymnosperms and even ferns and mosses furnish their glycoproteins with complex *N*-glycans sporting the very same structural features. The default plant *N*-glycans contain a xylose linked to the β-mannose of the core and an α-1,3-linked fucose linked to innermost GlcNAc of the core. Antennae—if present—form the Lewis A trisaccharide. Oligomannosidic *N*-glycans resemble those found in mammals. Investigations of the green alga *Chlorella pyrenoidosa* revealed the occurrence of an as yet undescribed type of *N*-glycan. The major glycan species in a MALDI spectrum displayed a mass as if it would embody a variation of the typical complex-type *N*-glycan of plants, *i.e*. the heptasaccharide MMXF3 with one additional pentose. The mass of the permethylated glycan, however, pointed at a glycan with three pentoses and no deoxyhexose. Contrary to our expectations, monosaccharide analysis revealed the presence of arabinose rather than xylose with one arabinose being methylated. Yet another unusual feature was the occurrence of galactose in an *N*-glycan containing only the two core GlcNAc and two mannose residues. ESI-CID-MS/MS of the permethylated glycan pointed at a linear structure of three hexoses carrying a linear chain of three pentoses on the middle hexose. Further permethylation and MS/MS analysis located the methyl group on the terminal arabinose residue. Our work on protein glycosylation in green algae suggests the existence of a completely novel type of *N*-glycans containing arabinose.

310. Characterization of bacterial lectins with novel seven-bladed beta-propeller fold

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Burkholderia pseudomallei and *Chromobacterium violaceum* are bacteria of tropical and subtropical soil

and water that occasionally can cause fatal infections in human. *Burkholderia pseudomallei* is connected to febrile illness Melioidosis which is endemic in East Asia and north Australia. *Chromobacterium violaceum* can cause sepsis and abscesses of liver and lung with high mortality. Due to unusual resistance of the both bacteria to antibiotics, the treatment is often very problematic and mortality rate of the infections is high. Microbial lectins are in many cases surface proteins and mediate the adhesion of organisms to host cells which is the first phase for the development of infection. The functional studies of carbohydrate-binding proteins should clarify their biological role in host-microbe interactions and could be used in potential anti-adhesive therapy of bacterial diseases. In this contribution we describe new lectins BP39L and CV39L from the bacteria *B. pseudomallei* and *C. violaceum*. The structure of the proteins was solved using X-ray diffraction and revealed seven-bladed beta-propeller fold with possibly seven binding sites per monomer. According to its structure and sequence, the proteins belong to a not yet described family of lectins. Surface plasmon resonance (SPR) and titration microcalorimetry (ITC) were used to characterize the interactions between the lectins and saccharides. The functional studies indicate the specificity of lectin BP39L towards D-mannose and CV39L towards L-fucose and L-galactose. This work was supported by the Masaryk University Program, project no. MUNI/A/1548/2014

311. Structural analysis of monosaccharide derivatives by vibrational circular dichroism

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The conformation of sugars governs their biological activity and chemical reactivity. However, there have been few methods that can elucidate the detailed conformation of sugars in solution. Here, we show the effectiveness of vibrational circular dichroism (VCD) spectroscopy to the structural analysis of monosaccharide derivatives. VCD measures the differential absorption of left- versus right-circularly polarized infrared radiation of sample molecules. Because all sugars exhibit infrared absorption, VCD can extract structural information from sugars, unlike conventional CD that measures the UV region. We have applied VCD spectroscopy to various monosaccharide derivatives, and have demonstrated that it clarifies their conformational ensembles. Some sugars were found to exist as a mixture of chair conformations and twist-boat conformations. We have also examined the validity of VCD technique to furanoses as well. Although it is sometimes difficult to determine the configuration and conformation of furanose, we have found that VCD spectroscopy is capable of determining both the structural properties simultaneously. Although applicability of this approach to oligosaccharide is yet to be examined, this work has proven the promiscuity of VCD technique to the structural analysis of sugars. In this paper, particular emphasis is placed on the structural analysis of glycosyl sulfoxides and several furanosides. Other examples of the use of VCD spectroscopy to glycoconjugates will also be presented.

312. Stable isotope-labeled aqua glycans for correct relative quantitation of glycans in LC-ESI-MS with porous graphitic carbon columns

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Structural analysis of isomeric glycans is a veritable challenge, which can be met by separation on porous graphitic carbon and detection with ESI-MS or ESI–MS/MS. One important reason for structural variation is sialylation, which leads to analytes with different numbers of charges. Together with large mass differences of various glycans this charge heterogeneity brings about that peak heights do not truly represent the molar ratios of glycans. For PGC-LC-ESI-MS, reduced but otherwise underivatized glycans are analyzed. Hence, stable isotopes cannot only be incorporated within the sugar constituents themselves. We have undertaken to label *N*-glycans by two different routes leading to stable isotope containing glycans suitable for relative and Absolute QUAntification akin to AQUA peptides. First, complex type AQUA glycans were generated with the help of UDP-^{13}C6-galactose that had been synthesized using galactokinase and galactose-1-phosphate uridyltransferase. This nucleotide sugar was used to enzymatically generate ^{13}C-labeled di- and triantennary *N*-glycans with no, one, two and three sialic acids and a mass difference to unlabeled glycans of 12 Da. The second approach made use of the cheap and easily available hydrazine

hydrate that served to de-acetylate reduced $Man_6GlcNAc_2$. Re-*N*-acetylation with perdeutero acetic anhydride yielded a Man_6 AQUA glycan with a mass difference of 6 Da. Positive and negative mode PGC-LC-ESI-MS of antibody and serum *N*-glycans demonstrated the requirement and usefulness of the addition of an equimolar mixture of the above mentioned AQUA glycans wherever true quantative ratios of glycan species are needed. Needless to say that AQUA glycans also serve to define the exact elution position of a particular glycan in a PGC-LC run and thus support the assignment of isomers.

313. Mannopyranosylthioureido calix[4]arenes in the cone geometry: a DFT modeling study

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Multivalent presentation of carbohydrates using suitable conjugation chemistry and scaffold selection may result in a better efficiency and a higher selectivity in the interaction with receptors such as lectins and immunoglobulines. Calix[n]arenes have been successfully used as scaffolds for multivalent presentation of glycosyl moieties. The smallest ones (n=4) have the lowest mobility and, if sufficiently steric demanding groups are present on their lower rim, stable conformational isomers exist that allow a controlled display of the saccharide units in the space. Several linkers have been exploited to connect sugars to calixarenes. When relatively rigid, they can heavily constrain the orientation options of the substituents and strongly affect their presentation. Several glycocalix[n]arenes have been reported where glycosyl moieties such as glucose, galactose, or lactose are linked as beta anomers to the upper rim through a thiourea unit. They have shown in some cases very interesting inhibition properties towards specific carbohydrate recognition proteins. Only one glycosylthioureidocalix[4]arene, with mannosyl moieties linked as alpha anomers, has been recently reported. We have investigated from a conformational point of view α- and β-D-mannosylthioureido calix[4]arene (1 and 2) both exposing mannosyl moieties but differing for the anomeric configuration. In this investigation the two potential multivalent ligands have been compared in order to evaluate the effects of the anomeric configuration on the presentation mode of the glycosyls. This in-depth theoretical study of 1 and 2 started with the choice of the suitable level of calculation and the evaluation of the properties of each monomeric component of 1 and 2. Then, after a deep investigation of the intrinsic conformational properties of cone-calix[4]arenes, the modeling of the whole macrocycles was carried out through a DFT approach.

314. Chemical approaches for connecting missing links in discrimination between folded and unfolded glycoprotein in the ER

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Glycoprotein oligosaccharide functions as a tag for glycoprotein quality control in the endoplasmic reticulum (ER). We have been working on functional analysis of the related glycan-processing pathway connecting with protein folding states. In spite of various efforts including us, how to distinguish between folded and unfolded glycoproteins through the glycan processings is still unclear. Our recent chemical approaches using synthetic glycoprobes revealed that folding acceleration of $Glc_1Man_9GlcNAc_2$ (G1M9)-protein by Calnexin/Calreticulin, the lectin like molecular chaperones, weakens the binding of Calnexin/Calreticulin to G1M9-protein and enhances glucosidase II mediated de-glucosylation to deliver the glycoproteins to proper secretion pathway. Furthermore, unfolded G1M9-protein was found to be specifically hydrolyzed the terminal glucosyl-mannose residues with ER-endomannosidase that has been discovered by us, contributing degradation of unfolded glycoproteins. Through these findings, we propose the novel notion that G1M9-glycan functions as an interfacial tag for glycoprotein-fate determination.

