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MASS SPECTROMETRY SUMMER SCHOOL

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Poster Abstracts

Parabola of Neon (1913)

Featured on the cover is an early 20th century parabola mass spectrograph. These devices, pioneered by J. J. Thomson, used electric and magnetic fields to disperse ion populations on photographic plates. Depending on their masses, the ions were dispersed along parabolic lines with those of the highest energy landing in the center and those with the least extending to the outermost edges. Positive ions are imaged on the upper half of the parabola while negative ions are deflected to the bottom half. Note that Ne produces two lines in the spectrum. Francis Aston, a former Thomson student, concluded from these data that stable elements also must have isotopes. These observations won Aston the Nobel Prize in Chemistry in 1922.

Grayson, M.A. Measuring Mass: From Positive Rays to Proteins. **2002**. Chemical Heritage Press, Philadelphia.

National Center for Quantitative Biology of Complex Systems

https://www.ncqbcs.com/

National Center for Quantitative Biology of Complex Systems (NCQBCS) was founded in 2016 through a grant from the National Institute of General Medical Sciences and is located on the campus of the University of Wisconsin-Madison. The development, application, and dissemination of mass spectrometry technology for proteomic analysis is our core mission.

At NCQBCS we are developing next-generation protein measurement technologies for a wide variety of biomedical applications. Our technologies are focused on making whole proteome analysis faster and broadly accessible. In addition to our technology development mission, we apply these technologies to various biomedical problems through collaboration. For those seeking to learn basic proteomic methodology or advanced training in our techniques, we offer hands-on learning experiences.

Innovate

New technologies that will permit faster, more thorough, proteome analysis.

Collaborate

Work with us to apply NCQBCS technologies to your research.

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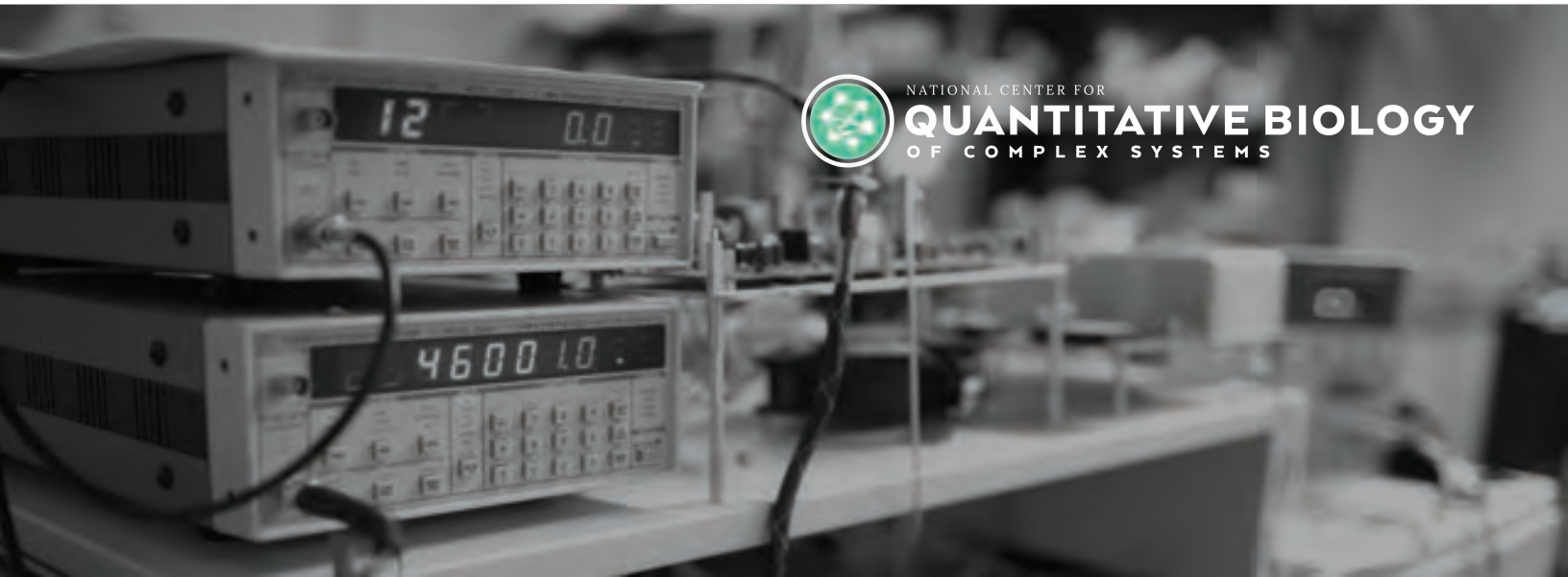
Visit the NCQBCS for hands-on proteomics training and workshops.



Dear Fellow Mass Spectrometrists,

Thank you for attending the **2nd Annual North American Mass Spectrometry Summer School** at the University of Wisconsin-Madison.

We would like to thank everyone who has worked diligently to organize this program and our instructors, many who have traveled far to participate. And we are grateful to all of our students for making this possible. Finally, we could not put on this program without the support of our sponsors - NSF, NIH, Promega, Thermo Fisher, and the Morgridge Institute for Research.

We look forward to hosting this summer school again in 2019. We hope you will recommend the program to a colleague.

Best wishes,

Josh Coon
Evgenia Shishkova
Laura VanToll

Poster Abstracts

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1. Scott Abernathy

Enzymatic Labeling of Oligonucleotides for Multiplexed LC-MS/MS

Scott Abernathy (Presenter) — University of Cincinnati, Cincinnati, OH, United States; Kayla M. Borland — Ludwig-Maximilians-University Munich, Munich, Germany, Germany; Peter A. Lobue — University of Cincinnati, Cincinnati, OH, United States; Patrick A. Limbach — University of Cincinnati, Cincinnati, OH, United States

Introduction

Multiplexing is an experimental approach that allows multiple samples to be run simultaneously, which not only saves time but also allows for relative quantification between samples. While multiplexing is particularly popular in proteomics, very few methods have been developed for the multiplexed analysis of oligonucleotides by mass spectrometry. Most common have been those approaches that enable duplexing of samples prior to MALDI- or LC-MS analysis. We have been examining the use of various enzymatic strategies that could be adapted for oligonucleotide labeling, enabling multiplexing by LC-MS/MS.

Methods

Developmental work was performed using a series of synthetic oligoribonucleotides of fixed sequence and length. Enzymatic labeling is performed by using E. coli polyadenosine polymerase (PAP) to extend the 3'-termini of the sample by a single nucleotide. PAP extension was tested using guanosine, uridine, and cytidine triphosphates. The resulting reaction products were then purified prior to be analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The extension reaction efficiency was monitored using LC-MS peak height data for the starting and final products of the PAP reaction. Once optimized, similar studies were performed using RNase T1 digested RNA samples. In those cases, special care must be taken to remove residual RNase.

Preliminary Data

Previously, poly adenosine polymerase (PAP) was used to extend oligonucleotides with an azide modified uridine triphosphate. The resulting extended oligonucleotides were then clicked with an alkyne tag, tandem mass tag zero (TMT0), of phenyl acetylene. While this showed promise, the TMT5 tag of deuterated phenyl acetylene proved difficult to analyze. Instead of extending with the azide and then clicking to increase the mass, isotopically labeled nucleotides are available in tri-phosphorilated forms. This would potentially give the mass unit difference while eliminating a step. Initial tests show that GTP and UTP both can get added by PAP enzyme, but the percentage of extension is low (about 8%) at initial incubation conditions. While this percentage of extension is unacceptable for multiplexing, the data also does not seem to show any poly extension. This means that as long as the yield can increase for the extension by manipulating the reaction conditions, the process should be applicable for multiplexing with the isotopically labelled GTP or UTP. Solid Phase extraction (SPE) was used to clean up the sample by removing the PAP enzyme buffer and some excess salts. Additionally, the SPE eliminates leftover substrate from the sample. Because of this, the substrate can be increased significantly in order to make sure that the process is not substrate limited. Once the reaction yield is increased, the strategy of multiplexing oligonucleotides can be further explored.

Novel Aspect

Multiplexing oligonucleotides could produce relative quantification between different samples while reducing run to run variability.

2. Dalal Alonazy

Proteome and Phosphoproteome Analysis of Rice Panicle in Response to Heat Stress

Dalal Alonazy[1*], Ramesh Katam[1†], Peter Scott[2], and Raja Reddy Kambham[3]

Introduction

Rice considered is world's third and most common agricultural consumer product. Annual increase in the global temperature has an adverse effect on the crop productivity and nutritional quality. Relatively, the study on temperature sensitivity in rice is less studied. During the temperature, there will be a lot of physiological, biochemical changes occur in the plant to survive to high temperature level.

Methods

Two cultivars of rice were grown in three sets of conditions. The cultivars selected were the heat tolerant Cultivar 5 and the heat susceptible Cultivar 13. They were grown in controlled chambers in a moderate heat stress, intense heat stress, or control conditions. Protein samples were collected from each plant according to the method of Yang et al. There were three biological replicates for each and two technical replicates, a total proteomics study and a phosphoprotein enriched study. iTRAQ labeling was used for quantification. Peptides were assigned to the spectra with Proteome Discoverer 2.2 using the Uniprot combined database. FDR confidence of .05 was used. Expression data was analyzed using AgriGOv2, Orange data mining, Cytoscape and String database. Selected proteins were modeled according to the method of Roy et all 2010 using I-TASSER and Chimera.

Preliminary Data

Out of total identified 779 Proteins, differentially expressed in heat stress vs control were 477 in Cv5 and 472 in Cv13. Twenty-seven were phosphoproteins. As indicated in the heatmaps below, Cv5 raises total protein abundance levels slightly in heat stress. On the other hand, there is reduction in Cv13 total protein abundance in temperature one but not in temperature two.

Novel Aspect

Drought induced various phosphorylation sites differentially expressed in tolerant and susceptible rice cultivars.

3. Yasmín Álvarez García

The Effects of Microbial Volatiles in an Organotypic Lung Model

Yasmin Alvarez-Garcia [1], Heidi Schoen [4], David J Beebe [2,3], Nancy P Keller [4,5]
[1] Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin
[2] Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, Wisconsin
[3] Carbone Cancer Center, University of Wisconsin-Madison, Madison, Wisconsin
[4] Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin
[5] Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin

Introduction

Polymicrobial infections in the lung are common. To understand these infections, it is crucial to unravel the communication among host and microbe, which is thought to occur via microbial volatile compounds. Previous research has shown that interkingdom volatile communication among the pathogens Aspergillus fumigatus and Pseudomonas aeruginosa causes changes in cytokine response within a human bronchiole lung model (Barkal et al., 2017). Here, we show that cytokines released from a volatile emitting co-microbial environment differ from those released when the pathogens were in mono-culture.

Methods

For the volatile microbial co-cultures, agar media was pipetted into each microbial culture insert. Then, either fungal spores or bacteria was flowed over the surface of the solid media. Cultures were kept at 37°C and transferred into the microscale organotypic devices, bringing the microbial cultures into volatile contact with the bronchial epithelial cells. VOCs were measured using Head Space Solid Phase Microextraction with a GCxGC-TOFMS (Pegasus 4D, LECO). Data were analyzed using ChromaTof software (LECO) and compounds were putatively identified using by comparison to the NIST database. Tentatively identified compounds had similarities of 700 or higher. Analytical standards of 3-octanone, 1-octen-3-ol, 3-methylbutanol, and 2,5 dimethylpyrazine were also tested.

Preliminary Data

Using head space solid phase microextraction gas chromatography-liquid chromatography, volatiles from A. fumigatus and P. aeruginosa in co- and mono-culture were identified at the macro scale and the microscale. Analytical standards of the volatile compounds identified from microbial cultures were used to mimic the volatile interactions by exposing the organotypic lung model to the gases using a customizable, clickable insert. After 24 h of volatile coculture, the media was harvested from the organotypic device for immunological readouts (cytokines, PMN migration, etc).

Novel Aspect

An organotypic lung model can show microbial volatiles' interactions, and their effect on host immune response.

4. Souad Amiar

Bulk lipidomics approach for characterization of Ebola virus protein expressed-cells: investigation of a novel and potential target to break Ebola virus replication cycle

Christina R Ferreira, Metabolite Profiling Facility, Bindley Bioscience Center, Purdue University
Zhuoer Xie, Department of Chemistry, Purdue University
Edwin Gonzalez, Department of Chemistry, Purdue University
Robert V. Stahelin, Medicinal Chemistry and Molecular Pharmacology, Purdue University

Introduction

Ebola virus (EBOV) causes an acute and serious illness, which is often fatal if untreated. Last year, two Ebola infection outbreaks were declared with over than 591. EBOV disease is listed as an urgent need for accelerated research and development by World Health Organisation because at this date there are no Food and Drug Administration-approved efficient drugs. Phosphatidylserine and phosphoinositides are required and sequestered at EBOV assembly and budding sites affecting probably the distribution of these two lipids at the cellular membrane architecture that may have a drastic consequence on host cell pathways and functions that require these two phospholipids. We aim to investigate the modification of cell lipidome post transfection with different EBOV proteins and post virus-like particle entry.

Methods

In order to investigate further potential lipid groups/species were identified by preliminary Multiple reaction monitoring (MRM) profiling, a specific acidic lipid extraction using HCl in addition to Blight and Dyer extraction protocol will be performed from Mock, or EBOV proteins transfected mammalian cells (HEK293) cells. This extraction will allow an enrichment of acidic lipids such as PS in the total lipid fraction. A one directional thin layer chromatography (TLC) will be then performed to separate different lipid classes post lipid extractions and selectively analyse the lipids with DESI-MS in collaboration with Prof. Graham Cooks lab. Then, extract lipids of interest for a deeper and target analysis using High Performance Liquid Chromatography HPLC-MS in collaboration with Purdue Metabolite Profiling Facility.