315. Comparative study of fucosylated chondroitin sulfates from four sea cucumbers species

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Anionic polysaccharides fucosylated chondroitin sulfates (FCS) isolated from sea cucumbers possess a wide spectrum of biological activities such as

anticoagulant, antithrombotic, anti-inflammatory, immunostimulatory. These biopolymers are built up of alternating→4)-linked β-D-glucuronic acid and→3)-linked *N*-acetyl β-D-galactosamine residues in a backbone. Some of glucuronic acid units are substituted at O-3 by side chains contained of sulfated α-L-fucosyl residues. It is known, that structures of branches vary accordingly with sea cucumber species and strongly influence the level of biological activity. In this communication the results of comparative study of FCS from four different sea cucumbers species are presented. Separation of FCS from other anionic polysaccharides was performed by ion-exchange chromatography. Then FCS were characterized in terms of monosaccharide content and degree of sulfation, as well as NMR analysis was performed. The level of anticoagulant activity of FCS was assessed in APTT and TT clotting assay using normal plasma. The ability of FCS to inhibit platelets aggregation was studied by turbidimetric analysis using platelet-rich plasma. The results indicated that level of biological activity was strongly depended on the structure of branches of FCS. The most potent effect was shown for the samples enriched by 2,4-sulfated α-L-fucose units. This work was supported by RSF grant 14-13-01325.

316. Structural and metabolic analyses of glycoconjugates in *Acanthamoebae* species

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Acanthamoeba is a eukaryotic microorganism (protozoa); while these amoebae are free-living bacterivores, some of them are opportunistic parasites that can cause severe infections such as amoebic keratitis and granulomatous amoebic encephalitis (GAE). This model organism is particularly interesting to study molecular host-pathogen interactions for several reasons: It is a free-living organism, it grows rapidly, the genomic data is available, it is human pathogen and there exist similarities with other protozoan parasites. For these reasons, we started the structural and metabolic analyses of glycoconjugates in *Acanthamoeba* focusing on glycoproteins (*N*-glycans and *O*-glycans) and glycolipids (lipophosphonoglycans). Our glycomic strategy is based on enzymatic or chemical release of glycans followed by high performance liquid chromatography (HPLC) and mass spectrometry (MS). We already discovered the presence of unusual substitutions on high-mannose like phosphorylation, methylation and core-xylosylation. For the metabolic analysis, we analysed the composition of nucleotide-sugars in *Acanthamoebae* species by HPLC and the activity of native enzymes after supernatant extraction on synthetic and natural substrate. Moreover, we are producing a set of recombinant glycoenzymes and have proved the biochemical function of the two enzymes required for GDP-fucose synthesis. Altogether, these pieces of information establish the basis for the structural glycobiology and the understanding of the metabolic pathways of glycoconjugates in *Acanthamoeba* species.

317. Rational design of mannac analogs as ligands for human Langerin

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Myeloid C-type lectin receptors (CTLR) are a family of pattern recognition receptors involved in pathogen defense and immune homeostasis. Many CTLRs represent potential drug targets *e.g.* in immunomodulatory or antiviral therapy. However, carbohydrate recognition typically is highly promiscuous and characterized by low affinities. This impedes targeting of individual CTLRs or a defined population of DCs. Moreover, targeting carbohydrate binding sites with small molecules is considered a challenging task, largely due to their hydrophilicity and high solvent exposure. Yet, rationally evolving carbohydrate scaffolds has led to the design of specific and potent ligands for CTLRs. The derivatization of carbohydrate scaffolds provides excellent three-dimensional diversity while key interactions formed by the natural ligand are maintained. Along these lines, we present a first approach to identify novel ligands for human Langerin, a Man-binding CTLR predominantly expressed on Langerhans cells. To this end, a structure-based in silico screening was implemented to compose a focused library of ManNAc analogs. The designed analogs were synthesized and their affinity was determined in a novel 19 F R2-filtered NMR-based competitive binding assay. The established structure-

activity relationship will be discussed with respect to the generated docking poses.

Society for Glycobiology (SFG) Lectures

Keynote Lectures

318. Insights into the enzymology and structural biology of mammalian glycosylation enzymes coming from large-scale eukaryotic expression platforms

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Glycan structures on cell surface and secreted glycoproteins play critical roles in biological recognition and targeting events in animal systems. The enzymes that synthesize these structures reside in membranes of the secretory pathway and modify glycoproteins in transit to the cell surface. These enzymes are generally poorly understood largely because they are challenging targets for functional expression even in eukaryotic hosts. We have established library of expression constructs encoding all human glycosylation enzymes (target gene list of ~350 coding regions) as secreted catalytic domain fusion proteins for recombinant production in HEK293 cells and baculovirus-infected insect cells. Comparison of expression levels in each host system indicates similar trends where most proteins are well-expressed, but a subset is efficiently secreted. This presentation will summarize our strategies for expression and downstream workflows for biochemical and structural studies. Discussion of resulting structures and insights into catalytic mechanisms and substrate recognition will focus on recently determined structures of sialyltransferases and fucosyltransferases coming from the mammalian expression platform. (supported by NIH grants P41GM103390 and P01GM107012 and BESC, a Bioenergy Research Center supported by the US DOE Office of Science.

319. Roles for glycans in mammalian development

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In order to define roles for glycans in the development of different cell types and tissues, we have developed mouse models that are globally or conditionally defective in a specific glycosyltransferase gene. Recently we have explored roles for complex *N*-glycans in spermatogenesis. We previously showed that when complex *N*-glycan synthesis is prevented by conditional deletion of Mgat1 in spermatogonia using Stra8-iCre recombinase, spermatogenesis is blocked due to the fusion of round and elongated spermatids. We also showed that, paradoxically, the testis contains a physiological inhibitor of MGAT1 termed GnT1IP-L that is expressed highly in spermatocytes during spermatogenesis. We hypothesize that MGAT1 and the GnT1IP-L inhibitor play complementary roles in modulating complex *N*-glycan synthesis during spermatogenesis. We have found that GnT1IP-L inhibits MGAT1 in the Golgi, and that inhibition is mediated by the luminal, and not the transmembrane, domain of GnT1IP-L. The activity of other GlcNAc-transferases of the medial Golgi is not inhibited by GnT1IP-L. Fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC) strategies have been used to investigate interactions between GnT1IP-L and MGAT1, MGAT2, MGAT3, MGAT4 or MGAT5 in the endoplasmic reticulum (ER) and Golgi. GnT1IP-L forms homomers in the ER, and specific heteromers with MGAT1 in the Golgi. Minimal interactions of GnT1IP-L with MGAT2, MGAT3, MGAT4 or MGAT5 were observed. The consequences of deleting or overexpressing the GnT1IP-L gene in male germ cells are currently under investigation. This work was supported by grants RO1 CA036434, RO1 GM105399 and the Mizutani Foundation to PS and funds from Oulu University to SK.

Lectures

320. Role of the conserved oligomeric complex (COG) and its partners in glycosylation in human cells

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Congenital disorders of glycosylation (CDG) are a rapidly growing family of rare diseases involving deficiencies in protein glycosylation. Glycosylation within the Golgi is crucial for normal function of all proteins in secretory pathway. The Conserved Oligomeric Golgi (COG) complex is a major player in recycling of Golgi enzymes. COG is composed of 8 subunits in two subcomplexes: lobe A (COG1-4), lobe B (COG5-8). COG defects are linked with CDG type II, which involves defects in the processing of *N*- and *O*-linked glycans. The major drawback of COG studies is a lack of knockout (KO) cell lines for functional assays. COG HEK293 KOs were obtained by using CRISPR technology. Staining with fluorescent GNL lectin was employed as a selection strategy. Individual COG KO colonies were made for all subunits and characterized via lectin staining, electron microscopy, western blot and sequencing. Gel filtration and native IP of COG subunits indicate that in the cytosol the predominant arrangement of the COG complex is the full octameric assembly, whereas in the membrane pool a 50 % of COGs elutes in two smaller sub-complexes. Using native IP and superresolution microscopy we demonstrate that these subcomplexes are spatially separated on Golgi and vesicle membranes with the abundant presence of lobe B on vesicles. Furthermore, we found the selectivity of the sub-complexes to interact with the major components of vesicle fusion machinery, suggesting their different roles in vesicle tethering cycle. Lobe A interacts with p115 and Rab30 on the Golgi while lobe B displays novel interaction with v-SNARE GS15 on vesicles. We propose a mechanistic model that employs association/disassociation of COG sub-complexes as a major mechanism that directs vesicle tethering at Golgi membranes. The COG complex's involvement in human diseases emphasizes the importance of Golgi trafficking. Understanding COG's role will lead to the discovery of potential therapies for COG-related diseases.