Preliminary Data

Our preliminary lipidomics analysis was performed on mock, or EBOV proteins: matrix protein VP40, glycoprotein GP, or VP40 and GP or minigenome transfected HEK293 cells using direct injection with a triple quadrupole MS after total lipid extraction using Bligh and Dyer extraction protocol. The minigenome is shortened version of the viral genome that expresses all the virus genes separately which allows a safe study of the virus in a biosafety level 2 conditions. MRM profiling was then performed to analyse more than 10 lipid classes according to their functional groups: phosphatidylcholine(PC)/ sphingolipids (SM), phosphatidylserine, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), cholesterol esters (Chol), ceramides (Cers), triacylglycerols (TAG), free fatty acids (FFA) and acyl-carnitines (AC). Principal component analysis using MetaboAnalyst 4.0, indicated a clear discrimination of mock transfected cells from EBOV protein transfected cells based on the lipids with ion intensity 30% or higher than a blank sample and also on lipid ratios. Indeed, analysed lipid classes in GP and minigenome transfected cells

exhibit distinct pools from mock transfected cells suggesting a dysregulation of lipid metabolism by the expression these two systems. In contrast, VP40 expressed alone, does not affect significantly the lipid profile of select lipid species, 48 hours post-transfection. This preliminary analysis provided us information that specific PS, Chol, Cers, and TAGs were mostly be affected by the expression of VP40, GP and the minigenome. Furthermore, the analysis of ion intensities of different lipid species indicated that PS (34:1) that could be more abundant during VP40 expression in transfected cells. Similarly, TAG and Cers showed an increase of ion intensity of specific species : TAG(48:0)_FA16:0, TAG(50:2)_FA16:0 and Cer(d18:1/16:0), where the 48, 16, 50, 18 correspond to the carbon chain length of the fatty acids and 0, 1 or 2 indicate the number of unsaturation on the carbon chain (double bounds).

Novel Aspect

We aim to determine how the virus proteins regulate host lipid metabolism that may impact virus replication, inflammation, and disease.

5. Shannon Andrews

Cancer Pharmacology Lab, A UW Madison core facility

Shannon Andrews Ph.D., Heather Green Ph.D., Song Guo M.S., Gene Ananiev Ph.D., Prof. F Michael Hoffmann, Prof Ronald Burnette

Introduction

The mission of the Cancer Pharmacology Lab is to advance cancer research by providing expertise; leadership; and bioanalytical assay development, validation and performance in support of the clinical, translational and laboratory research endeavors of UWCCC investigators. Services include: Sample Acquisition, Storage and Shipping, Pharmacokinetic Analytical Assays, Pharmacodynamic Assays, Preclinical Analytic Services.

Methods

Preclinical in vitro pharmacology assays and in vivo (mouse) pharmacokinetics assays are available through contracts with external CROs maintained by the UWCCC Drug Development Core.

The Drug Development Core's Cancer Pharmacology Lab provides analytical services to evaluate the pharmacokinetic and pharmacodynamic properties of new agents and known drugs. We analyze for drug concentrations on plasma, serum, urine and other tissues. We have validated HPLC, LC/MS and LC/MS/MS methods to assay a variety of compounds, including ABT-888, Capecitabine, Cotinine, Perillyl alcohol, polyamines, Phenylephrine, Sorafenib, UAB30, Paclitaxel, pomegranate fruit extract metabolites and other compounds. We also offer method development services for the analysis of novel compounds. In addition, we conduct the data analysis and interpretation of these assays, and report research findings to PIs. We recently added Phoenix WinNonlin software to our systems, which will expand our pharmacokinetic capabilities.

Preliminary Data

Cancer is the second leading cause of death in the United States. The prognosis associated with distant metastases from adult solid tumors is poor for most malignancies. The goal of treatment with systemic therapies, either chemotherapy and/or biologic agents, is primarily palliative for these

patients. There is a need to develop more efficacious and less toxic therapies for the treatment of cancer.

BMN 673 is an orally available inhibitor of poly (ADP-ribose) polymerase (PARP), with an approved generic name of talazoparib. BMN 673 is a novel, high purity, single enantiomer, methylbenzene sulfonate compound. It is the most potent and specific inhibitor of PARP1/2 in clinical development (IC50<1nM) and designed to have an improved therapeutic index relative to existing PARP inhibitors in development.

Measurements of DNA damage and PARP inhibition can be used to evaluate the effect of combination therapy with platinum and PARP inhibitors. Activation of DNA-damage signaling path–ways occurs through phosphorylation of histone H2AX (γ ≥H2AX). RAD51 is involved in homologous recombination repair. In response to DNA damage, RAD51 is re-localized within the cell nucleus to form distinct foci which can be visualized. The product of PARP 1 and 2 enzyme activity, PAR, has been utilized in previous studies as a measure of PARP activity.

These assays have been performed previously in PBMCs and circulating tumor cells (CTC)s.

Many questions about this combination remain. The exploratory correlative studies performed in the CP lab will examine these questions. The plasma pharmacokinetics of BMN 673 will be evaluated as a potential marker of toxicity, specifically thrombocytopenia as well as correlated with PD changes observed in PBMCs by an indirect PK/PD model.

(A Phase 1 Study of BMN 673 in Combination with Carboplatin and Paclitaxel in Patients with Advanced Solid Tumors, Kari Wisinski, MD, and Ticiana Leal, MD.)

Novel Aspect

BMN 673 represents a promising PARP1/2 inhibitor with potentially advantageous features as a therapy for the treatment of human cancers.

6. Pritha Bagchi

A Label-free Quantification Approach to Identify Differentially Expressed Proteins between Wild Type and Transgenic Alzheimer Rat Brains

Pritha Bagchi[1], Eric B. Dammer[2], Geng M. Wang[2], Robert M. Cohen[3], Nicholas T. Seyfried[2]
[1]Emory Integrated Proteomics Core (EIPC), Emory University, Atlanta, GA 30322, USA
[2]Department of Biochemistry, Emory University, Atlanta, GA 30322, USA
[3]Department of Psychiatry and Behavioral Sciences, Emory University, Atlanta, GA 30322, USA

Introduction

Alzheimer's disease (AD) is the most common form of dementia with age being the largest risk factor. A transgenic AD (Tg-AD) rat model was recently developed expressing mutant human amyloid precursor protein (APPsw) and presenilin1 (PS1ΔE9) genes, which are known to cause early-onset familial AD. These rats manifest age-dependent AD pathologies (e.g., deposition of amyloid-beta (Aβ) plaques and formation of neurofibrillary tangles (NFTs) in the brain) as well as cognitive dysfunction. We hypothesized that expression of mutant APP and PSEN1 might also alter the global brain proteome in Tg-AD rats. Therefore, in this study, we combined label-free mass-spectrometry

(MS)-based proteomics with systems biology to investigate protein network alterations in these transgenic AD rats at different ages.

Methods

Tg-AD (n = 13) and wild-type (WT; n = 10) rat brain tissues (frontal cortex) were categorized into three age groups — 6, 12, and 20-month-old. The tissues were lysed in presence of 8 M urea followed by trypsin digestion. Peptides were separated by liquid chromatography (LC) and mass spectra were acquired on an Orbitrap Fusion Tribrid mass spectrometer. Protein abundance was calculated using the label-free quantification (LFQ) algorithm in MaxQuant. Furthermore, we applied weighted gene co-expression network analysis (WGCNA) to identify the correlation patterns among the proteins. Protein modules were defined by dynamic branch cutting. DAVID gene ontology analysis was used to understand biological functions and/or cellular organelles associated with these modules.

Preliminary Data

Our proteomics data verify presence of the human isoform of amyloid beta precursor protein (APP) in Tg-AD rats, the expression of which is positively correlated with age. WGCNA analysis also identifies modules (with hub proteins GFAP, MSN, MBP, etc.) that show strong positive correlation with age. Additionally, some of these modules are upregulated in aged Tg-AD rats compared to wild-type controls. DAVID gene ontology analysis reveals that these proteins are mainly located in mitochondria and are involved in stress response, redox processes, and aging. Conversely, some modules (with hub proteins TMEM33, SEPT6, HSPH1, etc.) show reduced expression of proteins with age; however, most of them are not significantly different between WT and Tg-AD rats. These modules consist of synaptic proteins and those responsible for proper protein folding. There is another module where proteins (e.g., PLD3, VARS, MCEE) are significantly upregulated in Tg-AD rats, but do not show any age dependence. These proteins participate in small molecule catabolic processes and cofactor binding.

Novel Aspect

We identified genotype and age-dependent differential expression of proteins in Tg-AD rat model that are relevant to AD pathogenesis.

7. Nicholas Banahene

O-Acylated Trehalose Probes for Proteomic Profiling of the O-Mycoloylation Post-Translational Modification in Corynebacterineae

Taylor J. Fiolek (Michigan State University)
Nate Holmes (Michigan State University)
Herbert W Kavunja (Michigan State University)
Benjamin M. Swarts (Central Michigan University, PI)

Introduction

Mycobacterium tuberculosis (Mtb), the pathogen that causes tuberculosis (TB), is a member of the Corynebacterineae suborder, which comprises hundreds of important species. TB is a global health crisis with about 10 million new cases and 1.5 million deaths in 2017. The mycobacterial cell envelope includes a unique outer membrane, also known as the mycomembrane, which is the

major defense barrier that confers intrinsic drug tolerance to Mtb. The mycomembrane is typified by long-chain (up to 100 carbons), α-branched, β-hydroxy fatty acids called mycolic acids, which are esterified to various acceptors, including trehalose, forming trehalose mono- and dimycolate (TMM and TDM), as well as protein serine residues, forming O-mycoloylated proteins.

Methods

These structures are generated by a process called mycoloylation, which refers to the Ag85-catalyzed transfer of mycolic acid from the donor molecule TMM to the acceptor molecule. We recently reported an O-acylated TMM analogue containing a terminal alkyne group on the acyl chain (O-AlkTMM-C7), which allowed for metabolic incorporation of the alkyne handle into myco–membrane components and enabled their visualization using click chemistry. In addition, we demonstrated the utility of this probe for metabolic labeling of O-mycoloylated proteins, which facilitated their detection and identification.

Preliminary Data

Here, we describe an expanded toolbox of O-acylated TMM analogues bearing alkyne, azide, trans-cyclooctene, and fluorescent tags, which allowed us to test the substrate tolerance of Ag85 enzymes at the cellular level. In addition, these compounds provide significantly expanded experimental versatility including one- or two-step cell labeling, live cell labeling and rapid cell labeling via tetrazene ligation. Moreover, these compounds allow for the labeling and analysis O-mycoloylated proteins on the whole-proteome level. We have developed a two-step enrichment method that uses a “clickable” TMM analogue (O-AlkTMM-C11) in combination with a previously reported azido biotin reagent bearing an acid-cleavable linker (DADPS) in preparation for mass spectrometry analysis of O-mycoloylated proteins

Novel Aspect

The methods we have developed will facilitate rapid and quantitative global analysis of protein O-mycoloylation in Mtb and related organisms.

8. João Banha Oliveira

Exploiting activity-based probes to facilitate the discovery of novel deubiquitinase (DUB) inhibitors

João Banha Oliveira, Sian Armour, Sheelagh Frame, Jason Mundin and Jason Brown; Ubiquigent Ltd., Sir James Black Centre, Dow Street, Dundee DD1 5EH.

Introduction

The underlying concept of targeting intractable, undruggable proteins by modulating their half-life is an important emerging strategy in drug discovery. Ubiquitination controls the stability of most cellular proteins, and its deregulation contributes to human diseases including cancer, Parkinson’s and Alzheimer’s disease. Deubiquitinases (DUBs) remove ubiquitin from proteins, and their inhibition can induce the degradation of selected proteins, potentially including otherwise ‘undruggable’ targets. The identification of highly selective DUB inhibitors demonstrates that the DUB enzymes are tractable drug targets for a variety of diseases and is an area of intense focus.

Ubiquigent. is dedicated to providing a range of state-of-the-art Drug Discovery Services to support the rapidly growing academic and commercial drug discovery interests in the ubiquitin field.

Methods

Activity-based protein profiling (ABPP) is a powerful chemoproteomic strategy to (i) establish compound-target engagement and (ii) evaluate compound selectivity on a proteome-wide basis within complex biological samples. However, it can also be used to (iii) interrogate the functionality of an entire enzyme class within a given biological condition, and to (iv) map sites within proteins that can be pharmacologically interrogated for drug discovery.

Ubiquitin-Propargylamide (UbPA) is a potent, irreversible and specific inhibitor of all three major DUB families, C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), and Otubains (OUT's). UbPA is N-terminally tagged with the HA peptide sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein and allows for the sensitive identification or purification of DUBs by anti-HA antibodies and/or anti-HA-agarose beads.

Preliminary Data

To define the active 'DUBome', lysates were prepared from cell lines relevant to oncology and neurodegeneration, including MCF7 (breast cancer) and SH-SY5Y (Parkinson's and Alzheimer's disease). Lysates were incubated with the HA–UbPA probe and the active 'DUBome' was characterised by Western blot and by mass spectrometry. We have successfully enriched DUBs using the HA-UbPA probe from lysates prepared from MCF7 cells compared with "no probe" controls and succeeded in identifying DUBs from different families using this approach. Data characterizing the active 'DUBome' in multiple cell lines will be presented.

To evaluate compound specificity, crude cell extracts or live cells were pre-incubated with or without small-molecule compounds, followed by incubation with HA-UbPA. The profile of HA-UbPA-modified DUBs (in the presence or absence of compound pre-treatment) revealed the selectivity of the compounds by both Western blotting and mass spectrometry. The data obtained using the ABPP methodology is compared with the output from an in vitro biochemical DUB activity assay using a ubiquitin-rhodamine cleavage readout.

Ongoing assay development is focussing on optimising the detection of the active 'DUBome' using a variety of experimental and analytical MS strategies, evaluating other probes, and assessing different enrichment strategies.

In conclusion, ABPPs not only assess the activity of the protein target (and target class) of interest, but also help to demonstrate target engagement and assess the selectivity of inhibitors on a proteome-wide scale. Exciting next steps for this strategy are to utilise the important structural information from these studies to inform the design of novel DUB inhibitors, and to provide confirmation of target engagement (and selectivity) in pre-clinical and clinical testing paradigms in animal models and humans when these novel DUB inhibitors reach the clinic. The data obtained within this project will inform our own drug discovery efforts and those of our clients, as we pursue the design of novel DUB inhibitors.

Novel Aspect

The first characterization of the active 'DUBome' in cells relevant to the study of DUBs in disease.

9. Aida Barreiro Alonso

Changes in membrane protein complexes in the absence of PBRM1

Aida Barreiro-Alonso[1], Mercedes Pardo[1], Lu Yu[1] and Jyoti Choudhary[1]
[1]The Institute of Cancer Research, London, UK.