321. An unusual, protein-specific *N*-glycan with high sensitivity and specificity for pancreatic adenocarcinoma

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We have identified an unusual Asn-linked glycan structure that is expressed on only a single protein, CEACAM6, (CD66c), in pancreatic adenocarcinoma cell lines and tissues, as evidenced by its sensitivity to PNGase F. There are twelve peptide sites that express *N*-linked glycans on this protein. The glycan of interest, which is the epitope for monoclonal antibody 109, is expressed on only one site on CEACAM6. Two, independent studies have shown its specificity and sensitivity for pancreatic carcinoma to both be>89 %, n=230. The site-specificity of the antibody is explained by the identification of a three amino acid sequence, 300Q-301A-302H, which is required for synthesis of the epitope. The glycan epitope is also synthesized and secreted when a fragment of CEACAM6 is expressed in both Sf9 and S2 insect cell lines. The epitope is also expressed in several other human cancers, including small-cell lung cancer, on CEACAM5, a glycoprotein whose sequence is closely-related. The glycan epitope is synthesized and secreted at low levels by Lec1 CHO and HEK cells, but only after transfection with a cDNA encoding a fragment of CEACAM6. Expressed in the Lec1 background, however, the glycan is resistant to endo F(H) treatment by contrast to the remaining $Man_5GlcNAc_2$ glycans. The epitope is likely an onco-fetal glycan that functions during early development, conserved from insect to man, and re-expressed during oncogenesis of some cell types.

322. Distribution and expression of Siglec-8 and Siglec-9 ligands in human airways

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Siglecs are a family of 14 sialoglycan binding proteins (in humans), most of which are expressed on immune cells and most of which carry an immunoreceptor tyrosine based inhibitor motif (ITIM). Among these are Siglec-8, on human allergic inflammatory cells (eosinophils, mast cells, and basophils) and Siglec-9 on neutrophils (as well as monocytes, dendritic cells and natural killer cells). Crosslinking Siglec-8 results in apoptosis of human eosinophils, whereas crosslinking Siglec-9 results in apoptosis of neutrophils. In both cases, activated immune cells are more susceptible to siglec-mediated apoptosis than resting cells. In the physiological balance of activation and inhibition of immune responses, selective sialoglycan ligands for Siglec-8 and Siglec-9 on tissues may engage siglecs on the surface of activated eosinophils and neutrophils respectively, inhibiting ongoing immune responses. The identity of endogenous human siglec ligands is largely unknown. Siglec-8-Fc and Siglec-9-Fc chimeric lectin overlay of human upper airway tissue sections revealed a high density of Siglec-8 ligands in serosal cells of submucosal glands. Siglec-9 ligands were also found in serosal cells, as well as on the airway epithelium and connective tissue. Ligands for both siglecs were expressed at significantly higher levels in airway sections from patients with chronic rhinosinusitis, an inflammatory disease of the upper airways. Inflammation-mediated increases of Siglec-9 were also documented in a human airway

cell line, Calu-3. Treatment of Calu-3 cells with immune mediators LPS, TNF-α or IL-13 increased expression of sialoglycan Siglec-9 ligands via an NFκB-mediated pathway. Siglec-9-Fc lectin pull-down from extracts of human airway tissue and Calu-3 cells indicated that a portion of the Siglec-9 ligand is carried on MUC5B. Molecular knowledge of siglec ligands and their expression may provide insights into the control of airway inflammation. Supp by US NIH grant HL107151.

Asian Community of Glycoscience and Glycotechnology (ACGG) Lectures

Keynote Lectures

323. Functional significance of glycan-mediated interactions on sperm microdomains during fertilization

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Membrane microdomains (or lipid rafts) are recognized as the cell surface sites involved in cellular interactions and signal transductions. They are unique areas of biomembranes that are enriched in sphingolipids, cholesterol, and glycosylphosphatidylinositol (GPI)-anchored proteins. Although not so well recognized, a unique feature of microdomains is an enrichment of particular glycan chains derived from glycoproteins and glycolipids. Few studies, however, have focused on those microdomain-localized glycan chains. In 1999, we first reported biochemical characterization of sperm microdomains using sea urchin (Ohta *et al*. 1999), and have since demonstrated the importance of microdomain-localized glycans during fertilization. In this study, we found two highly glycosylated proteins, WGA16 and WGA-gp, on microdomains of uncapacitated boar sperm: (1) WGA16 was a prostate-derived seminal plasma protein, which was deposited initially on the sperm surface, then removed during capacitation. WGA16 is the Jacalin-like family lectin, and can bind to heparin with high affinity. Unlike other seminal plasma glycoproteins, WGA16 *N*-glycans are terminated by GalNAc or GlcNAc residues. The GlcNAc/GalNAc residues can work as binding ligands for a sperm surface galactosyltransferase, which actually galactosylates WGA16 *in situ* in the presence of UDP-Gal. Interestingly, surface removal of WGA16 is induced by either UDP-Gal or heparin. In the crystal structure, *N*-glycosylated sites and the heparin-binding site face opposite sides. (2) WGA-gp is localized mainly in flagella and structural analyses of purified protein identified that it is CD52, a GPI-anchored protein secreted from epididymis. Interestingly, WGA-gp may be involved in intracellular Ca ion regulation. Presence of carbohydrate-enriched flagellar proteins involved in intracellular Ca ion regulation may be a common feature among animal sperm.

324. Rationales and strategies for cancer immunotherapy targeting glycolipids

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GD2 is a glycolipid overexpressed in neuroectodermal tumors and sarcomas. Dr. Alice Yu has pursued immunotherapy of neuroblastoma with a chimeric anti-GD2, Dinutuximab, from preclinical studies, IND to phase III trial, culminating in its FDA approval in March 2015, marking the first immunotherapeutics targeting a non-protein antigen. Another prevalent cancer associated glycolipid is Globo H ceramide (GHCer) which is overexpressed in cancers of lung, prostate, breast, ovary, colon, gastric, pancreatic, etc.. They found Globo H to be present in breast cancer stem cells (BCSCs) of clinical specimens. Notably, Gb5, the precursor of Globo H, was also expressed in BCSCs of >60 % of tumors. Injection of mice with Globo H-KLH vaccine induced both anti- Globo H and anti-Gb5, suggesting BCSC targeting by Globo H vaccine. Recently, they demonstrated immunosuppressive effects of GHCer which facilitate the escape of cancer cells from immune surveillance. This is due to down-regulation of Notch1 signaling by increased ID3 and egr2/3 controlled itch expression. They also showed uptake of GHCer shed by breast cancer cells into endothelial cells, leading to enhanced angiogenesis in vitro and *in vivo*. Clinically, Globo-H^+ breast cancer specimens contained higher vessel density than Globo-H^- tumors. Mechanistic investigations linked the angiogenic effects of GHCer to its endocytosis and binding to TRAX, with consequent release of PLCb1 from TRAX to trigger Ca^{2+} mobilization. Thus, GHCer plays triple roles in serving as a CSC antigen, as an immune checkpoint and as an angiogenic factor, thereby driving the ongoing multi-national randomized trial of globo H vaccine in breast cancer. Meanwhile, a new generation of Globo H vaccine was generated in collaboration with Dr. Wong by conjugating Globo H to diphtheria toxoid and use of C34, an analog of α-GalCer, as an adjuvant. The latter is superior to α-GalCer by its lack of α-GalCer- induced anergy and myloid dervived suppressor cells.

Lectures

325. Antiproliferative and cytotoxic effect of *Areca catechu* (betel nut) agglutinin on B16F10 melnoma cancer cell

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Betel nut (*Areca catechu*) lectin (ACA) was purified by DEAE cellulose, gel filtration on Superose 6 and affinity chromatography on fetuin Sepharose columns. ACA (MW~75 kDa) showed strong binding to *N*-linked serum glycoproteins by SPR analysis. Quantum dot-ACA conjugate detected high expression of transferrin in alcoholic liver cirrhosis patients' sera than those of normal individuals. Immunomodulatory potential of ACA against B16F10 melnoma cancer cells was assessed, which showed ACA treatment caused prominent suppression of the anti inflammatory cytokines such as IL 10 and TGFβ. B16F10 cancer cell proliferation was found to be suppressed on treatment with ACA as monitored by cancer proliferation markers such as Cox-2, Akt and cyclin D1 (G1-S transition check point). The expression of acid sphingomyelinase was found to be enhanced on treatment of B16F10 cells with ACA, which triggered the generation of ceramide in B16F10 cells leading to apoptosis. ACA also reduced the production of pERK and pAkt indicating that ACA hindered B16F10 cell proliferation. ACA also reduced procaspase-3 and procaspase-8 indicating activation of caspase pathway and hence apoptosis. The expression of the anti-apoptotic protein Bcl-2 was down regulated without altering the expression of pro-apoptotic proteins Bax. The results highlight the cytotoxic effect of ACA towards B16F10 melanoma cancer cells and suggest that ACA may be explored for therapeutic applications in melanoma.

326. Bifidogenic prebiotics: comparison between sialyllactose and fucosyllactose and their impact on the bacterial metabolic network

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Intestinal microbiota are diverse and dynamic depending on the food or personal variations. Meanwhile, homeostasis of the beneficial population is key to the good health. While *Bifidobacterium infantis* and *Bifidobacterium bifidum* are considerably beneficial populations in infant gut microbiota, human milk oligosaccharides (HMO) and their bifidogenic impact play an essential role in nurturing infants. However, due to the structural complexity and the compositional diversity, mass production of HMOs are still problematic. Two small oligosaccharides, sialyllactose and fucosyllactose which exist in human and bovine milk, are currently synthesized in plant scale. Therefore, we evaluate the bifidogenic and prebiotic capability of sialyllactose and fucosyllactose and their impact on the bacterial metabolism by comparing to the overall protein expression pattern of HMO grown cells. Six representative *Lactobacilli* and *Lactococci*, *Leuconostoc* sp. three *Bidfidobacterium* sp. and nine food pathogens were grown anaerobically on sialylactose and fucosyllactose as a single carbon source. Only *B. infantis* and *B. bifidum* could grow on fucosyllactose showing the bifidogenic impact, however, three major food pathogens *Escherichia coli* O157:H7, *Salmonella typhimurium* and *Clostridium perfringens* were able to grow and utilize sialylactose. Interestingly, *E. coli* DH5a, TOP10, and K-12 were unable to grow with sialylactose on M9 minimal media suggesting the possible correlation between bacterial pathogenesis and the sialyl-utilization. Protein expression profiles of *B. infantis* and *B. bifidum* grown on fucosyllactose exhibited the strong potential of fucosyllactose as a HMO supplementary.

327. Semi-quantitative measurement of a specific glycoform using a DNA-tagged antibody and lectin affinity chromatography for glyco-biomarker development

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Aberrant glycosylation-targeted disease biomarker development is based on cumulative evidence that certain glycoform are mass-produced in a disease-specific manner. However, the development process has been hampered by the absence of an efficient validation method based on a sensitive and multiplexed platform. In particular, ELISA-based analytical tools are not adequate for this purpose, mainly due to the presence of a pair of *N*-glycans of IgG-type antibodies. To overcome the associated hurdles in this study, antibodies were tagged with oligonucleotides with T7 promoter and then allowed to form a complex with

corresponding antigens. An antibody-bound specific glycoform was isolated by lectin chromatography and quantitatively measured on a DNA microarray chip following production of fluorescent RNA by T7-trascription. This tool ensured measurement of targeted glycoforms of multiple biomarkers with high sensitivity and multiplexity. This analytical method was applied to an *in vitro* diagnostics multivariate index assay where a panel of HCC biomarkers comprising α-fetoprotein, hemopexin and α-2-macroglobulin was examined in terms of the serum level and their fucofractions. The results indicated that the tests using the multiplexed fuco-biomarkers provided improved discriminatory power between non-HCC and HCC subjects compared to the AFP level or fuco-AFP test alone. The developed method is expected to facilitate the validation of disease-specific glycan biomarker candidates.

328. A comprehensive glycomic approach to overview the causal relationships between various phases of multistep tumorigenesis and glycosylation status by using a human brain tumor/glioma progression model

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Although cancer cells frequently display glycans at different levels or with fundamentally different structures from normal cells, the relationship between altered glycosylation and causal genetic alteration(s) is poorly understood. Based on the multistep theory of tumorigenesis (characterized by tumor initiation, promotion, and progression), we used an original brain tumor model in which normal human astrocytes were transformed through the serial introduction of hTERT, SV40ER, H-RasV12, and myrAKT, thereby mimicking human brain tumor grades I–IV. Alterations were then explored in three major classes of cell surface glycans/glycoconjugates: *N*- and *O*-glycans on glycoproteins, and glycosphingolipids by recently established analytical techniques. Specific glycomic alterations associated with particular cancerous properties were observed as well as various previously described glioma-related glycomic alterations. These observations uniquely dissect the contribution of specific glycosylation changes to each tumor grade, highlighting the importance of a systematic global overview of glycomic expression for the discovery and validation of disease-related biomarkers, and for predicting prognosis.

329. Galectin-3 protects intracellular listeria monocytogenes by suppressing autophagy activation via host *N*-glycan-dependent and -independent pathways

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Glycans are generally confined in the lumens of intracellular organelles, but can become exposed to the cytosolic milieu when the organelles are ruptured by pathogens. It has become known recently that host glycans exposed to the cytosol are recognized as stress signals by galectins, which are a family of β-galactoside-binding animal lectins synthesized in the cytosol. Several galectin family proteins have been shown to accumulate at endosomes initially containing intracellular bacteria, but subsequently ruptured by the bacteria as the latter escape from the vacuoles. Galectin-8 was shown to promote antibacterial autophagy by recognizing host glycans exposed on ruptured vacuolar membrane and recruiting the autophagy adaptor protein NDP52. The functional consequences of the accumulation of other galectins on ruptured bacteria-containing vacuoles remained unknown. Here, we infected macrophages from wild-type ($gal3^{+/+}$) and galectin-3 knockout ($gal3^{-/-}$) mice with the intracellular bacterium Listeria monocytogenes (LM). We show that endogenous galectin-3 protects LM by suppressing antibacterial autophagy. The autophagy-inhibitory activity of galectin-3 is largely mediated through a host *N*-glycan-dependent pathway, although a host *N*-glycan-independent mechanism involving inhibition of bacterial ubiquitination also plays a minor role. Furthermore, galectin-3 directly binds to cytosolic LM and may interfere with bacterial ubiquitination. Altogether, the results indicate that in contrast to galectin-8, galectin-3 suppresses antibacterial autophagy and is therefore beneficial for intracellular bacterial survival. The discovery that galectin-3 and galectin-8 have disparate functions during host-pathogen interaction highlights the versatility of the galectin family in regulation of cell-autonomous immunity. This work also further strengthens the notion that galectins are uniquely suited for sensing stress signals associated with the appearance of host glycans in the cytosol.

Young Glycoscientists Symposium Lectures

Keynote Lectures

330. The structural role of antibody *N*-glycosylation in receptor interactions

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Asparagine (N) 297-linked glycosylation of Immunoglobulin G Fc is required for binding to FcγRIIa, IIb and IIIa though it is unclear how it contributes. We found the quaternary structure of glycosylated Fc was indistinguishable from aglycosylated Fc indicating *N*-glycosylation does not maintain relative Fc Cγ2/Cγ3 domain orientation. However, the conformation of the C'E loop, which contains N297, was significantly perturbed in the aglycosylated Fc variant. The conformation of the C'E loop as measured with a range of Fc variants shows a strong correlation with FcγRIIIa affinity. These results indicate the primary role of the IgG1 Fc *N*-glycan is to stabilize the C'E loop through intramolecular interactions between carbohydrate and amino acid residues and preorganize the FcγRIIIa interface for optimal binding affinity.

331. Challenges of high-throughput glycomics - what to expect with large datasets

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Understanding complex molecular mechanisms underlying common diseases requires an integrated analysis of large scale multiomics datasets. While genomics and proteomics have advanced rapidly, because of a limited set of tools, techniques and resources glycomics was for a long time lagging behind. Recent technological and methodological developments have enabled glycomics to join the other high-throughput omics. With a growing number of high-throughput profiling methods it is becoming increasingly evident that glycosylation analysis does not necessarily have to be time-consuming, labor-intensive, expensive and generally overwhelming. However, processing a large number of samples often reveals methodological weaknesses (like problems with rare events and outliers) which usually do not appear in small-scale studies. Therefore, generating high-quality glycomics data in a high-throughput fashion requires protocols dealing with the aforementioned problems while satisfying time constraints. Furthermore, combining different fields and their expert knowledge (computer science, statistics, chemistry, biology, etc.) with as much as possible automatization is of utmost importance. Based on our experience in dealing with large datasets, the most common problems and appropriate solutions will be discussed, including: proper study design (randomization and blocking), reproducible and robust sample preparation (experimental vs. biological variation), accurate glycan identification (data processing of UPLC/LC-MS data), quantification (automatic integration), and thorough quality control (normalization and batch correction). Identification and appropriate management of these critical steps has greatly improved the quality of our glycomics data and facilitated large-scale glycoprofiling.