Introduction

PBRM1 or BAF180 is a specific protein from the chromatin remodeling complex PBAF. PBRM1 has been related to key cellular processes as cell division, cell cycle progression and transcription, moreover absence/alteration of PBRM1 expression has been related to cancer development. Likewise, this protein has been considered a tumor suppressor in cancers such a renal and breast cancer however its role in tumoral hallmarks is not clear and can be contradictory depending on the cell type. Cancer associated processes as migration, EMT and invasion are tightly related to membrane changes so in this work we aim to study how the absence of PBRM1 can influence protein membrane complexes. This result may bring to light new PBRM1 cancer-related roles.

Methods

Membrane fractions were isolated from wild type cells and cells lacking PBRM1 using either commercial kits or sucrose cushion and ultracentrifugation procedures. Native membrane protein complexes were separated using Blue Native PAGE and proteins were identified by mass spectrometry. Protein correlation profiles were analysed to study the changes in protein complexes when PBRM1 levels are diminished.

Preliminary Data

Results from a previous study (Roumeliotis et al., 2017) show that knock out of BAF or PBAF components as PBRM1 impair general changes in the cell proteome. To study these changes in the membrane fraction we have developed a strategy that allows us to isolate native membrane protein complexes.

Novel Aspect

Changes in membrane composition in absence of PBRM1.

10. Tynan Becker

Using CFRAPS: a cell free antigen processing system to elucidate the generation of immunodominant peptides

Tynan A. Becker[1] and Thomas Kuhn[2]
[1]Department of Biology and Wildlife and [2]Department of Chemistry and Biochemistry, University of Alaska Fairbanks, Fairbanks, Alaska USA

Introduction

A quintessential step of adaptive immune responses entails the interaction of T cell receptors with pathogen-derived epitopes presented on major histocompatibility complex class II (MHCII) molecules by antigen presenting cells (APCs). Epitopes exhibit a hierarchy of immunodominance, based on the strength of T cell response. The molecular selection process of immunodominant peptides remains elusive. After uptake of pathogens, proteolytic enzymes degrade the proteins in an

increasingly reducing and acidic environment. In the late endosomal MHCII compartment (MIIC), antigenic peptides form complexes with MHCII prior to being shuttled to the APC surface. Determinants of epitope selection may result from a combination of protease actions and the thermodynamics of peptide-MHCII complex formation.

Methods

The essential components of CFRAPS include the protein of interest (influenza H1N1 hemagglutinin (HA) protein), MHCII and three cathepsins (B, H and S) at pH 5.0 or 6.4. Three scenarios are envisioned; first, HA binding to MHCII is executed prior to cathepsin exposure. Second, HA will be exposed to cathepsins, prior to MHCII binding. Third, all components (cathepsins, HA and MHCII) will be incubated simultaneously. In all scenarios, peptides captured by MHCII will be recovered and characterized by mass spectrometry followed by comparison to experimentally established and published immunodominant hierarchy and the peptide library established in-house through previous cathepsin cleavage experiments.

Preliminary Data

Enzymatic digestion of HA was performed at pH 6.4 and 5.0 using cathepsins B, H and S at various time points over a 24-hour time period. One aliquot of each digest was subjected to SDS-PAGE electrophoresis, followed by tryptic in-gel digestion, and peptide sequences were determined using mass spectrometry (MS). The remainder of the in-solution cathepsin digest was separated on a 10k MW cut off filter and both the retentate and the filtrate were analyzed with MS. Data analyses were performed using MaxQuant. The cleavage library was compared to published data on microdomain flexibility of the HA protein as pH changes and the established immunodominance hierarchy.

We discovered that some immunodominant and subdominant peptides were resistant to proteolytic degradation, but not all. There was a slight pH effect and the kinetics appeared slightly slower at pH 6.4 than pH 5.0 possibly due to differences in cathepsin activity. Supporting the idea that immunodominant peptides are determined by the enzymatic activity is the finding that HA peptides, from regions of high hydrophobicity and microdomain flexibility (therefore are degraded early), correspond to peptides that elicit at best, a weak T cell response when applied exogenously. Additionally, two immunodominant and one subdominant peptide exhibit no change or a protective change in flexibility as the pH lowers. However, there are also examples from this same protein that indicate that the factors of cathepsin specificity and microdomain flexibility are not the only variables that determine resistance to enzymatic activity and the selection of immunodominant epitopes. One subdominant peptide is both hydrophobic and highly flexible, yet it remains intact at 24 hours at both pH. For some of the immunodominant and subdominant peptides, the interplay between microdomain accessibility and cathepsin specificity may explain the cathepsin resistant “survival” of the peptides.

Novel Aspect

This work directly examines the intersection of proteolytic activity and thermodynamics in generating the immunodominant peptidome presented to T cells.

11. Christian Beusch

Proteome-wide identification of human protein-metal interactome

Roman A. Zubarev
Division of Physiological Chemistry I, Department of Medical Biochemistry and Biophysics
Karolinska Institutet, SE-17 165 Stockholm, Sweden

Introduction

Protein conformation is an independent regulator of biological systems on top of protein expression. The conformation of a protein is dynamic and defined by the surrounding environment as well as permanent and transient interactions with other biological components such as proteins and small molecules. In this respect, metal ions are known to play a crucial role in regulating protein stability and function and are deemed to be required for proper activity of almost half of all enzymes. Therefore, proteome-wide knowledge on protein-metal interactions can be of significant importance in understanding biology in homeostasis and disease. However, proteome-wide experimental data of protein-metal interactions is currently lacking.

Methods

We have recently enhanced and reformatted thermal proteome profiling (TPP) for 10x higher throughput and increased depth of quantified proteins. The new method called Proteome Integral Stability Alteration (PISA) assay significantly reduces the instrumental time for sample analysis and circumvents the need for curve fitting, thus facilitating thermal stability measurements. We applied PISA to determine the thermal stability of human cell lysate upon treatment with various metal salts (calcium, magnesium, sodium, and potassium).

Preliminary Data

Applying Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA), contrasting the melting points of proteins after exposure to various salts resulted in the identification of specific protein-metal interactome for each treatment. Comparisons of binding events (shifts in melting points of proteins) uncovered by PISA with available databases on metal-protein interaction showed a high overlap, indirectly validating PISA as a suitable method to study the human protein-metal interactome.

Additionally, multiple proteins with altered thermal stability upon treatment, could not be cross-confirmed in any database or by literature, revealing novel protein-metal interactions. Based on these proof-of-principle results, a follow-up experiment was performed extending the list of treatments to 10 metal salts, aiming to build a comprehensive human protein-metal interactome database.

Novel Aspect

Proteome-wide identification of proteins interacting with multiple biologically relevant metal ions by PISA.

12. Kyle Biegas

Development of Clickable Photoaffinity Probes to Identify Outer Membrane Proteins in Mycobacteria

Herbert Kavunja (Central Michigan University)
Benjamin Swarts (Central Michigan University)

Introduction

The Corynebacterineae suborder of bacteria comprises hundreds of species, many of which are of high significance to human health, notably the causative agent of tuberculosis, Mycobacterium tuberculosis. These bacteria are characterized by presence of a glycolipid-rich barrier, known as the mycomembrane that is predicted to enlist a substantial proteome to balance various functions in the cell. However, due to the structural complexity of the mycobacterial envelope and lack of effective tools, the proteomic composition is poorly defined.

Methods

We developed a chemical strategy that permits selective labeling of mycomembrane proteins in living cells followed by analysis on the whole-proteome level. Trehalose analogues were used to metabolically install photoactivatable and click chemistry enabling groups. Upon exposure of live cells to UV irradiation, the dual-labeled glycolipids underwent photocrosslinking with myco-membrane proteins in their native setting, thus covalently tagging the proteins with a terminal alkyne that allowed subsequent click chemistry-mediated protein detection, affinity enrichment, and mass spectrometry-based identification. Peak lists were searched against an M. smegmatis database from Uniprot by MaxQuant and identified by Andromeda. The output was analyzed using Scaffold to validate protein identifications and perform quantitative analysis.

Preliminary Data

When deployed in the model organism M. smegmatis in conjunction with label-free quantitative proteomics, this strategy identified approximately 100 proteins that were statistically over-represented in probe-treated, UV-exposed bacteria, including the known mycomembrane porin MspA and other proteins with known mycomembrane synthesis or remodeling functions, including antigen 85 (Ag85) mycoloyltransferases, and trehalose dimycolate hydrolase (Tdmh). Additional proteins of known and unknown function were identified as candidate mycomembrane proteins, which are of considerable interest for future structural and functional investigation.

Novel Aspect

This strategy is applicable to discovery of mycomembrane proteins across the Corynebacterineae suborder and will serve as a valuable tool.

13. Mehdi Bouhaddou

Unbiased Proteomics and Network Propagation Reveals Cancer Drug Targets

Mehdi Bouhaddou[1,2], Neil Bhola[3], Rachel O,ÄôKeefe[3], Margaret Soucheray[1,2], Hua Li[3], Tian Zhu[3], Kelechi Nwachuku[3], Toni Brand[3], Gordon Mills[4], Dan Johnson[3], Danielle L Swaney[1,2], Jennifer Grandis[3], Nevan J Krogan[1,2]

[1] Cellular and Molecular Pharmacology, University of California, San Francisco.
[2] Gladstone Institutes, San Francisco.
[3] Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco.
[4] Oregon Health and Sciences University, Portland, OR.

Introduction

Head and neck cancer is the seventh most common malignancy worldwide with few treatment options. The only FDA-approved targeted kinase inhibitor to treat the disease is cetuximab, a monoclonal antibody against EGFR to which patients often develop lethal resistance. There is a critical need to understand the mechanisms of drug resistance and discover novel targets whose inhibition could provide synergy with current therapy.

Methods

Here, we integrate proteomic data from cell lines and patients to reveal novel factors underlying cetuximab resistance. To collect proteomics data in cell lines, we first cultured Cal-33 cells (a tongue squamous cell carcinoma) with cetuximab for several months until resistance developed. We then performed global phosphoproteomics and abundance proteomics on both the drug-naïve and resistant models. In addition, for each model, we performed affinity purification mass spectrometry (AP-MS) for 28 of the most commonly mutated proteins in head and neck cancer (including several protein mutants). High confidence interacting proteins were identified and quantified using a multi-step bioinformatics pipeline. First, the open source MaxQuant software was used to convert raw MS data into a list of identified proteins. Next, the CompPASS (Comparative Proteomic Analysis Software Suite) software was used to identify high confidence protein-protein interactions (PPIs).

Preliminary Data

The result is a comprehensive map of changes in protein-protein interactions between the drug-naïve and resistant cell contexts. Phosphoproteomics revealed increased activation of PI3K signaling. Abundance proteomics revealed enhanced recruitment of metabolic pathways. AP-MS data reveals several novel oncogene interactions—many of which drastically change upon drug-induced rewiring. Lastly, an integrative network propagation technique, which incorporates all layers of proteomics data including reverse phase protein array (RPPA) data from head and neck cancer PDX models, reveals a subnetwork of ~100 genes underlying drug resistance and sensitivity. Ongoing studies aim to perform CRISPRa/i screens of identified targets in combination with cetuximab to assess synergistic potential. In sum, the current study reveals proteins underlying the cetuximab resistance phenotype in head and neck cancer. We create a resource map of altered protein-protein interactions and reveal a protein subnetwork signature of drug resistance using a network

propagation procedure to overlay distinct datatypes and extract overlapping features. Potential applications from this study span to other cancer types and drug targets.

Novel Aspect

Mass of proteomics data on isoclonal resistant/sensitive cell line and data integration across five distinct data sources using network propagation.

14. James Byrnes

Characterizing and Targeting the Hypoxic T Cell Surfaceome to Promote Immune Function in Cancer

Lisa Kirkemo[1], Amy M. Weeks[1], and James A. Wells[1,2]

[1]Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA, USA

[2]Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, USA

Introduction

Recent cancer treatment research efforts have focused on developing T cell-based immuno–therapies. However, these therapies have minimal efficacy in solid tumors. One characteristic of the solid tumor microenvironment is low oxygen availability, or hypoxia. Previous studies investigating the effects of tumor hypoxia on T cell function suggest that hypoxia suppresses the anti-tumor immune response. We hypothesize that hypoxia alters the T cell surface protein profile (the "surfaceome") and T cell function in a manner consistent with a net immunosuppressive effect. Using proteomics-based approaches, we aim to reveal new strategies for increasing the anti-tumor function of hypoxic T cells. These findings will inform the engineering of antibody-based T cell therapies to enhance T cell function in regions of immunosuppressive tumor hypoxia.

Methods

Primary CD4+ or CD8+ effector T cells (Teffs), as well as immunosuppressive regulatory T cells (Tregs) were isolated from peripheral blood using magnetic beads. Expanded or naïve T cells were activated using anti-CD3/CD28 stimulation and cultured for 3 days in either normoxia (20% O2) or hypoxia (1% O2). Surface proteins were profiled using an established biocytin hydrazide surface glycoproteomics pipeline and LC-MS/MS. Both forward/reverse SILAC and label-free quantitation approaches were used to monitor hypoxia-induced surfaceomic changes. Future studies will use small-input proteomic strategies to characterize T cells isolated from mouse and human tumor samples. Furthermore, recombinant antibodies against hypoxia-induced proteins will be developed via phage-display and used to probe the role of hypoxia-induced proteins in modulating T cell function.

Preliminary Data

To examine how hypoxia affects T cell surfaceomes in vitro, primary Teffs and Tregs were expanded for two weeks in heavy or light lysine/arginine containing media to ensure complete isotope labeling. LC-MS/MS of surface-enriched proteins from either CD4+ or CD8+ Teffs cultured in normoxia or hypoxia identified over 700 surface proteins, many of which were significantly

repressed or induced by hypoxia. CD4+ and CD8+ Teffs responded similarly to hypoxia (R=0.7, P<0.0001) and responses were similar between two donors (R=0.7, P<0.0001). Consistent with previous reports suggesting hypoxia is immunosuppressive, hypoxia significantly downregulated numerous Teff stimulatory proteins (cytokine receptors, co-stimulatory proteins). In addition to observing previously reported hypoxia-induced proteins, many proteins involved in protein glycosylation and carbohydrate metabolism were upregulated on hypoxic Teffs. Additionally, hypoxia significantly increased expression of established inhibitory proteins PD-1 and TIM-3 in one of the two donors. A preliminary Treg experiment suggested that the Treg response to hypoxia was inversely correlated with that of Teffs (R = –0.6, P<0.0001), with induction of cytokine receptors and co-stimulatory proteins. This suggests hypoxia may enhance Treg activity and contribute to suppressed Teff function in hypoxic tumors.