332. Glycans & cancer: applications of mass spectrometry

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Protein glycosylation has been proposed as a new source of potential biomarkers for diseases as diverse as cancer and infection. Thus far, studies that are aimed towards the identification of glycomics based biomarkers have, however, primarily focused on released glycans, thereby eliminating protein- and/or site- specific information. In this presentation, we describe a targeted method for extensive site-specific analysis of protein glycosylation. Multiple reaction monitoring on QQQ-MS is an excellent tool for quantification of protein glycosylation. We developed transitions for ten abundant serum proteins, and glycopeptide signals were normalized to the absolute protein amount to determine the protein- and site- specific glycosylation patterns. The method has been applied to determine the glycan changes associated with lung and ovarian cancer.

333. The role of sialylation for the anti-inflammatory activity of intravenous immunoglobulin

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IgG antibodies play a dual role during autoimmune diseases since they are responsible for tissue destruction and inflammation while at the same time, they are known to have anti-inflammatory properties when applied in high doses. Research has highlighted the role of specific sugar residues in modulating pro- and anti-inflammatory activities of the IgG molecule. IgG exhibits highly conserved glycosylation patterns attached to the

asparagine 297 of the IgG-Fc fragment which were shown to be responsible for IgG activity *in vivo*. Altering the conformational flexibility of IgG through different glycovariants was demonstrated to result in divergent binding specificities of IgG to its receptors and thereby to enhance or reduce antibody effector functions. Moreover, several glycosylation patterns containing low levels of terminal galactose or sialic acid residues have been associated with active autoimmune disease in rheumatoid arthritis (RA) or SLE for example. In contrast, sialic acid rich IgG glycovariants were found to be associated with disease remission and during anti-inflammatory conditions. The most famous scenario of anti-inflammatory IgG activity is high dose Intravenous Immunoglobulin (IVIg) therapy. IVIg is a polyclonal IgG preparation with potent immunomodulatory properties. Originally developed to treat immunodeficient patients, it is nowadays used for the therapy of various autoimmune and inflammatory diseases including thrombocytopenia (ITP), chronic inflammatory demyelinating polyneuropathy (CIDP) and other immune-mediated neurologic, hematologic or dermatologic disorders. Despite this well-documented clinical success, the precise molecular and cellular mechanisms underlying the immunomodulatory activity are not completely understood and discussed controversially. In several murine models of autoimmunity we were able to show a loss of anti-inflammatory IVIg activity by enzymatic removal of terminal sialic acids within the asparagine 297 linked IgG glycan.

Lectures

334. IgCarbKB: a glycomics and glycoproteomics focused immunoglobulin knowledge base

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Immunoglobulins (Ig's) and Fc receptors are complex glycoproteins and key components of both, the innate and adaptive immune systems. The specific glycosylation of both, immunoglobulin and their receptors is well known to be crucial for maintaining and modulating effector functions. Thus combination of both, glycomics and glycoproteomics analyses of Ig isotypes such as IgG, IgA, IgD, IgE and IgM is required to investigate the functional role(s) of individual sugar modifications. As a first step towards a comprehensive Ig-carbohydrate knowledgebase (IgCarbKB) we present a detailed glycomic and glycoproteomic map of the entire human Immunoglobulome (IgA, sIgA, IgD, IgE, IgG and IgM) acquired by a variety of analytical platforms and retrieved from the literature. Immunoglobulins were SDS-PAGE separated, the heavy and light chains were proteolytically digested and the resulting (glyco)peptides in-depth analysed by nano-reversed-phase-LC–ESI iontrap MS/MS and RP-LC-Orbitrap MS/MS to study site-specific glycan microheterogeneity in polyclonal immunoglobulins derived from healthy donors and myeloma patients. PGC nanoLC ESI-MS/MS & xCGE-LIF provided detailed glycomic profiles of the released *N*-glycans from the respective Ig's heavy chains, whereas the *O*-glycans were analysed by PGC nanoLC ESI-MS/MS. Each individual Ig class showed distinct glycomic profiles, which furthermore differed on the individual sites of glycosylation of the respective Ig's. Not surprisingly, glycomic and glycoproteomic profiles differed significantly in all myeloma Ig's analysed. In order to make glycomic and glycoproteomic knowledge on Ig's accessible we have also developed an open source knowledge base IgCarbKB (www.IgCarbKB.org) where literature as well as acquired data on Ig's specific carbohydrate information is stored and made accessible. This knowledge base will provide one step forward to gain a more complete understanding on the functional role of Ig glycosylation.

335. Comprehensive profiling of glycosphingolipid head groups using recombinant endoglycoceramidases in a high-throughput semi-automated glycan analytical platform

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The biological functioning of glycosphingolipids (GSLs) is largely determined by their glycan part which has placed renewed emphasis on their detailed analysis. Comprehensive profiling of GSL head groups in biological samples requires the use of endoglycoceramidases with broad substrate specificity and a robust workflow that enables their high-throughput analysis. To compensate for the current lack in such tools we here present a semi-automated glyco-analytical platform for GSL head group profiling as adapted from a recently established robotic *N*-glycan analysis workflow including selective glycan capture on hydrazide beads, 2AB-fluorescent glycan labelling and

analysis by UPLC-HILIC-FLD. The workflow accommodated for the efficient evaluation of the GSL hydrolysing activity of two recombinant endoglycoceramidases – a novel endoglycoceramidase I (EGCase I) from *Rhodococcus triatomea* and an endogalactosylceramidase (EGALC) from *Rhodococcus equi*. Superior activity on a broad substrate range including gangliosides, globosides, (n)Lc-type GSLs and cerebrosides was observed for EGCase I which was complemented by the avidity of EGALC towards structures which are linked to the ceramide unit by (β1-1)-galactose. Our integrated strategy was successfully applied for the systematic profiling of glycolipid head groups from human serum which should facilitate its use in functional glycomics studies and biomarker discovery.

336. Glycomic & glycoproteomic characterisation of an important gastrointestinal tumour marker for the analysis of cancer-specific glycosylation features

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Gastrointestinal carcinomas such as colorectal and gastric cancer are among the top 5 diagnosed cancers associated with a high mortality rate. Thus, many efforts have been made to comprehend the pathophysiological events in onset and progression of the disease, resulting in the finding of numerous marker (glyco)proteins for cancer screening and/or treatment monitoring. The vast majority of these tumour markers currently in use are glycoproteins, nevertheless detailed information on the specific glycosylation of these proteins is sparse. Therefore we set out to on the comprehensive glycoproteomic investigation of a highly *N*-glycosylated, commonly used tumour marker applied in the follow-up diagnosis of gastrointestinal cancer patients to map its specific *N*-glycan macro- and micro-heterogeneity. The purified glycoprotein, which was obtained from different sources such as human colon adenocarcinoma, colon adenocarcinoma cells and ascites fluid was subjected to the comprehensive glycoproteomic characterisation by specifically adapted glycomic & glycoproteomic protocols ensuring a highly sensitive and selective analysis. Glycopeptides (before and after enzymatic deglycosylation) were obtained after selective proteolytic digestions and analysed by reversed phase nano-LC-ESI MS/MS for microheterogeneity investigation. The data obtained revealed distinct site-specific glycosylation patterns and site-occupancy profiles. The purified protein was also dot-blotted onto PVDF membranes for subsequent porous graphitized carbon nano-LC-ESI MS/MS of released *N*- and *O*-glycans. Man5 was found to be the major *N*-glycan present on all samples analysed from this glycoprotein, besides core fucosylated, paucimannosidic ones. Distinct glycosylation differences such as branching, degree of sialylation and level of bisecting *N*-glycans were identified between the different sources of the purified protein.

337. Sweet escape: sialic acids in tumor immune evasion

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Tumor cells decorate their surface with a dense layer of sialoglycans that confers resistance to apoptosis, promotes tumor cell adhesion and migration and mediates resistance to therapy. Recent insights suggest that sialoglycans protect tumor cells from recognition and eradication by the immune system through the interaction with immunosuppressive Siglec receptors on immune cells. Thus, blocking aberrant sialylation on tumor cells might significantly interfere with tumor growth at multiple levels and could enhance tumor immunotherapy. We have recently demonstrated that the sialic acid analogue 3Fax-Neu5Ac developed by Rillahan *et al.* potently blocks sialic acid expression in cancer cells. As a consequence of sialic acid blockade, the migratory and adhesive capacity of cancer cells was impaired as well as their outgrowth *in vivo*. Furthermore, we found that targeted delivery of 3Fax-Neu5Ac-loaded nanoparticles to melanoma cells *in vivo* strongly reduced their metastatic spread. Currently, we are in the process of unraveling the dominant effects of sialic acid blockade on the formation of the immunosuppressive tumor microenvironment and anti-tumor immunity. Our data indicate that sialic acid blockade in the tumor microenvironment is feasible *in vivo* without apparent side effects. Strikingly, sialic acid blockade results in increased infiltration of effector immune cells (T cells, NK cells) and surprisingly, decreased numbers in immunosuppressive regulatory T cells. Moreover, we found that blocking tumor sialoglycan expression positively influenced the

outcome of cancer immunotherapy in mouse tumor models. We will present our data on how sialic acid blockade influences tumor growth with particular focus on the tumor-immune interface and the beneficial effects for tumor immunotherapy. Altogether, these studies should help to understand the diverse role of sialic acids in immunobiology of cancer and provide rationale for the development of new cancer (immuno)therapies.

338. Enrichment of breast cancer stem cells with globo-series epitopes

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Cancer stem cells, which are critical to maintain the cancer cell population and tumor development, become the target of the therapeutics and early stage diagnosis. To know more about them, several protein marker systems, particularly CD24/CD44 and ESA/PROCR, were established for breast cancer stem cells (BCSCs) enrichment but the purity of obtained BCSCs is low. In the previous work, BCSCs with $ESA^{hi}PROCR^{hi}$ or $CD24^{lo}CD44^{+}$ have a significant expression level of globo-series molecules including SSEA-3, SSEA-4 and globo-H. In order to investigate the tumorigenicity of cells expressed with conventional marker systems and carbohydrates epitopes, subpopulation carrying ESA/PROCR or CD24/CD44, along with SSEA-3, SSEA-4 or globo-H, their ability of forming cell colonies, mammospheres or *in vivo* tumor was detected. Surprisingly, $ESA^{hi}PROCR^{hi}SSEA\text{-}3^{+}$ or $CD24^{lo}CD44^{+}SSEA3^{+}$ cells had better self-renewal ability than others such as ESAhi PROCRhi SSEA-3- and $CD24^{lo}CD44^{+}SSEA3^{-}$ cells. Similarity, in the animal study, it is found that $ESA^{hi}PROCR^{hi}SSEA\text{-}3^{+}$ or $CD24^{lo}CD44^{+}SSEA3^{+}$ cells formed tumor in the mammary gland effectively. Also, cells carrying solely $SSEA\text{-}3^{hi}$ had a higher tumorigenic potential than those carrying $SSEA\text{-}3^{lo}$ in both MDA-MB-231 and MCF-7. On the other hand, it is also found that SSEA-3 is important to breast cancer apoptosis and survival. Thus, it can be concluded that SSEA-3 is a potential BCSC marker and essential to the proliferation of breast cancer.

339. Interplay between E-cadherin and IR/IGF-IR signaling in the regulation of tumor cell invasion. a mechanism mediated by changes in bisected *N*-glycans expression

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Changes in glycosylation are considered a hallmark of cancer, and one of the key targets of glycosylation modifications is E-cadherin. Previous studies have shown that E-cadherin has a role in the regulation of bisected *N*-glycans expression, remaining to be determined the Ecadherin-dependent signaling pathway involved in this *N*-glycans expression regulation. In this study, we analysed the impact of E-cadherin expression in the activation profile of receptor tyrosine kinases such as insulin receptor (IR) and IGF-I receptor (IGF-IR). We demonstrated that exogenous E-cadherin expression inhibits IR, IGF-IR and ERK 1/2 phosphorylation. Stimulation with insulin and IGF-I in MDA-MD-435 cancer cells overexpressing E-cadherin induces a decrease of bisected *N*-glycans that was accompanied with alterations on E-cadherin cellular localization. Concomitantly, IR/IGF-IR signaling activation induced a mesenchymal-like phenotype of cancer cells together with an increased tumor cell invasion capability. Altogether, these results demonstrate an interplay between E-cadherin and IR/IGF-IR signaling as major networking players in the regulation of bisected *N*-glycans expression, with important effects in the modulation of epithelial characteristics and tumor cell invasion. Here we provide new insights into the role that Insulin/IGF-I signaling play during cancer progression through glycosylation modifications.

340. Total plasma *N*-glycome changes in diabetes mellitus type 2

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Diabetes mellitus type 2 (T2D) is a global public health problem affecting over 300 million people. T2D is characterized by high blood glucose levels due to insulin resistance and may lead to numerous complications, including cardiovascular disease and increased risk for blindness and death. Whereas the non-enzymatic glycation of plasma proteins in T2D has been investigated extensively, little is known about enzymatic protein glycosylation in T2D. Recently, changes in serum *N*-glycome between T2D patients and healthy controls were found by analyzing 10*N*-glycan species via CGE-LIF after desialylation. However, sialylation of protein *N*-glycans is known to be implicated in various (patho)physiological conditions. Therefore, we detected more than 100*N*-glycan species in plasma samples from 221 T2D patients and 113 healthy controls by using a recently developed high-throughput approach, which allows sialic acid stabilization and differentiation of its linkages in MALDI-TOF-MS. Among others, multiple glycans bearing fucose and/or bisecting *N*-acetylglucosamine were found to be decreased significantly in T2D patients vs. controls. Sialylation of various glycans increased, driven by an increase of α-2,6-sialylation, while α-2,3-sialylation decreased with T2D. The latter might be explained by elevated fibrinogen levels known to be present in T2D patients. Various *N*-glycans were correlated with the body mass index and/or age of patients, and showed sex-specific variations. In summary, our data indicate that – among others – specific changes in *N*-glycan sialylation occur in T2D. New advances in analytical MS-techniques as demonstrated by our new method will enable unraveling even isomer-specific glycomic changes in disease and, thus, promote clinical research. Further studies are needed to elucidate the possible pathophysiological role and clinical usefulness of glycomic changes in T2D.

341. The sweet characterization of therapeutic protein *N*-glycosylation using xCGE-LIF

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The use of valuable protein-based therapeutics to fight various human diseases, like diabetes, multiple sclerosis, anemia and cancer, became an integral part in modern medicine. More than 100 biopharmaceuticals (originators and biosimilars) have already been approved, whereas many more are under development and in clinical trials. Thereby, quality attributes like biological activity, bioavailability, immunogenicity, efficiency and function are important parameters for their successful approval by the authorities. Their quality attributes are often affected by one of the most common posttranslational modification of proteins, the *N*-glycosylation. Its variability and heterogeneity is challenging for R&D and production as it impacts the therapeutic properties of these glycoproteins. This makes it indispensable to characterize and monitor their *N*-glycosylation along R&D and during production. Common glycoanalytical techniques therefore are chromatography and/or mass spectrometry based. In contrast, we have developed a toolbox for the in depth characterization of *N*-glycosylation, utilizing multiplexed capillary gel electrophoresis with laser induced fluorescence detection (xCGE-LIF) as core technology. By using this miniaturized electrokinetic separation technique with up to 96 capillaries in parallel, assisted by sample preparation in 96-well format, a massive reduction of analysis time and cost per sample could be achieved. This is presented by an exemplary set of (therapeutic) glycoproteins. Further, the high sensitivity of xCGE-LIF is demonstrated by the glycoanalysis of less than 0.1 μg of such glycoproteins. Beyond this, its impressive separation performance is shown to enable the discrimination of sialylated *N*-glycans containing either *N*-acetylneuraminic acid (NANA), *N*-glycolylneuraminic acid (NGNA) or both sialic acid species. All this makes xCGE-LIF to a powerful and valuable tool for the characterization of the glycosylation of biopharmaceuticals.