In an attempt to eliminate the need for long-term in vitro expansion of T cells for SILAC labeling, we utilized a small-scale surface enrichment protocol that permits the use of freshly-isolated naïve T cells. In a pilot experiment using ~10% of the cell input needed for a full-scale SILAC experiment, label-free analysis with MaxQuant identified 273 membrane proteins. Intriguingly, preliminary data from this experiment suggest the surfaceomes of fresh Teffs are less affected by hypoxia than expanded Teffs. Ongoing efforts aim to increase the number of identified membrane proteins using this scaled-down approach and to collect additional data to examine donor-donor variability. These studies will help further illuminate potentially targetable hypoxia-induced proteins on both Teffs and Tregs.

Novel Aspect

This work uses surface-specific proteomics to characterize hypoxic T cell biology and identify new strategies to enhance anti-tumor immune responses.

15. Nicolas Calo

Roles of the Sortilin K269E mutation in systemic glucose and lipid homeostasis

Calo, N.[1], Mitok, K.[1], Schueler, K.[1], Rabaglia, M.[1], Mitchell, B.[2], Keller, M.[1], Shuldiner, A.[2,3], Attie, A[1]
[1]University of Wisconsin-Madison, Madison, WI, USA 53706
[2]University of Maryland School of Medicine, Baltimore, MD 21201
[3]Regeneron Genetics Center, Tarrytown, NY, USA 10591

Introduction

Obesity and type 2 diabetes (T2D), its commonly associated co-morbidity, both greatly increase the odds of developing life-threatening pathologies and have reached pandemic status. No efficient treatments against these illnesses currently exist due to our poor understanding of their complex molecular pathogenesis. The locus of the SORT1 gene, which encodes Sortilin, is one of the strongest hits in genome-wide association studies for elevated circulating Low-density lipoprotein cholesterol, an obesity/T2D-associated trait. Sortilin actively participates in white adipose tissue (WAT), liver, and muscle metabolic homeostasis. How Sortilin participates in the control of whole-body energy balance is still highly debated and unclear. There is an important need to better understand Sortilin physiological and molecular roles, given its potential as a therapeutic target.

Methods

In collaboration with Dr. Alan Shuldiner (University of Maryland, USA), the Attie group identified in an Amish community the first documented human Sortilin coding mutation (K269E) associated with elevated fasting insulin and LDL-C. This mutation changes a lysine (K) to a glutamate (E) at the highly-conserved residue 269 in the luminal cargo-binding domain of Sortilin. We generated a mouse expressing the K269E Sortilin and characterized its metabolic response to a chow (CD) and a high-fat, high-sucrose, cholesterol-enriched western-style diet (WD) (44.6% kcal from fat, 40.7% kcal from carbohydrate, 34% sucrose) for 20 weeks. We used wild-type (WT) and Sortilin knock-out (KO) animals as controls.

Preliminary Data

K269E mice have the same Sortilin protein abundance in WAT, liver and muscles but are predisposed to metabolic imbalance upon WD feeding. Despite unchanged food intake and intestinal fat absorption, K269E male mice exhibit adipocyte hypertrophy and increased adiposity as compared to WT upon long-term WD feeding. Conversely, KO animals are leaner than WT mice. K269E mice show increased WAT insulin sensitivity, suggesting a potentiated adipogenic effect of insulin in these mice that could lead to enhanced fat mass gain.

K269E animals display fasting hypercholesterolemia, hyperglycemia and hyperinsulinemia. They however have normal glucose tolerance upon WD feeding, and an unexpected much higher rise in plasma insulin than WT in response to glucose challenge. Their drastically reduced insulin/C-peptide ratio during the glucose tolerance test (GTT) hints at a decreased insulin clearance. The requirement for more insulin to maintain normal glycemia suggests K269E Sortilin promotes systemic insulin resistance upon chronic metabolic stress.

Hepatic glucose output is increased in the mutant mice, as determined by pyruvate and alanine tolerance tests in CD- and WD-fed K269E animals respectively. This occurring upon standard feeding shows Sortilin K269E exhibit altered metabolic function even under normal physiological conditions. Insulin receptor (InsR) levels are decreased on the hepatocyte surface of K269E animals despite similar total cellular protein expression. The decreased InsR on the hepatocyte membrane likely explains defects in insulin clearance during GTT. It is the first time such a role for Sortilin in insulin homeostasis has been identified.

Overall, the current data obtained in K269E mice is consistent with those observed in humans harboring the mutation (fasting hypercholesterolemia and hyperinsulinemia). KO and K269E animals generally showed opposite behaviors as compared to WT, suggesting the K269E variant is a gain-of-function mutation. Sortilin K269E appears to control InsR localization in hepatocytes, hence causing hyperinsulinemia that could promote weight gain.

Novel Aspect

We identified the first coding Sortilin mutation altering systemic energy balance and potential new Sortilin functions in insulin homeostasis.

16. Dan Castro

Multiscale Approach Provides a New Axis for Single Organelle Peptidomics and Transcriptomics

Penn State University

Introduction

RNA localization and translation within individual axons is an integral process in the guidance of dendritic processes for the formation of new synaptic connections. Aberrant synaptic connections or lack thereof, underlie a multitude of neuropathological disorders. The ability to analyze sub-cellular dysregulations within both the transciptome and peptidome of individual neurons is a vital step in understanding the nuclear transcriptional changes that lead to disease phenotypes. By taking a multi-combinatorial approach we hope to use parallel fluidic devices for high-throughput selective capture to prepare single organelles for mass spectrometry analysis followed by transcriptomics.

Methods

By using microMS, a software program that uses microscopy images to generate coordinate positions of tagged organelles on an ITO-glass slide, we can perform mass spectrometry analysis using a variety of different instruments, such as MALDI MS and capillary electrophoresis MS. A combinatorial approach can be taken by initially performing high-throughput MALDI-MS profiling of organelles and the material remaining from the organelles of interest may be sufficient for follow up characterization using other MS platforms, greatly broadening our analyte detection range.

Preliminary Data

Initial efforts have been directed towards optimizing high-throughput MALDI-MS screening to both establish and optimize analyte detectability, as well as generate compatible MALDI matrices for subsequent transcriptomic analysis. Preliminary experiments involved detection of neuro–transmitters in single cell rat hippocampal dissociates. Hippocampal dissociates are stained with Hoecsht 33342 for fluorescent detection using a Zies Axio Observer. Translatable cell coordinates are then generated using microMS before MALDI-MS analysis. MALDI matrices 1,5-Diamino–naphthalene (DAN), N-Phenyl-2-naphthylamine (PNA), dihydroxybenzoicacid (DHB), and 9-amino-acridine have been tested using a custom-built autosprayer, with PNA showing the best detection of neurotransmitters thus far. Chosen matrices will have to be optimized using a sublimation procedure due to the relatively wet deposition performed by the autosprayer. Further efforts are being directed toward detecting peptides in dense core vesicles with the idea of subsequently moving towards smaller organelle structures.

Novel Aspect

First single organelle measurement.

17. Joseph Cesare

Novel UHPLC-MRM-MS approach allows for absolute quantification of Histone PTMs in as little as 20 minutes

Joseph Cesare[1,2], Zuo Fei Yuan[2], Steven Zhao[3,4], Peder Lund[2], Josue Baeza[2], Yekatarina Kori[2], Simone Sidoli[2], Hee Jong Kim[2], Hyoungjoo Lee[1,2], Kathryn E. Wellen[3,4], Benjamin A. Garcia[1,2]

[1]Quantitative Proteomics Resource Core, Department of Biochemistry and Molecular Biophysics, University of Pennsylvania, Philadelphia PA, 19104 USA

[2]Epigenetics Program, Department of Biochemistry and Molecular Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104 USA

[3]Department of Cancer Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104 USA

[4]Abramson Family Cancer Research Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia PA, 19104 USA

Introduction

Histones are structural proteins that modulate chromatin structure and gene expression. Their post translational modifications (PTMs) recruit transcription factors and directly affect chromatin state through chemical interactions. These modifications have been found in several disease states and are important for development and aging. Mass spectrometry has been the method of choice for global histone PTM analysis. However, current methods available have their limitations (Creech et al. Methods 2015, Sidoli et al. JOVE 2016) including extensive instrument time, batch effects as a result of nano-chromatography, and interreference ions affecting the quantification of low-level marks. To address these challenges, we outline a UHPLC-MRM-MS method for targeted histone quantification that is more reproducible, robust, and allows for absolute quantification in high throughput experiments.

Methods

A synthetic histone library of 93 peptides was scheduled on a TSQ Altis (Thermo Scientific) coupled to a Vanquish UHPLC with a 20-minute gradient at 300ul/min. Once scheduled, a calibration curve was generated using 3 technical replicates at each of 6 concentrations. To account for isobaric species, the synthetic peptide library incorporated heavy labeled amino acids so that each peptide had a unique precursor m/z. Product ions were selected based on unique m/z, linear fit, % coefficient of variation (CV), and sensitivity. Scheduled data acquisition was applied to cell lines and tissue prepared as previously described. A model to quantify isobaric species and other targets using the concentration curves was developed and incorporated into our EpiProfile 2.0 software.

Preliminary Data

Using analytical flow ultra-high-pressure liquid chromatography (UHPLC) coupled to a triple quadrupole mass spectrometer using multiple reaction monitoring (MRM) acquisition mode, 93 peptides and 80 known PTMs were analyzed in only 20 minutes. The calibration curves demonstrated a median LOD of 0.094, a median LOQ of 0.283, and a median R-squared of 0.98 across all product ions. The method obtained highly reproducible results with a median coefficient of variation 3.9%. A synthetic histone like peptide was used as an internal quality control to demonstrate efficient digestion and chemical derivatization. This method was applied to a human

derived cell lines and mouse tissue and was compared to nano-liquid chromatography coupled to data independent acquisition (DIA) demonstrating more robust quantification than previous methods being used.

Novel Aspect

Absolute quantification of histone PTMs can be accomplished in 20 minutes opening new opportunities in epigenetics and translational medicine.

18. Christopher Chermside-Scabbo

Proteomic Characterization of Young and Aged Murine Bones in Response to Mechanical Loading

Christopher Chermside-Scabbo and Matthew Silva
Department of Orthopaedic Surgery, Washington University in St. Louis, MO

Introduction

Fifty million people in the United States suffer from osteoporosis or low bone mass. Thus, there is need for additional strategies to increase bone mass and prevent osteoporotic fractures, which cost more than $22 billion annually. A potent physiological strategy to increase bone formation is mechanical stimulation, but with aging, bone becomes less responsive to the same stimulus. The reason for this decline remains unclear. Our overall goal is to restore the mechanoresponsiveness of the aged skeleton. Previous studies suggest impaired mechanosensation in aged bones, but little is known about the early protein-level changes following loading and if they are altered with aging. Our objective is to characterize these changes in young and aged mice using proteomics.

Methods

We will obtain young (5-month) and aged (22-month) C57BL/6 mice. Female mice will initially be studied. Right tibias will be loaded cyclically while left tibias will serve contralateral, non-loaded controls. Tibias will be harvested 6 hours after the loading bout, which represents a biological state of mechanosensation before bone is formed. At sacrifice, left and right tibial mid-diaphyses will be isolated, and centrifugation will remove the bone marrow. Next, the samples will be pulverized, and protein will be extracted in SDS buffer. The Proteomics Core will perform LC/MS-MS on the supernatant, and differentially expressed proteins between loaded and non-loaded tibias will be compared at both ages. These proteomics data will be compared to previously collected transcriptomic data.

Preliminary Data

Because proteomics on bone tissue is so novel, it was necessary to determine the optimal pulverization/protein extraction methods for murine bone for experiments. The considered methods used SDS buffer per standard Proteomics Core workflow and included: mincing with scissors, Covaris impactor, and bullet blender. At a protein confidence threshold of 99.9%, across the 3 methods, 2784 unique proteins were identified. All 3 methods identified the same 1534 proteins (1534/2758, 56%), but compared to the bullet blender (1715/2758, 62%), the Covaris impactor (2539/2758, 92%) and mincing with scissors (2251/2758, 82%) methods were far superior. Based on these results, the Covaris impactor method was chosen as the extraction method of choice for the

loading study. The next study will investigate the biological reproducibility of 3 different states in 3 different biological replicates. For the loading study, mice are being ordered, and these experiments will begin within 2 months (in time to have preliminary results for the program).

Novel Aspect

We will be the first to use proteomics study the age-related changes in the mechanical loading response of bone.

19. Natalie Clark

Generating multi-scale predictive networks of Northern Corn Leaf Blight resistance

Gaoyuan Song, Iowa State University; Nick Lauter, Iowa State University; Justin Walley, Iowa State University

Introduction

Northern Corn Leaf Blight (NLB) is one of the most significant corn diseases, causing the largest crop loss of any disease in the Northern United States from 2012-2015. Understanding how gene expression correlates with specific disease phenotypes is critical for developing disease-resistant crops that enhance global food security and limit economic losses. However, the underlying proteomic regulatory mechanisms mediating resistance to NLB are not well understood. Thus, the goal of this study is to integrate proteomics measurements, disease phenotyping, and compu–tational modeling to build predictive networks of NLB resistance.

Methods

Two independent replicate blocks of 220 Intermated B73xMo17 Doubled Haploid Lines (IBMDHLs) were planted. Each replicate block was planted in a randomized complete block design and each IBMDHL was planted as a twin plot of 15 plants per plot (30 plants per line total). Plants in one of the replicate blocks were treated with mock and NLB inoculum at the sixth and seventh vegetative stages. Seven days after inoculation, leaf material was collected from 3 mock and 3 infected plants for each of 96 of the IBMDHLs. Mass spectrometry will be performed on a Thermo QExactive Plus high-resolution quadruple Orbitrap mass spectrometer, using 11-plex Tandem Mass Tag (TMT) peptide labeling, where each multiplex contains 10 experimental samples and 1 pooled reference sample for normalization between runs.

Preliminary Data

We are currently processing the collected leaf material using mass spectrometry as described in the methods. Previously, we have coupled improved sample preparation with TMT labeling to quantify ~8,000-10,000 proteins, from up to 11 independent samples, in a single 2-dimensional liquid chromatography nanoelectrospray tandem mass spectrometry (2D-LC-MS/MS) run. Thus, we are confident that our methods will produce a large number of detected proteins for downstream analysis. After these data are obtained, I will first determine proteins that are differentially abundant between mock and infected plants within each IBMDHL, as well as between lines. These differentially abundant proteins will be combined with collected disease phenotype data on these lines to correlate changes in protein abundance with phenotype. I also expect to incorporate gene ontology

analysis (GO) of the corresponding coding genes for these proteins to gain a general understanding of their biological functions. After differential abundance analysis, I will perform QTL mapping to identify protein (pQTL) loci that significantly contribute to disease QTLs identified from our phenotypic analysis. This analysis will likely identify thousands of pQTLs that potentially contribute to NLB resistance. To narrow down this list of candidate QTLs to the best (i.e., highest confidence) candidates, I will use a combination of co-expression network analysis and machine-learning-based network construction to infer a protein regulatory network. From this network, I will use techniques such as network motif analysis to predict the proteins that are most important for regulating NLB resistance. The expected results from this work are a list of proteins that have been predicted, from protein abundance data and network inference, to be important for determining the degree of NLB resistance in maize.

Novel Aspect

This study will produce the first large-scale dataset of proteins that regulate NLB resistance in corn.

20. Caitlin Colleary

Microwave-assisted acid hydrolysis for whole bone proteomics and paleoproteomics

Timothy Cleland, Research Scientist, Smithsonian Museum Conservation Institute

Introduction

Paleoproteomic studies often demineralize fossil bone samples before enzymatic digestion, a process that first requires powdering samples and then an incubation period of up to several days. Recent studies have shown advantages to digesting demineralized bone pellet resulting in detection of less soluble parts of the bone proteome (e.g., glycosylation). Despite the advantage of digesting the solid, demineralized matrix, the demineralization process remains slow. A possible avenue to overcome the long incubation time is microwave-assisted acid hydrolysis that can do both demineralization and breakdown the proteins to peptides in a short time period. Here, we evaluate the formic acid and acetic acid hydrolysis for bone proteomics and paleoproteomics.

Methods

We hydrolyzed femur fragments (~50 mg) from an American moose (Alces alces) with either 12.5% formic acid or 12.5% acetic acid using an ETHOS UP High-Performance Microwave Digestion System for 30 minutes at 140 °C. An additional sample of moose bone was analyzed by homogenizing the bone into a non-demineralization buffer, followed by digestion of the proteins using single-pot, solid-phase-enhanced sample preparation (SP3) and digested using trypsin. Proteins were then separated on a self-packed Thermo BioBasic C18 column on an Ultimate 3000 and analyzed on a LTQ Orbitrap Velos. Spectra were searched in PEAKS 8.5 against Cervus (Uniprot), Bos taurus (Uniprot) and Odocoileus virginianus (NCBI) databases.

Preliminary Data

After 30 minutes, formic acid digestion was complete, but there were still pieces of bone in the acetic acid. 27 and 14 protein groups were detected from formic acid and acetic acid hydrolysis, respectively. Collagens (e.g., collagen I and XI), osteocalcin, proteoglycans (e.g., biglycan) and

glycoproteins (e.g., thrombospondin) were detected in both acids. Sequence coverage was an average of 10% higher in the formic acid sample. Microwave-assisted formic acid digestion has been previously shown to cleave at the C-terminal of Aspartic Acid (D), whereas acetic acid cleaves on either or both sides of aspartic residues. However, we found that in the formic acid sample, 52% of cleavages were on the C-terminal of D and 48% were on the N-terminal. This is in contrast to the acetic acid sample where 80% of cleavages were on the C-terminal, with 20% on the N-terminal. Other less specific hydrolysis was detected at glutamic acid, glycine and lysine. Formylation, a known problem with formic acid hydrolysis, was detected on 7% of the peptides.

Using the whitetail deer (Odocoileus virginianus) database, we compared the acid digested samples to the same bone that was prepared using trypsin digestion. The trypsin-digested bone had 37 protein groups exceeding both acid methods. Collagen I alpha 1 sequence coverage was higher for the trypsin digest (57%) than for the formic acid (48%) or acetic acid (33%) digestions, although they covered similar regions. Osteocalcin made up a significant amount of sequence coverage for both formic and acetic acid digestions (27% and 29% respectively), but was not detected in the trypsin digest.

Novel Aspect

Microwave-assisted acid hydrolysis simplifies preparation and minimizes the total amount of time needed for sampling in bone proteomic studies.

21. Valentine Courouble

Developing novel enrichment strategies to facilitate proteomic analysis of NR5A2 in triple negative breast cancer

Yuanjun He - The Scripps Research Institute
Ruben Garcia-Ordonez - The Scripps Research Institute
Patrick R. Griffin - The Scripps Research Institute

Introduction

Liver receptor homolog-1 (LRH-1) is a nuclear receptor expressed in tissues of endodermal origins controlling various cellular processes including lipid and cholesterol homeostasis. Disruption of the receptors regulatory mechanisms leads to aberrant activity resulting in diseases and cancers. LRH-1 dysregulation has been identified in triple negative breast cancer (TNBC). Considering TNBC tumors lack estrogen receptor, progesterone receptor and human epidermal growth factor receptor 2 making it resistant to traditional hormone therapies like Tamoxifen and Herceptin. As such, there is great interest in finding alternative treatments. Our lab discovered SR1848, a small molecule capable of reducing LRH-1 target gene expression and inhibiting tumor growth. An exact mechanism of action (MOA) of SR1848 remains unknown.

Methods

Synthesis of the SR1848 PROTAC probe was completed in collaboration with Dr. Kamenecka's lab at Scripps, Florida. MCF7 cells were treated with 10μM of PROTAC probe for 6h and 12h or 12h with prior 6h treatment with 10 μM MG132 a proteasome inhibitor. Whole cell lysates where separated on an SDS-PAGE gel into three fractions followed by reduction, alkylation, and in-gel digest.

Proteomic analysis of three injections per gel fractions was carried out using an UltiMate™ 3000 RSLCnano (Thermo Fisher) system with data acquisition on an Orbitrap Fusion™ Lumos™ (Thermo Fisher). Data processing was conducted on Proteome Discoverer™ 2.2 (Thermo Fisher). Changes in LRH-1 protein levels in response to PROTAC probe treatment was analyzed by western blot.

Preliminary Data

Preliminary analysis of SR1848 activity using confocal imaging and co-transfection promoter-reporter assays demonstrates that while LRH-1 does not reduce total LRH-1 protein expression levels it causes dispersion of LRH-1 resulting in functional inactivation of the receptor. Using a panel of biophysical techniques including HDX-MS, fluorescence polarization competition assay and NMR, we failed to see direct interaction between SR1848 and LRH-1 ligand binding domain (LBD). We thus hypothesized that the Michael Acceptor moiety present in the molecule may be forming covalent adduct with the receptor's DNA binding domain. After expressing full length LRH- 1 in *E.coli* our initial analysis using native MS failed to show covalent adduct formation between the molecule and full length protein. Current efforts are underway to complete the same panel of biophysical techniques described above. The lack of direct interaction between SR1848 and LRH- 1 suggests a novel MOA compared to other nuclear receptor antagonists. As such, we are attempting to identify an alternative molecular target for SR1848, using a proteolysis targeting chimera (PROTAC) probe. The heterobifunctional probe will bind to SR1848's molecular target as well as an E3 ligase resulting in the ubiquitination of the target and subsequent degradation. Preliminary analysis of PROTAC probe in a panel of TNBC cells as well as MC7 cells failed to show degradation of LRH-1 further suggesting that SR1848 has an alternative molecular target. Label-free quantification analysis of PROTAC probe treatment in MCF7 lines have generated a list of 13 significantly degraded protein, that are currently being further investigated as well as replicate label-free quantification analysis in MCF7 and TNBC cell lines.

Novel Aspect

We are elucidating a novel mechanism of action for a small molecule repressor of the nuclear receptor LRH-1.

22. Taylor Courtney

Optochemical Control of Biological Processes

Alexander Deiters, Department of Chemistry, University of Pittsburgh

Introduction

Protein phosphatases are critical components of signaling networks involved in embryonic development, metabolic homeostasis, stress response, cell cycle transitions, and other essential functions. Unlike their kinase counterparts, protein phosphatases remain understudied and are often poorly characterized. Traditional genetic and biochemical methods have contributed significantly to our current understanding; however, these methodologies lack precise spatial and temporal control. Thus, the development of tools to study these processes with high spatiotemporal resolution are desirable. The use of light as an external trigger provides the desired resolution needed; therefore, an optically-controlled protein phosphatase would be beneficial for dissecting complex signaling networks.

Methods

We have utilized established unnatural amino acid mutagenesis methods for incorporating caged cysteine and caged lysine into MKP3. Confirmation of unnatural amino acid incorporation has been confirmed via western blot analysis. Cellular testing of photoactivation of phosphatase function was performed in HEK293T cells with a fluorescently tagged reporter construct.

Preliminary Data

We have developed the first light-activated protein phosphatase, the dual specificity phosphatase 6 (DUSP6 or MKP3). Through genetic code expansion with unnatural amino acids, MKP3 was placed under optochemical control via two distinct approaches: (i) incorporation of a caged cysteine into the active site for controlling catalytic activity and (ii) incorporation of a caged lysine into the kinase interaction motif for controlling the phosphatase-substrate interaction. Applying the optically triggered MKP3 in conjunction with live cell reporters, we have discovered that ERK nuclear translocation is regulated in a graded manner in response to MKP3 activity.

Novel Aspect

Optical control of protein phosphatase function enables dissection of cell signaling networks.

23. Caitlin Cridland

Inositol pyrophosphates and the cross-talk between lipids and phosphate sensing

Eric Land; Dept. of Plant and Microbial Biology, North Carolina State
Sherry Hildreth; Dept. of Biochemistry, Virginia Tech
S. Phoebe Williams; Dept. of Biochemistry, Virginia Tech
Rich Helm; Dept. of Biochemistry, Virginia Tech
Imara Perera; Dept. of Plant and Microbial Biology, North Carolina State
Glenda Gillaspy; Dept. of Biochemistry, Virginia Tech

Introduction

Phosphate (Pi) is an essential nutrient for plants, required for plant growth and seed viability. Under Pi stress, plants undergo dynamic morphological and metabolism changes to leverage available Pi, including the breakdown of membrane phospholipids. Plants have been shown to “remodel” their lipid membrane profiles under phosphate starvation, degrading phospholipids in the cell membranes and utilizing the generated Pi for essential biological processes. The inositol phosphate (InsP) signaling pathway is a crucial element of the plant’s ability to respond to changing energy conditions. Inositol hexakisphosphate (InsP6) is the most abundant InsP signaling molecule and can be phosphorylated further by VIP kinases, resulting in inositol pyrophosphates (PPx-InsPs). PPx-InsPs have high energy bonds and have been linked to maintaining Pi and energy homeostasis in yeast.

Methods

Using liquid chromatography-mass spectrometry and tandem mass spectrometry, we are examining the lipid profile of an Arabidopsis vip double mutant, in response to phosphate depletion, to address the role of PPx-InsPs in Pi sensing.

Preliminary Data

By concomitantly inducing a phospholipid hydrolysis pathway and galactolipid biosynthetic pathway, membrane phospholipids are replaced by non-phosphorus containing galactolipids and sulfolipids under Pi stress. We have identified an altered lipid profile in Arabidopsis mutants with perturbations in PPx-InsPs.

Novel Aspect

Our results suggest that PPx-InsPs play a crucial role in Pi sensing and are involved in the regulation of lipid biosynthesis.

24. Emily Cushing

Investigating the role of Ptpn18 in regulating diet induced obesity

Mark Scalf, Shane Simonett, Donnie Stapleton, Kathy Schueler, Mark Keller, Lloyd Smith, Alan Attie

Introduction

Obesity is a complex disorder combining nutritional, physiological, behavioral, and neurological factors. It has become a global epidemic, affecting both adults and children. In a genetic screen, our laboratory recently identified a gene (Ptpn18) that demonstrated shared genetic architecture with the insulin secretion locus. Through CRISPR/Cas9 gene editing, our lab derived a knock-in mouse with a single amino acid substitution in Ptpn18: D181A that abolishes its phosphatase activity and causes the mutant protein to become covalently bound to substrate. The knock-in mice have a dramatic resistance to diet-induced obesity and improved insulin sensitivity. Therefore, I hypothesize that Ptpn18 is a critical regulator of weight gain and adipocyte or macrophage proliferation.

Methods

I will prepare adipose and pancreatic islet tissue lysates from Ptpn18D191A and control mice, and purify phosphotyrosyl peptides in an unbiased manner by Affinity Purification Mass Spectrometry (MS) using as an affinity reagent, a Src homology 2-domain-derived Superbinder, which has been shown to have higher specificity for phosphotyrosine-modified proteins than universal phosphotyrosine antibodies. Peptides will be characterized by MS/MS. These experiments will provide a catalog of proteins that show differential phosphorylation at specific tyrosine residues in adipose and islet tissue from Ptpn18D191A vs control mice. The positive control for this experiment will be EGFR, a previously identified substrate of Ptpn18.

Preliminary Data

The results of the proposed experiments will significantly advance our understanding of the function of Ptpn18 and its link to diet-induced obesity. Through the discovery of direct substrates of Ptpn18, I expect to find a novel pathway for regulation of adipose tissue function and its response to diet. While Ptpn18 is unlikely to be a direct therapeutic target, it is possible that the pathway I discover can be mined for therapeutic targets.

Novel Aspect

A physiological role for PTPN18 in obesity has not been described; identifying substrates may lead to therapeutic targets.

25. Li Dai

Quality Control for Biomarker Determination for the Early Diagnosis of Psoriatic Arthritis

Li Dai[1], Sophie Belman[2], Bingjian Feng[2]
[1]Department of Pediatrics, University of Utah; [2]Department of Dermatology, University of Utah

Introduction

Psoriasis (Ps) is a chronic, inflammatory skin disease affecting approximately 3% of Americans. Up to 30% of Ps patients develop psoriatic arthritis (PsA), which can lead to joint destruction, functional limitations, and increased mortality. The damaging effects of PsA can be minimized with appropriate early therapy, but early treatment is often impossible because PsA is frequently undiagnosed. Biomarkers, particularly those present in the early stage of the disease, will greatly facilitate the early diagnosis of PsA.