342. Human blood plasma *O*-glycoproteomics

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A site-specific glycosylation analysis is key to investigate structure-function relationships of glycoproteins,

e.g. in the context of antigenicity and disease progression. The analysis, though, is quite challenging and time consuming, in particular for *O*-glycosylated proteins. In consequence, despite their clinical and biopharmaceutical importance, many human blood plasma glycoproteins have not been characterized comprehensively with respect to their *O*-glycosylation. Here, we report on the site-specific *O*-glycosylation analysis of human blood plasma glycoproteins. To this end a human blood plasma of healthy donors was digested non-specifically using proteinase K, followed by a precipitation step, as well as a *O*-glycopeptide specific enrichment and fractionation step via HILIC-LC. Enriched glycopeptide fractions were subjected to nanoRP-LC-ESI-IT-MSn using an ion trap mass spectrometer operated in positive-ion mode. Peptide identity and glycan composition were derived from CID fragment spectra acquired in multistage mode (MS^2, MS^3). To pinpoint the *O*-glycosylation sites glycopeptides were fragmented using ETD. All acquired spectra were interpreted and annotated in parts database-assisted, but mostly manually. Overall, 31 *O*-glycosylation sites belonging to 22 proteins were identified, of which 23 *O*-glycosylation sites could be pinpointed. Also 11 previously unknown sites were registered, of which 8 sites could be pinpointed. In this regard, the use of proteinase K proved to be beneficial for pinpointing the *O*-glycosylation sites. The identified *O*-glycan compositions most probably correspond to mono- and disialylated core-1 mucin-type *O*-glycans.In summary the presented workflow allows the identification and characterization of intact *O*-glycopeptides derived from a complex sample, like human blood plasma. Our data provide novel insights into the human blood plasma mucin-type *O*-glycoproteome, which can help to unravel structure-function relationships of glycoproteins.

343. Evaluation of normalization methods for glycomics data analysis

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Recent technological advances in glycan quantification have enabled analyses of large number of people, but also introduced a novel challenges related with data analysis. Since signal intensities of glycan quantification methods can vary on a sample-to-sample basis, normalization methods are applied to raw glycan data to balance individual measurements across all samples and make them comparable. The most often used normalization method for glycan analyses is total area normalization, where glycan measurements of individual are constrained to the sum of a 100, resulting in so-called compositional data. While it is often claimed by the experts in the field that glycans are by their nature compositions and percentage of a glycan is biologically relevant information, these constraints imposed on the data result in spurious correlations that make many standard univariate statistical methods either inappropriate or inapplicable. One can follow two approaches to tackle this challenge – apply one of the compositional data approaches on the percentage data or apply different normalization method that does not introduce spurious correlations. In this study we simulated associations of IgG *N*-glycans with outcome variable and tested the influence of six normalization methods on downstream analyses, namely univariate associations and variable selection methods. The performance of normalization methods was assessed as accuracy of the estimates compared to that of the oracle (root mean squared error - RMSE) for univariate associations and prediction error and number of zero coefficients for variable selection methods. Quantile normalization resulted in the largest number of RMSEs and the best prediction in the variable selection setting. While quantile normalization is not necessary the best normalization method for other statistical methods not tested in this work, it is highly advisable to use this or other rank based methods for correlation structure dependent analyses.

344. Effect of abiraterone and ionizing radiation on the glycohydrolase activities in prostate cancer cells

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Prostate cancer (PC) is the most common malignancy and second leading cause of cancer-related deaths in

men. Among the different therapeutic options, abiraterone is a new promising drug recently approved for the treatment of PC, nevertheless it mechanism of action is almost unknown. PC is characterized by "aberrant glycosylation", caused by specific glycosyltransferases and glycohydrolases present both intracellularly and at the plasma-membrane (PM) level. Interestingly, changes in the activity of different glycohydrolases have been detected in different cell lines after proton irradiation. In particular, it has been recently demonstrated that in breast cancer cells the irradiation caused the production at the PM-level of pro-apoptotic ceramide through the in-situ hydrolysis of complex glycolipids. Based on these findings, this study addresses whether these enzymes are a target of abiraterone and of ionizing radiations in human PC. To this purpose, androgen-sensitive and androgen-insensitive PC cell lines were subjected to treatments with abiraterone and/or ionizing radiation and the activities of different PM-associated glycohydrolases as well as the ceramide level were evaluated. Interestingly, all the cell lines tested showed a marked increase in all the PM-associated glycohydrolases as well as in their ceramide content, especially after the combined treatment with abiraterone and ionizing radiation. These data demonstrate the involvement of the glycohydrolases in the mechanisms of abitaterone- and radiation- induced cell death in both androgen sensitive and insensitive PC cells and suggests that these enzymes, capable to evocate the production of ceramide at the PM-level, could represent new potential therapeutic targets for PC.

345. Special tricks and treats for the successful LC-MS/MS characterisation of glycopeptides

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The posttranslational glycosylation of proteins play key functional roles in cellular processes and the characterization of glycan structures, sites occupied, and glycan/protein interplays is critical to understand these events. In a recent study we showed that positive mode LC-MS/MS using collision-induced dissociation (CID) and higher energy collision-induced dissociation (HCD) could be used to distinguish between glycan and glycopeptide structural isomers. Oxonium ion fragmentation profiles of HexNAc residues derived from GalNAcα1-*O*- and GlcNAcβ-*O*- substituted peptides were characteristically different. To gain further mechanistic insights into differences in oxonium ion fragmentation profiles deuterium labelled *O*-glycopeptides and $(Man)_3(GlcNAc)_2$ substituted *N*-glycopeptides have now been prepared and analysed. In a separate project, Chondroitin sulphate (CS) substituted tryptic peptides were purified from urine samples using strong anion chromatography (SAX), and depolymerised using chondroitinase ABC. The resulting hexasaccharide CS-glycopeptides were subjected to HCD at low normalized collision energy (NCE), which resulted in glycosidic fragmentation to identify the glycan structure; and at higher NCE, which resulted in peptide fragmentation and prominent HexNAc derived oxonium ions, to identify the peptide. The identified CS glycopeptides carried 0–2 sulphate groups, which were labile during the HCD conditions. To facilitate the sulphate analysis sodium ions were added to the MS vial, which specifically complexed to the sulphate(s) and protected them from secondary fragmentation, and allowed for the pinpointing of sulphate groups on the CS glycan. The fragment analysis of oxonium ions derived from glycopeptides, and the use of sodium ions to study glycan sulphation add to our arsenal of glycopeptide analysis tools.

346. Ca^{2+} recognition of the C-type lectin receptor Langerin reveals an intradomain allosteric network

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Many C-type lectins are endocytic pattern recognition receptors that, upon glycan binding trigger internalization of their cargo. Hence, pathogen recognition and uptake are tightly coupled to immune cell activation and antigen processing. C-type lectin receptors recognize their carbohydrate ligands utilizing a central calcium ion harbored by conserved amino acids in the lectin binding site. The fate of the cargo is determined by the kinetics and endosomal routing of

the C-type lectin receptor. Endosomal calcium channels open to reduce the effective calcium concentration and additional acidification concertedly leads to cargo release. These mechanisms affect members of the C-type lectin family differently and also vary with cell type. Here, we chose Langerin as an endocytic model receptor. This homotrimeric C-type lectin is highly expressed on endothelial Langerhans cells capturing invading pathogens such as HIV. We studied the molecular mechanisms involved in calcium binding at atomic resolution applying protein NMR spectroscopy, isothermal titration calorimetry and molecular dynamics simulations. Biomolecular NMR provided intriguing insight into a network of amino acids involved in Ca^{2+} recognition, while Markov state models constructed from molecular dynamics simulations led to a detailed picture of the conformational dynamics of Langerin. Together these complementary techniques indicate a potential intradomain allosteric network, while overall no interdomain allostery of the trimeric protein could be observed. We discuss these results in light of the physiological role of Langerin.

347. Structual MUC1 alteration determined by 2D NMR against multiple glycosylated peptides

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MUC1 is a common type of mucin present on the epithelial cell surface. MUC1 is extended through many parts of the human body. *O*-glycosylation of theses MUC1 involves in body immune response, cell differentiation and other biological function, whereas altered *O*-glycosylation involves in tumorigenesis and cancer metastasis. The alteration from normal *O*-glycosylated MUC1 leads to the structural alteration due to which identification and treatment for such alteration becomes difficult. Therefore, recognised to be a good target as therapeutic agent. In this study initially variants of MUC1 peptide sequence, His-Gal-Val-Thr-Ser-Ala-Pro-Asp-Thr-Arg-Pro-Ala-Pro-Gly-Ser-Thr-Ala-Pro-Pro-Ala bearing five *O*-glycosylation sites were used for micro array studies against known antibodies Kl6, SM3 and DF3. Kl6 being a known marker for specific cancers due to its specific binding affinity against minimum core 1 sialylated Thr at the Pro-Asp-Thr-Arg motif was focussed to understand the structural conformation during the presence of asialylated core 1 (T/TF antigen), sialylated core 1 (Sialylated T/TF) antigen and single GalNAc residue (Tn antigen) at the same residue. This study reveals the structural changes and examines the conformational impact of these MUC1 glycopeptides using 2D NMR.