Methods

Profiling of proteomes from human blood samples is key for clinical research and biomarker discovery, and the successful biomarker determination is largely depended on the quality control of blood sample collection and processing. Furthermore, a consistent and validated protocol of blood sample collection and processing for proteomic profiling will benefit the data and sample sharing between multiple institutions, which is important to overcome the challenge of sample size in one institute, a usual problem for common diseases. In this study, we have started to develop the quality control protocol for mass spectra-based biomarker determination for the early diagnosis of PsA. We have evaluated the blood collection and processing methods by comparing the different procedures usually applied at clinical and research labs and use the mass spectra-based biomarker identification as output. These include the blood collection method evaluation (difference among EDTA-plasma, heparin-plasma, ACD-plasma, and serum), protein degradation evaluation during collection, transport and storage, and pipeline evaluation. Our preliminary data using Tandem Mass Tagging Mass Spectrometry (TMT MS) have identified more than 400 proteins across different sample collection and processing procedures. These proteins will be used to compare and integrate with other independent pathway based proteomic profiling and genomics dataset, and provide possible biomarkers present in the early stage of PsA.

Preliminary Data

Our preliminary data using Tandem Mass Tagging Mass Spectrometry (TMT MS) have identified more than 400 proteins across different sample collection and processing procedures. These proteins will be used to compare and integrate with other independent pathway based proteomic profiling and genomics dataset, and provide possible biomarkers present in the early stage of PsA.

Novel Aspect

This will be the first evaluation of proteomics profiling in PsA.

26. Geoffrey Dann

Investigating the mechanism of arginyltransferase enzyme 1 using mass spectrometry-based approaches

Benjamin A. Garcia, University of Pennsylvania

Introduction and Methods

Protein post-translational modifications (PTMs) provide an essential and dynamic layer of regulation that allows biological systems to operate at levels of complexity beyond that afforded by the genetic instructions hardwired into their DNA. Enzymes that install and remove, or proteins that interact with PTMs, all exist as therapeutic targets, underscoring the importance of PTM signaling pathways in cellular physiology and their widespread dysregulation in human disease. Arginylation is an emerging and poorly understood post-translational modification that involves the tRNA-mediated enzymatic addition of arginine to protein and polypeptide N-termini and internal acidic residue sidechains by the arginyltransferase ATE1. We aim to characterize the arginine transfer reaction using a variety of mass spectrometry-based approaches.

Preliminary Data

We are, for the first time, reconstituting in vitro the arginyltransferase reaction using entirely human components. The enzymatic transfer of arginine to protein substrates begins by aminoacylation of an arginine specific tRNA (tRNAArg) by the human arginine aminoacyl-tRNA synthetase (RARS). The aminoacylated tRNAArg (aatRNAArg) is then engaged by arginyltransferase enzyme 1 (ATE1) to transfer arginine to protein substrates. This mode of engagement and the mechanism of arginine transfer, however, are entirely unknown. Preliminarily, we have been able to obtain RARS and tRNAArg of sufficient purity for biochemical studies. Current and future work will employ hydrogen-deuterium exchange mass spectrometry (HDX-MS) to map the tRNAArg-ATE1 binding interface. This will be complemented by a mass spectrometry-driven in vitro RNA-protein crosslinking approach. We anticipate the resulting data will point to critical residues within ATE1 that are responsible for tRNAArg recognition as well as the arginine transfer reaction. The function of such residues will be tested both in vitro and in cell-based ATE1 activity assays using ATE1 mutants and model peptide and protein substrates.

Novel Aspect

We are mapping the interaction interface between arginine-specific tRNA and arginyltransferase enzyme 1 to better understand the arginine transfer reaction.

27. Chris Davis

Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry Analysis of the 13C-enrichment of Serum Retinol to Evaluate Efficacy and Effectiveness of Vitamin A Interventions

Christopher R. Davis – University of Wisconsin-Madison
Bryan M. Gannon - University of Wisconsin-Madison, Cornell University
Jesse Sheftel - University of Wisconsin-Madison
Sherry A. Tanumihardjo – University of Wisconsin-Madison

Introduction

Maize is a staple crop in many parts of Africa and the Americas and has been targeted for biofortification with provitamin A carotenoids to provide vitamin A (VA) to potentially deficient populations. VA assessment in humans can be challenging due to the homeostatic control of serum VA concentrations. Retinol synthesized to contain two to ten 13C atoms on the backbone of the retinol moiety has been utilized to estimate body stores of VA. Additionally, changes in the natural enrichment of 13C in serum VA can be used for effectiveness studies to compare the contribution to body VA of C4 plant-derived provitamin A to that from C3-plants due to differences in natural 13C enrichment in these sources.

Methods

A VA-biofortified maize efficacy trial was performed in Zambian children. Total body VA before and after the intervention was estimated using the 13C-retinol isotope dilution (13C-RID) test, assessed using gas chromatography-combustion-isotope ratio MS (GCCIRMS). A follow-up study in Mongolian gerbils (n = 55) investigated changes in the 13C-enrichment (measured asδ13C) of serum retinol following consumption of provitamin A-containing orange maize (a 13C-enriched C4 plant) and high-carotene carrots (a C3 plant). The design was 2x2x2: maize (orange vs. white) by carrot (orange vs. white) by VA fortificant (VA+ vs. VA-) with a 62-d treatment. Liver VA and serum retinol were quantified, purified by HPLC, and analyzed by GCCIRMS.

Preliminary Data

The biofortified orange maize fed to Zambian children was as efficacious as a daily VA supplement at the end of the trial. Furthermore, the 13C-RID test revealed that 59% of the children had hypervitaminosis A from the combination of five years of high-dose supplementation, consumption of VA-fortified sugar, and a diet rich in provitamin A carotenoids.

In the study with gerbils, treatments affected liver VA concentrations (0.048±0.039 to 0.79±0.24 μmol/g, $P<0.0001$). 13C-enrichment of serum retinol and liver VA were significantly correlated ($R2=0.92$, $P<0.0001$). Serum retinol δ13C was significantly different in control groups consuming white maize and white carrots (–27.1±1.2 δ13C ‰) from treated groups consuming orange maize and white carrots (–21.6±1.4 δ13C‰, $P<0.0001$) or white maize and orange carrots (–30.6±0.7 δ13C ‰, $P<0.0001$). Using these results a prediction model was established which can estimate the relative contribution of carotenoids from orange maize to the total dietary VA for groups consuming VA from mixed sources.

In a previous trial where orange maize was also fed to rural 3-5 year old Zambian children, 13C natural enrichment in the retinol from serum was greater in the group fed orange maize (n = 38) than that what was found in the white maize control group (n = 38, P = 0.049). The predictive model from the aforementioned gerbil study was applied to this set of data, which estimated that the orange maize was 11% (2-21%, 95% confidence interval) of the recent dietary VA these children consumed.

Novel Aspect

Establishment of predictive equation for proportion of VA from C4 plants, and elevated VA stores found in assumed-deficient Zambian children.

28. Nicholas Del Grosso

"Lean" Mass Spectrometry: Using Kanbans to Uncover the Hidden Recording Capacity of Existing Laboratory Hardware

Matthias Mann, Max Planck Institute of Biochemistry

Introduction

In many mass spectrometry workflows, the combined elution time of the HPLC and mass analyzer represent the primary bottlenecks. Long lead times between the start and end of an experiment limit the total number of feasible pilots in a project, samples in an experiment, and complexity of a research question. To compensate for long lead times during instrumentation bookings, researchers "overbook" (reduce risk of running out of time before recordings are complete), "phantom book" (reduce lead times by making bookings before experiments are planned), and "shotgun-book" (reduce risk of bad recordings by making large recording batches); these efforts, however, only further increase lead time. To uncover the "hidden capacity" of our mass spectrometry laboratory, we implemented techniques from the Lean Manufacturing field to build an instrument booking system that more effectively manages our mass spectrometers.

Methods

We reduced the lead time introduced by our lab's calendar-based instrument booking system by changing it to a single-queue kanban system. Autosampler sequence files were used as the batch submission data structure, which further increased the transparency of the recording sequence planned; besides reducing overbookings, this served to increase knowledge of the various experimental designs and methods used between projects and increase the reliability of metadata for downstream data analysis. Finally, weekly scheduling meetings were used as a discussion platform to spread awareness of new techniques and potential bottlenecks between group members; these meetings used kanbans as an agenda system to emphasize the relationship between batch size, capacity, and the need for prioritization during high demand.

Preliminary Data

We began piloting this booking system on a small subset of the total instruments available in the pool. In the two months since we began this project, we've seen a number of improvements in our total capacity. Overbooking was completely eliminated, and average batch sizes were reduced by almost half, resulting in a new demand level that stayed in line with our capacity. We also saw increased acceptance of new, efficient recording methods, as the productivity benefits of the new system were made visible on the kanban queue. The group rescheduling meetings produced fruitful discussions that resulted in a new focus on iterative piloting and methods for fairly distributing resources among researchers. Finally, this method allowed maintenance, weekend and evening instrument scheduling, and repairs to be made smoothly even during unplanned events; as a result, over 80% of the batches booked in this system were completed within 8 days. This kanban booking system has recently been expanded to include a much larger pool of our instruments, with further visual management innovations expected to uncover more capacity in our existing system.

Novel Aspect

Increasing awareness of Lean Manufacturing fundamentals is an unusual, yet effective, method for making advances in the mass spectrometry field.

29. Kalina Dimova

Sarcoplasmic Proteome Changes in Response to Aging and Diet

Marguerite Pacheco[1], Emily Morris[1], Kalina Dimova[1,2], Sean Garvey[3], David Russ[4], Trevor Hawks[4], Stylianos Scordilis[1],[2].

[1]Smith College Department of Biochemistry, [2]Smith College Center for Proteomics, [3]R&D Abbott Nutrition, [4]Ohio University Laboratory for Integrative Muscle Biology, Division of Physical Therapy.

Introduction

Age-related loss of muscle function affects as many as 30% of adults over the age of 60 and can greatly increase their risk of injury and lower quality of life as it hinders their mobility and independence. Insights into the molecular mechanisms of muscle aging can be gained by looking at changes in the skeletal muscle proteome with age and may lead to clues about meaningful intervention. Dietary intervention by omega-3 fatty acid enrichment (O3, from fish oil) has been anecdotally endorsed to have an effect on these mechanisms. A number of anti-inflammatory effects known to reduce oxidative injury have been ascribed to fish oil and it has therefore been suggested that O3 may reduce the age-associated accumulation of markers of inflammation and oxidative injury in skeletal muscle, where they may contribute to loss of mass and force production.

Methods

It was predicted that an O3 diet would result in an increased expression of oxidative stress protection proteins due to observed trends in other studies and links between FA reducing inflammatory responses The increase in protective proteins should lead to less inflammation-induced muscle damage, a damage seen in aging, and an increase in muscle quality.

Preliminary Data

The tetanic strength of skeletal muscle was positively impacted by an O3 diet, but that change is not explained by the gel-based proteomics data. This positive impact may be due to sarcomere proteome changes rather than changes in the sarcoplasm.

Aging showed a decrease in expression of oxidative stress protection proteins, which has been studied before. This effect held true for aging with and without the O3 diet. O3 diet had a slight negative effect on oxidative stress protection proteins, opposite to our predictions. Interestingly, only superoxide dismutase increased with O3 diet, which was one of the proteins cited to increase in the presence of polyunsaturated fatty acids. O3 diet could be decreasing oxidative stress protection expression by inducing the generation of reactive oxygen species (ROS) as it is catabolized through the mitochondrial ETC. The increase in ROS damages proteins of all kinds, including oxidative stress protection proteins.

The effect of the O3 diet on metabolism was surprising. Glycolytic proteins decreased with age but increased slightly with O3 diet. Interestingly, FA catabolism proteins markedly decreased with the O3

diet, which was not expected. This could be due to the fact that oxidative capacity has been shown to decrease in aged skeletal muscle.

Protein transport regulators increased in younger rats as a function of diet, suggesting that an increase in protein turnover might be linked to the strength increases that were demonstrated and underscoring the importance of autophagy in skeletal muscle repair.

Novel Aspect

Correlation of proteomic and physiological effects of high omega-3 diet on aging skeletal muscle.

30. Sean Dunham

Cysteine-selective middle-down proteomics with ultraviolet photodissociation analysis

Jennifer S. Brodbelt, University of Texas at Austin

Introduction

Although bottom-up proteomic strategies are widely used, they are not without drawbacks. Many small redundant peptides are often generated. Middle-down proteomic strategies are an alternative approach that create larger and more informative peptides. In this study, middle-down peptides are generated by targeting cysteine, one of the least abundant amino acids, as a cleavage site. Selective cleavage at cysteine residues is accomplished by aminoethylating cysteine residues to convert them into lysine-like analogs that are recognized and cleaved by LysC protease. Owing to the large size of the peptides, ultraviolet photodissociation (UVPD) provides an ideal activation method to provide informative fragmentation patterns. This cysteine-selective/UVPD strategy is readily incorporated into a middle-down proteomics workflow.

Methods

Proteins were carbamylated in 8M urea prior to reduction of cysteines with dithiothreitol and aminoethylation using 2-bromoethylamine (pH 10). Proteins were buffer exchanged into 50 mM ammonium bicarbonate by using molecular weight cutoff filters prior to LysC digestion. MWCO filters were also used to desalt samples. Liquid chromatography was performed on a Dionex UltiMate 3000 nanoLC system equipped with PLRP trap and analytical columns coupled to a Thermo Orbitrap EliteTM mass spectrometer modified to implement UVPD via a 193 nm excimer laser. Peptide ions were activated by either higher-energy collisional dissociation (HCD) or UVPD. MS/MS spectra were analyzed with Byonic. Fixed modifications on lysine (+43.0058 Da) and cysteine (+43.0421 Da) residues were implemented to accommodate carbamylation and aminoethylation.

Preliminary Data

A key linchpin of middle-down proteomics strategies is the production of large peptides, ones that cover longer stretches of a protein sequence than the small peptides produced by trypsin in bottom-up workflows. Trypsin targets basic amino acids (Arg, Lys), both of which are in the middle of the frequency range of amino acids in the proteome. In contrast, cysteine is one of the least frequent amino acids, making it an excellent target for generating larger peptides during proteolysis. Since cysteine-selective proteases are rare, cysteine can be converted into a recognized, cleavable residue by aminoethylation. Aminoethylation converts cysteine into a lysine-like structure that is recognized

and cleaved by LysC, an endoproteinase that cleaves on the C-terminal side of lysine residues. To prevent cleavage at the lysine residues in a protein (which would defeat the purpose of a cysteine-selective strategy), all lysines were converted to non-cleavable residues via a highly efficient carbamylation reaction. For reaction optimization and evaluation of the efficiency of LysC proteolysis, initial efforts focused on two proteins, hemoglobin and bovine serum albumin. The alpha and beta subunits of hemoglobin have only one and two cysteine residues, respectively, making them excellent candidates for production of large peptides. For example, one 11.6 kDa peptide detected corresponded to a large peptide from the alpha subunit. The net coverage obtained using this middle-down approach for the alpha subunit of hemoglobin was 99%. The beta unit of hemoglobin yielded a sequence coverage of only 23%, indicating greater obstacles to generating or detecting the expected peptides. For example, one prominent 11.5 kDa peptide could not be assigned, representing a yet unsolved challenge. For bovine serum albumin, 85% sequence coverage was achieved with peptides ranging from 1 kDa to 9 kDa. For this protein, 67% of the peptides had masses of 3 kDa or larger.