348. Advances in high-throughput glycosylation analysis by MALDI-TOF-MS

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Glycosylation is an important co- and post-translational modification which has been associated with a growing number of diseases, may serve as biomarker, and influences the activity and specificity of biopharmaceuticals. Advantageously, compositional glycan analysis can rapidly be performed by MALDI-TOF-MS, with added information on sequence, branching and linkage with chemical derivatization and/or fragmentation. For the analysis of sialylated glycan species, derivatization is particularly useful. In unmodified form, sialic acids are frequently lost in mass spectrometric analysis by in-source and metastable decay. To cope with this, we have recently published a derivatization method to stabilize and neutralize sialylated glycan species, which allows for reflectron positive ion mode MALDI-TOF-MS. The method has the added benefit of leading to mass-based separation of α-2,3-linked and α-2,6-linked isomers, as these selectively form intra- and intermolecular esters during the reaction. We have since extended the method to the linkage-specific derivatization of IgG tryptic glycopeptides, providing the added benefit of site-specific glycosylation analysis. The various steps of the released glycan protocol have been automated, comprising robotic sample preparation, MALDI-TOF-MS measurement, and data preprocessing. Current throughput allows for the analysis of 384 samples during a single working day, and leads to the detection of 80 distinct *N*-glycan compositions in human plasma. Main peak CV is around 5 % across intra- and interday repeat measurements, and lower CV values are observed for the biologically more

interesting derived traits (*e.g.* fucosylation, galactosylation and α-2,3-linked sialylation per galactose). Using this high-throughput automated workflow, we have studied the *N*-glycosylation of 1944 serum samples, providing insight into glycosylation changes associated with the amelioration of rheumatoid arthritis during pregnancy.

349. Expression of OGT correlates with migration and proliferation of colon cell lines

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The *O*-GlcNAc transferase (OGT) is a key regulator of the post-translational modification of proteins by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) onto Ser/Thr residues. OGT uses the end product of the hexosamine biosynthetic pathway (HBP), UDP-GlcNAc, as a donor for *O*-GlcNAcylation processes. It is reported that OGT and *O*-GlcNAcylation levels are increased in cancers. We showed that in the colorectal cancers (CRC) cell lines (HT29, HCT116) the expression of OGT and *O*-GlcNAcylation level were elevated, and that *O*-GlcNAcylation directly interfered with β-catenin stability and proliferation of cells. Previous studies showed that oncogenic factors such as p53, MYC or β-catenin are *O*-GlcNAcylated. The Wnt/β-catenin pathway is modified in most CRC by genetic alteration of β-catenin or one member of the destruction complex. Consequently, β-catenin is protected from proteasomal degradation and therefore induces cell proliferation. A similar observation was made when HBP flux was increased by culturing cells in high glucose medium. In these conditions, β-catenin was protected against the degradation thus accelerating cell proliferation. In a recent study, we identified four *O*-GlcNAcylation sites at the N-terminus of β-catenin, one of those (T41) localized in the destruction box is crucial for the control of β-catenin degradation. In that context we studied the effect of OGT silencing in CRC cell lines and non cancer cells CCD841CoN. We reported that silencing of OGT halved proliferative and migratory capacities of cancer cells. OGT knock-down also diminished cell adhesion corroborating previous observations that inhibiting *O*-GlcNAcylation decreases β-catenin/α-catenin interactions necessary for mucosa integrity, which suggests that *O*-GlcNAcylation also affects localization of β-catenin at adherens junction level.

350. Structures and membrane microdomain association of Shiga toxin glycosphingolipid receptors from African green monkey kidney epithelial (VERO B4) cells

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Enterohemorrhagic *Escherichia coli* (EHEC) are responsible for severe intestinal infections that can cause heavy diarrhea, hemorrhagic colitis, and the life threatening hemolytic uremic syndrome in humans. The pathogenic effect of EHEC is primarily based on its ability to produce and release Shiga toxins (Stxs). Stxs bind to certain globo-series glycosphingolipid (GSL) receptors of human cells and preferentially target endothelial cells of human kidneys and the brain. Although cytotoxic effects of Stxs have been described for the various Vero cell lines, which originate from the kidney of an African green monkey and represent the gold standard for Stx cytotoxicity studies, knowledge about Stx receptor GSLs and their membrane localization is poor. Here we present the first study on the structural analysis of globo-series GSLs isolated from the Vero-B4 cell line. We identified the GSL receptors of this renal epithelial cell type for various Stx subtypes using thin-layer chromatography overlay detection combined with electrospray ionization quadrupole time-of-flight mass spectrometry. Moreover, investigation of GSL association with membrane microdomains revealed their preferential enrichment in detergent-resistant membranes, which represent supramolecular lipid raft analogue structures. In addition, Stx GSL receptors were found to co-distribute with sphingomyelin and cholesterol known as lipid raft markers. Our comprehensive study suggests functional implication of lipid raft association of receptor GSLs for Stx-mediated cellular damage of Vero-B4 cells and demonstrate the binding

potential of globo-series GSLs to Stx1a, Stx2a and Stx2e subtypes.

351. Bisecting galactose as a feature of *N*-glycans of *Caenorhabditis elegans*

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Nematodes are one of the most diverse animal phyla on earth with a large number of discovered species defined as parasites. Contrary to the common knowledge, simple organisms can produce rather complicated glycomes. Our research was focused on decoding the *N*-glycome of the free-living nematode *Caenorhabditis elegans* as well as characterising novel glyco-epitopes shared by other nematodes. Previously we have proved the *in vivo* enzymatic activities of three fucosyltransferases (FUT-1, FUT-6 and FUT-8) which are the responsible enzymes for generating the highly fucosylated *N*-glycan core. However, *N*-glycomic studies indicated that one *N*-glycan can carry maximally four fucose residues. To simplify the *N*-glycome, we prepared a triple FUT knockout lacking *fut-1*, *fut-6* and *fut-8* genes. Glycans from both wildtype and triple knockout were enzymatically released from pepsinised glycopeptides, derivatised with a fluorescent tag prior to HPLC fractionation and finally analysed with MALDI-TOF MS. Our data revealed that the triple mutant composes maximally one (methyl-)fucose residue on its *N*-glycans, whereas the FUT-1, FUT-6 and FUT-8 products are completely absent as expected. To investigate where the "last" fucose is attached, NMR assays were performed on a glycan pool of reversed phase HPLC fractions. Together with enzymatic and chemical treatments as well as LC-MS data, evidences indicated that the (methyl-)fucose is α-1,2-linked via a bisecting β-galacose to the core region of the *N*-glycans. Furthermore, α-linked galactose was also present on *N*-glycans as judged by its sensitivity to coffee bean α-galactosidase. In conclusion, bisecting galactose is another common feature of *C. elegans* *N*-glycans indicating unknown *N*-glycan biosynthetic pathways, possibly shared with other nematodes.

352. Fluorinated carbon tag derivatization combined with FSPE for highly sensitive and selective analysis of *N*-glycans by mass spectrometry

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Glycans represent key disease biomarkers and have been associated with a variety of diseases, such as cancer, inflammation and degenerative diseases. Currently, mass spectrometry (MS) has evolved as a robust tool for glycan profiling due to its ability to provide glycan structural information. The sensitive and specific detection of glycans via mass spectrometry (MS) remains a significant challenge because of their low abundance in complex biological mixtures, inherent lack of hydrophobicity and suppression by other, more abundant biological molecules (proteins/peptides) or contaminants. A new strategy for the sensitive and selective MS analysis of glycans based on fluorous chemistry is reported. Glycan reducing ends were derivatized with a hydrophobic fluorinated carbon tag. Because the fluorous tag greatly increased glycan hydrophobicity, and obvious ionization efficiency enhancements were achieved, as demonstrated by over 40-fold S/N enhancement after derivatization relative to the native glycan. After derivatization, the strong affinity between perfluoroalkyl groups and fluorous solid phase through their dipole-dipole interactions allows fluorous solid-phase extraction (FSPE) to be used for isolating highly fluorinated-glycans from contaminated solutions (including inorganic salt 2.6 M NH_4HCO_3, 6.2 M NaCl, and chaotrope 2 M urea) and protein mixtures. Finally, we successfully analyzed the *N*-glycome in human serum using this new method. A total of 34 derivatized *N*-glycans were sensitively identified from only 0.25 μL human serum sample.

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