Novel Aspect

The combination of carbamylation and aminoethylation reactions afford a new way to produce large peptides for middle-down analysis.

31. Amarjeet Flora

Large Scale EasyPepTM MS Sample Preparation for Phosphopeptide Enrichment Workflows

Ryan D. Bomgarden, Ph.D.[1], Sergei I. Snovida, Ph.D.[1], Ashok Salunkhe, Ph.D.[1], John C. Rogers, Ph.D.[1]
[1]Thermo Fisher Scientific, Rockford, IL USA

Introduction

Phosphorylation is a critical post translational modification that modulates the function of numerous proteins, and recent advances in mass spectrometry (MS) instrumentation have enable studying phosphorylation at proteome-wide scale in complex biological samples. However, due to the low stoichiometic abundance of phosphorylated peptides in protein digests, affinity-based phosphopeptide enrichment from milligrams of protein digest represents is required for MS detection and quantification. Recently, we developed a new, simplified sample prep kit containing pre-formulated reagents and a standardized protocol for processing 10 to 100µg protein samples in less than 2 hours. In this study, we assessed the scalability of our chemistry larger protein amounts (> 1mg) for subsequent enrichment using immobilized metal affinity chromatography (IMAC) of phosphopeptides using different Fe-NTA resins.

Methods

Cellular protein extracts were diluted in lysis buffer with a phosphatase inhibitor mixture. A universal nuclease was added to cellular extracts to reduce sample viscosity. Protein samples were heated at 50°C for 10 minutes in the presence of a combined reduction/alkylation solution before digestion using a trypsin/LysC protease mixture at 1:25, w:w. A mixed mode peptide clean-up procedure was used to remove detergent removal before IMAC enrichment using Thermo Scientific Pierce Hi-Select TM Fe-NTA phophopeptide enrichment kit or a novel, magnetic Fe-NTA poly–

acrylamide particle. Peptides were quantified and normalized using the Pierce™ Quantitative Colorimetric Peptide Assay prior to LC-MS analysis using a Thermo Scientific Orbitrap Fusion mass spectrometer. Thermo Scientific Proteome Discoverer 2.2 software was used to localize the phosphorylation sites.

Preliminary Data

Due to the relative low stoichiometry of phosphorylated peptides in complex protein digests, phosphopeptide enrichment from large scale protein digests (> 1mg) is typically required to identify and localize significant numbers of phosphopeptide sites by mass spectrometry. Although our optimized chemistry developed for significantly reduces both hands-on and total sample processing time, peptide clean up using microcentrifuge spin columns capacity is limited by the amount of resin and device volume. We assessed various centrifugation-based and vacuum-based column formats for peptide clean-up and identified a column large-scaled format that showed nearly identical performance in terms of peptide yield, phosphopeptide specificity, identification rates, alkylation efficiency and digestion efficiency compared to the smaller spin column protocol. We also assessed phosphopeptide yield and specificity using both agarose and a novel, magnetic polyacrylamide FE-NTA particle. Our optimized chemistry combined with the large-scaled format and subsequent phosphopeptide enrichment was completed in less than 6 hours but could be further improved by elimination of speed vac before IMAC enrichment. Overall, we demonstrate that our chemistry is readily adaptable to large scale enrichment for phosphoproteome mass spectrometry analysis.

Novel Aspect

Large scale sample preparation using optimized chemistry for phosphopeptide enrichment using novel magnetic particles.

32. Nathaniel Fortney

Microbial metabolism of gamma-valerolactone

Steven D. Karlen - The Great Lakes Bioenergy Research Center and University of Wisconsin-Madison Department of Biochemistry
Timothy J. Donohue - The Great Lakes Bioenergy Research Center and University of Wisconsin-Madison Department of Bacteriology
Daniel R. Noguera - The Great Lakes Bioenergy Research Center and University of Wisconsin-Madison Department of Civil and Environmental Engineering

Introduction

Treatment of lignocellulosic biomass with the organic solvent γ-valerolactone (GVL) is a promising method for biomass deconstruction for biofuel production. Current models predict a nontrivial amount of GVL to persist in the biorefinery processing streams from the hydrolysate to the conversion residue (CR). Although GVL is highly toxic to fermentative organisms, it also presents a potential carbon source for biofuel production. On a per-mole basis, GVL contains a comparable amount of chemical energy as glucose. Ultimately, we seek to better understand the mechanisms of aerobic and anaerobic biodegradation of GVL and engineer these pathways into fermentative organisms to produce GVL-tolerant and – utilizing organisms for increased lignocellulosic biofuel production.

Methods

The metabolism of GVL was studied using a pure culture of Rhodopseudomonas palustris, a bacterium with the known ability to metabolize GVL under photoheterotrophic conditions. A mixed community of microorganisms derived from a wastewater treatment plant was grown on CR and GVL to identify novel organisms capable of GVL metabolism. Metabolites in the supernatant from growth media and cell lysate analyzed using HPLC to track the decrease in GVL over time. Samples were derivatized using bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA-TMCS) in pyridine solvent for analysis by GC-MS to identify metabolic intermediates. Multiple-reaction-monitoring (MRM) methods were developed for HPLC-MS/MS for metabolite quantification.

Preliminary Data

In both the pure culture of Rp. palustris and the mixed culture bioreactors we have observed a decrease in GVL concentration over time coupled to an increase in biomass suggesting the ability of these organisms to utilize GVL for anabolic and catabolic processes. Furthermore, we have observed the ability of Rp. palustris to grow on higher concentrations of GVL to a higher cell density than in previous studies. Interestingly, we have observed the accumulation of an as-yet unidentified metabolite coinciding with the depletion of GVL. An increase in concentration of this compound over time in both bioreactor experiments suggests it is a metabolic byproduct of GVL degradation, rather than a byproduct of CR metabolism, or a constituent of the Rp. palustris growth media. The use of HPLC with RID has thus far proven ineffective in characterizing this compound, necessitating the use of more advanced chromatography and mass spectrometry techniques. Analyzing the metabolites in the supernatant following Rp. palustris growth via GC-MS had limited success due to the potential of compounds of interest eluting when the MS detector was off during the solvent delay (first 4.5 minutes of analysis). Derivatization is necessary for detection of organic acids similar to those expected as intermediates of GVL degradation (Figure 2), however further investigation using alternative derivatization reagents is warranted. MRM method development on HPLC-MS/MS has been effective at identifying and quantifying GVL, and efforts are underway to develop MRM methods for additional predicted intermediate metabolites to GVL degradation. Additionally, we are exploring purification strategies to identify unknown byproducts of GVL metabolism. Ultimately through these techniques we seek to track the accumulation and depletion of predicted intermediate compounds to GVL degradation in order to fully characterize the pathway.

Novel Aspect

Elucidating the metabolic pathway of GVL degradation allows us to engineer better biofuel producing microorganisms.

33. Erika Foster

From sand to soil: Microbial functional development across a chronosequence of irrigated fields in the desert of southern Peru

Purdue University: Zachary Brecheisen, Lucia Zuniga, Alex Ccanccapa, Darrell Schulze, Tim Filley
Universidad National de San Agustin: Martin Villalta Soto, Juan Lopa Bolivar

Introduction

Recent agricultural development in the desert region of Arequipa Peru has created 40,000ha of newly irrigated lands. The addition of irrigation has reinitiated soil development. With new water inputs and carbon inputs from crop roots and residues, we can study the precise evolution of soil physical, chemical, and biologic properties. As the living component of the soil, the fungi and bacteria serve as the drivers of both carbon and nutrient cycling within this system. The soil microbial community both controls biogeochemical process rates and also serves as a rapid indicator of soil response to management. By understanding the function of the microbial community, we can uncover the mechanisms behind changes in both soil chemistry and physical soil structure.

Methods

The agricultural research station in Majes, Arequipa contains three fields of continual grape production each with a different start date of cultivation (5, 15, and 35 years). The grape fields contain three distinct ages of carbon input, but similar irrigation and fertilizer inputs. The research project will include collaboration with local students and professors. The piolet soil sampling to a depth of 80cm has already occurred in two plots and sampling the soil surface from 0-20 and 20-40cm continues. We will monitor soil geochemistry, by measuring pH, EC, total C and N content, and metal contamination via ICP-OES.

Additionally, we will measure soil biological properties, including microbial biomass, in situ activity via CO2 measurements, and microbial function using proteomic techniques.

Preliminary Data

The initial soil sampling to depth in the vineyards currently is being analyzed in the laboratory. Preliminary geochemical results show accumulation of salts in these fields after a coarse textured sand layer, which could have repercussions for root and microbial development. Additionally, we have data graphed showing distribution of CO2 production at depth within two vineyard profiles.

Novel Aspect

Measurement of microbial functional development from a highly limited desert system to a productive agricultural region, elucidates soil biogeochemical evolution.

34. Sheelagh Frame

Exploiting activity-based probes to facilitate the discovery of novel deubiquitinase (DUB) inhibitors

João Banha Oliveira, Sian Armour, Sheelagh Frame, Jason Mundin and Jason Brown; Ubiquigent Ltd., Sir James Black Centre, Dow Street, Dundee DD1 5EH.

Introduction

The underlying concept of targeting intractable, undruggable proteins by modulating their half-life is an important emerging strategy in drug discovery. Ubiquitination controls the stability of most cellular proteins, and its deregulation contributes to human diseases including cancer, Parkinson's and Alzheimer's disease. Deubiquitinases (DUBs) remove ubiquitin from proteins, and their inhibition can induce the degradation of selected proteins, potentially including otherwise

'undruggable' targets. The identification of highly selective DUB inhibitors demonstrates that the DUB enzymes are tractable drug targets for a variety of diseases and is an area of intense focus. Ubiquigent. is dedicated to providing a range of state-of-the-art Drug Discovery Services to support the rapidly growing academic and commercial drug discovery interests in the ubiquitin field.

Methods

Activity-based protein profiling (ABPP) is a powerful chemoproteomic strategy to (i) establish compound-target engagement and (ii) evaluate compound selectivity on a proteome-wide basis within complex biological samples. However, it can also be used to (iii) interrogate the functionality of an entire enzyme class within a given biological condition, and to (iv) map sites within proteins that can be pharmacologically interrogated for drug discovery. Ubiquitin-Propargylamide (UbPA) is a potent, irreversible and specific inhibitor of all three major DUB families, C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), and Otubains (OUT's). UbPA is N-terminally tagged with the HA peptide sequence (YPYDVPDYA) derived from the influenza hemagglutinin protein and allows for the sensitive identification or purification of DUBs by anti-HA antibodies and/or anti-HA-agarose beads.

Preliminary Data

To define the active 'DUBome', lysates were prepared from cell lines relevant to oncology and neurodegeneration, including MCF7 (breast cancer) and SH-SY5Y (Parkinson's and Alzheimer's disease). Lysates were incubated with the HA–UbPA probe and the active 'DUBome' was characterised by Western blot and by mass spectrometry. We have successfully enriched DUBs using the HA-UbPA probe from lysates prepared from MCF7 cells compared with "no probe" controls and succeeded in identifying DUBs from different families using this approach. Data characterizing the active 'DUBome' in multiple cell lines will be presented. To evaluate compound specificity, crude cell extracts or live cells were pre-incubated with or without small-molecule compounds, followed by incubation with HA-UbPA. The profile of HA-UbPA-modified DUBs (in the presence or absence of compound pre-treatment) revealed the selectivity of the compounds by both Western blotting and mass spectrometry. The data obtained using the ABPP methodology is compared with the output from an in vitro biochemical DUB activity assay using a ubiquitin-rhodamine cleavage readout. Ongoing assay development is focussing on optimising the detection of the active 'DUBome' using a variety of experimental and analytical MS strategies, evaluating other probes, and assessing different enrichment strategies. In conclusion, ABPPs not only assess the activity of the protein target (and target class) of interest, but also help to demonstrate target engagement and assess the selectivity of inhibitors on a proteome-wide scale. Exciting next steps for this strategy are to utilise the important structural information from these studies to inform the design of novel DUB inhibitors, and to provide confirmation of target engagement (and selectivity) in pre-clinical and clinical testing paradigms in animal models and humans when these novel DUB inhibitors reach the clinic. The data obtained within this project will inform our own drug discovery efforts and those of our clients, as we pursue the design of novel DUB inhibitors.

Novel Aspect

The first characterization of the active 'DUBome' in cells relevant to the study of DUBs in disease.

35. Matthew Fry

Novel Rabbit Monoclonal Antibodies for Profiling of Ser/Thr O-GlcNAc modified proteins

Fry MD, Najjar R, Yiying Zhu, Stokes MP Lee K - Cell Signaling Technology
Schweppe, Devin Karl, Gygi, SP - Harvard Medical School

Introduction

A distinct form of protein glycosylation, beta-linked N-acetyl-glucosamine (GlcNAc) moieties can be added to serine or threonine residues of proteins. This differs from other forms of glycosylation, as it typically is a single moiety rather than the complex branched sugars that are more commonly studied. It is thought that these modifications happen in a dynamic cycle reminiscent of phosphorylation. GlcNAc modified proteins are found in the cytoplasm and nucleus and are modulated by means of specific O-GlcNAc transferases (OGT) as well as GlcNAcase activity. O-GlcNAc modification of proteins plays an important role in many cellular processes, including metabolism, growth, morphogenesis, apoptosis, transcription, and cancer.

Methods

Polyclonal antibodies were produced by modifying serine and threonine residues with O-linked GlcNAc randomized peptide libraries coupled to KLH. Rabbits were selected for monoclonal antibody development based on reactivity in ELISA and western blot assays. Rabbit monoclonal clones were then produced, tested, and selected for scale-up and additional testing. ELISA and western blot assays were performed as described (5). GlcNAcase activity was inhibited using Thiamet-G (TMG) on COLO205 and HeLa cells at 10 µM for 6-8 hr at 37°C. PTMScan^{TM} analysis was performed using immunoaffinity enrichment with a mixture of OGlcNAc positive clones on trypsin digested peptides from HeLa cells treated with TMG. This enrichment was followed by LC-MS/MS analysis for identification and quantification of hundreds of non-redundant peptides.

Novel Aspect

Novel antibodies recognize Ser/Thr O-GlcNAc modifications for western blot and LC-MS/MS to enable site identification and quantification.

36. Joseph Gage

The genomic basis for dysregulation of protein abundance in maize

Edward S. Buckler, USDA-ARS, Ithaca, NY; Richard Vierstra, Washington University, St. Louis, MO

Introduction

An organism's proteomic state is directly responsible for phenotype and can be considered a phenotype itself. The low correlation between transcript abundance and protein abundance indicates that in addition to transcript abundance, protein abundance is likely dependent on other genomic, cellular, temporal, or environmental influences. Genomic influences include DNA variants associated with differential protein abundance between individuals (pQTL), and the apparent relationship between transcript and protein abundance is complicated by differential degradation rates, differential transport between tissues, and temporal cycling. We propose to model genome-

wide patterns of how rare or deleterious variants cause changes in protein structure and function that disrupt protein translation, accumulation, and degradation.

Methods

Germplasm utilized in this study will be 27 diverse maize inbred lines that have been genotyped at more than 80 million genomic positions. Leaf tissue will be collected at eight time points over the course of 24 hours during the three-leaf stage. We will extract RNA and protein from the latest-sampled tissue, and RNA alone from tissues sampled at earlier time points. By extracting proteins and RNA from the same final tissue samples, we will reduce latent variability that could complicate comparisons of transcript and protein from different samples. Transcript abundance will be assayed by 3' RNAseq, while protein quantification will be performed using a high-throughput tandem mass spectroscopy protocol developed by the Vierstra Lab at Washington University.

Preliminary Data

In collaboration with Dr. Richard Vierstra at Washington University, we are currently performing a pilot study in which we will produce proteomic profiles for four diverse maize inbred lines. Members of the Vierstra lab have developed a method for shotgun quantifying the maize leaf proteome, using tandem mass spectrometry in high-energy collision-induced dissociation mode coupled with label-free quantification. We are applying this technique to the maize inbred lines B73, Mo17, CML103, and P39. These lines were chosen for their genetic diversity and anticipated proteomic diversity. The first two represent temperate maize germplasm from the US Corn Belt, whereas P39 is a sweet corn line and CML103 is adapted to tropical conditions. Samples are currently at the Vierstra lab for processing and, based on previous studies, are anticipated to produce tens of thousands of unique peptides and thousands of maize protein groups per sample. These data will be combined with existing genomic and time-series transcriptomic data to develop biological models of how rare genetic variants impact transcription, translation, and protein accumulation in maize.

Novel Aspect

Assessing genome wide differences in proteome profiles and circadian RNA abundance across diverse maize accessions.

37. Sanjeewa Gamagedara

Investigation of Urinary Metabolites as Potential Biomarkers for Prostate Cancer Detection

Tyler Cook, Department of Statistics, University of Central Oklahoma, Edmond, OK 73034; Yinfa Ma, California State University - Sacramento, Sacramento, CA 95819

Introduction

Urinary metabolomic profiles recently drew a lot of attention because a debate regarding their possible role as potential clinical markers for prostate cancer. In this study, levels of proline, kynurenine, uracil, and glycerol-3-phosphate in 126 patients with genitourinary malignancies were analyzed using a validated method and compared with no evidence of malignancy. Sarcosine was thoroughly studied as a prostate cancer biomarker, but it was not included in this study due to the difficulties of analysis due to structural isomer L- alanine and its contradictions as a biomarker.

Methods

A novel method was developed to separate and quantify these five metabolites including creatinine using LC/MS/MS. The five metabolites were detected in the multiple reaction monitoring (MRM) with ESI positive mode. Chromatographic characteristics of the analytes were determined using a phenyl-hexyl column with 0.1 % formic acid in water and acetonitrile respectively under a gradient program. Above metabolites levels were determined in urological cancers such as prostate (n=63), bladder (n=63), as well as in healthy subjects (n=68) to identify whether there are any differences between them. The amounts of metabolites were divided by creatinine in order to account for physiological concentration. Statistical hypothesis testing, MANOVA, PCA, CART, and seven different statistical normalization techniques were conducted to analyze the data.

Preliminary Data

The statistical results show that these biomarkers cannot differentiate prostate cancer from no evidence of malignancy or from other related cancer types, such as bladder cancer. Also, for prostate cancer, there is no significant difference in biomarker levels for T1, T2 stages and Gleason scores <7, ≥7. From the correlation study, we can see that age or serum PSA levels do not influence these metabolite concentrations in urine. However, the strong correlation between these metabolites and urinary creatinine concentrations implies that their occurrence is mainly due to renal excretion. This detailed study shows that above urinary metabolites are not reliable biomarkers for prostate cancer detection or for differentiating the aggressiveness of prostate cancer.

Novel Aspect

A novel LC/MS/MS method was developed used to separate and quantify these five metabolites in urine samples.

38. Rodolfo García-Villegas

Using the TFAM-FLAG mice to study mtDNA expression in vivo

Rodolfo García-Villegas[1], Nina A. Bonekamp[2], Henrik Spår[1] and Nils-Göran Larsson[1].
[1]Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 77 Stockholm, Sweden; [2]Max Planck Institute for Biology of Ageing, Joseph-Stelzmann-Str. 9b, D-50931 Cologne, Germany

Introduction

The mammalian mtDNA encodes 13 subunits of the respiratory chain complexes that are essential for the function of the oxidative phosphorylation system (OXPHOS). In mammals, mtDNA is packed in a nucleoid structure of around 100 um. The main structural protein of the nucleoid is the mitochondrial transcription factor A (TFAM), in vitro experiments demonstrated that TFAM alone is able to pack mtDNA. Moreover, in highly compacted nucleoids TFAM can block transcription and replication of mtDNA. The TFAM-to-mtDNA ratio is an important mechanism to regulate mtDNA copy number and expression. However, how this ratio is maintained is poorly understood.

Methods

We generated a TFAM-FLAG mouse model to investigate by mass spectrometry how the nucleoid composition affects mtDNA expression in vivo. Additionally, we will use a targeted proteomic

strategy investigated if post-translational modifications of TFAM control the mitochondrial genome expression.

Preliminary Data

The TFAM-FLAG rescue mice born at Mendelian ratios. In heart tissue, the expression of TFAM-FLAG protein was similar to WT TFAM. In concordance, we observed normal levels of mtDNA by Southern Blot or qPCR, stable mitochondrial transcripts and no changes in the OXPHOS protein levels in the heart tissue. In the liver, we observed a lower TFAM-FLAG protein expression that correlates with a reduction in mtDNA. In addition, the levels of light strand promoter transcripts were reduced. However, OXPHOS protein levels remain stable in the liver of TFAM-FLAG rescue mice. Taken together, our results showed that mitochondrial function is not compromised in TFAM-FLAG rescue mice. We obtained purified mitochondrial nucleoids with the immunoprecipitation of TFAM-FLAG protein from isolated liver mitochondria. Preliminary mass spectrometry data from this preparation identified expected nucleoid interacting proteins such as the catalytic subunit of DNA polymerase gamma. The characterization of nucleoid composition in different tissues from the TFAM-FLAG rescue mice is being planned.

Novel Aspect

Use of a TFAM-FLAG mouse model for the study of nucleoid composition and mtDNA expression in vivo.

39. Heather Green

Cancer Pharmacology Lab, A UW Madison core facility

Shannon Andrews Ph.D., Heather Green Ph.D., Song Guo M.S., Gene Ananiev Ph.D., Prof. F Michael Hoffmann, Prof Ronald Burnette.

Introduction

The mission of the Cancer Pharmacology Lab is to advance cancer research by providing expertise; leadership; and bioanalytical assay development, validation and performance in support of the clinical, translational and laboratory research endeavors of UWCCC investigators. Services include: Sample Acquisition, Storage and Shipping, Pharmacokinetic Analytical Assays, Pharmacodynamic Assays, Preclinical Analytic Services, Preclinical in vitro pharmacology assays and in vivo (mouse) pharmacokinetics assays are available through contracts with external CROs maintained by the UWCCC Drug Development Core.

Methods

The Drug Development Core's Cancer Pharmacology Lab provides analytical services to evaluate the pharmacokinetic and pharmacodynamic properties of new agents and known drugs. We analyze for drug concentrations on plasma, serum, urine and other tissues. We have validated HPLC, LC/MS and LC/MS/MS methods to assay a variety of compounds, including ABT-888, Capecitabine, Cotinine, Perillyl alcohol, polyamines, Phenylephrine, Sorafenib, UAB30, Paclitaxel, pomegranate fruit extract metabolites, BMN 673 and other compounds. We also offer method development services for the analysis of novel compounds. In addition, we conduct the data analysis and interpretation of these

assays, and report research findings to PIs. We recently added Phoenix WinNonlin software to our systems, which will expand our pharmacokinetic capabilities.

Cancer is the second leading cause of death in the United States. The prognosis associated with distant metastases from adult solid tumors is poor for most malignancies. The goal of treatment with systemic therapies, either chemotherapy and/or biologic agents, is primarily palliative for these patients. There is a need to develop more efficacious and less toxic therapies for the treatment of cancer.

Preliminary Data

BMN 673 is an orally available inhibitor of poly (ADP-ribose) polymerase (PARP), with an approved generic name of talazoparib. BMN 673 is a novel, high purity, single enantiomer, methylbenzene sulfonate compound. It is the most potent and specific inhibitor of PARP1/2 in clinical development (IC50<1nM) and designed to have an improved therapeutic index relative to existing PARP inhibitors in development.

Measurements of DNA damage and PARP inhibition can be used to evaluate the effect of combination therapy with platinums and PARP inhibitors. Activation of DNA-damage signaling pathways occurs through phosphorylation of histone H2AX (γ ≥H2AX). RAD51 is involved in homologous recombination repair. In response to DNA damage, RAD51 is re-localized within the cell nucleus to form distinct foci which can be visualized. The product of PARP 1 and 2 enzyme activity, PAR, has been utilized in previous studies as a measure of PARP activity. These assays have been performed previously in PBMCs and circulating tumor cells (CTC)s. Many questions about this combination remain. The exploratory correlative studies performed in the CP lab will examine these questions. The plasma pharmacokinetics of BMN 673 will be evaluated as a potential marker of toxicity, specifically thrombocytopenia as well as correlated with PD changes observed in PBMCs by an indirect PK/PD model.

(A Phase 1 Study of BMN 673 in Combination with Carboplatin and Paclitaxel in Patients with Advanced Solid Tumors, Kari Wisinski, MD, and Ticiana Leal, MD.)

Novel Aspect

BMN 673 represents a promising PARP1/2 inhibitor with potentially advantageous features as a therapy for the treatment of human cancers.

40. Sara Hazinia

Natural products and ultrastructural specifications of mycoheterotrophic plant, M.uniflora (ghost plant)

Harry Horner Institute: Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA, USA Email: hth@iastate.edu
Tracey Stewart Institute: Department of Genetics, Development, and Cell Biology, Iowa State University, Ames, IA, USA Email: tpepper@iastate.edu
Basil J. Nikolau Institute: Department of Biochemistry Biophysics and Molecular Biology, Iowa State University, Ames, IA, USA Email: dimmas@iastate.edu

Introduction

Mycoheterotrophic, non-photosynthetic plants exploit nutrients from their fungal symbiont throughout mycorrhiza root system. This advanced mycorrhizal communication is comprised of three species: vascular plant serving as the photosynthetic source, mycoheterotrophic plant, and mycorrhizal fungi serving as the intermediate in transferring nutrients from photosynthetic to mycoheterotrophic plant. Monotropa uniflora is non-photosynthetic, with significant adaptations to mycoheterotrophy including photosynthesis loss, but has maintained other features specific to plantae including fruit and seed development. Although the mycorrhiza root system is well characterized, the aerial specifications of mycoheterotrophic plants remains unclear. In this study, M. uniflora aerial tissues were ultrastructurally characterized and the metabolic compositions were identified and quantified applying GC/MS.

Methods

Plant material were collected from Ledges State Park, Iowa during late summer. Non-targeted and targeted analysis of metabolites were performed applying GC/MS based platforms. Based on the polarity, non-targeted metabolites were divided into polar and non-polar phases after being extracted with chloroform, hot methanol and water. Targeted metabolites include: 1) Epicuticular surface lipid crystals, extracted by submerging the tissue in chloroform for one minute, 2) Cutin monomers, beginning with tissue delipidation followed by cutin depolymerization via catalysis reaction, 3) Amino acids using the Phenomenex EZ:Faast kit for free amino acids. Transmission and Scanning electron microscopy (TEM and SEM) were applied to determine the ultrastructure of epidermal cells, cuticle, and epicuticular surface lipid crystals.

Preliminary Data

GC/MS analysis of untargeted (total metabolites) and targeted (epicuticular surface lipids, cutin monomers, and amino acids) compounds led to detection of 86 analytes which could be chemically identified. Compared to Arabidopsis, M.uniflora synthesizes high levels of coumaric acid, a phenolic molecule known to be involved in the biosynthesis of lignin1 and salicylic acid2. All other surface specified components, including epicuticular surface lipids and cutin monomers, are common in photosynthetic plants. In epicuticular surface lipid profile, the classes of metabolites from most to least concentrated are triterpenes, primary alcohols, long chain fatty acids (LCFAs), alkanes and sterols. Similarly, dihydroxy acids, LCFAs, 2-hydroxy acids and —†-hydroxy acids are metabolites detected in cutin monomers. Internal metabolites are composed of sugars, organic acids, phenolics, sugar alcohols, primary alcohols, LCFAs and terpenoids. Similar to the epicuticular surface lipid profile, coumaric acid is the most abundant analyte among non-polar compounds, offering again, the unique chemical composition of M.uniflora.

SEM analysis demonstrate that epicuticular wax crystals on the plant's stem are not uniformly distributed. Compared to Arabidopsis stem, wax crystals are small and smooth without rigid edges embedded into a thick and wrinkled cutin membrane. TEM analysis and comparison between upper and lower portions of stem show the vacuoles of upper stem epidermal cells are mainly filled with black materials which can indicate synthesis of polyphenolics. These observations are not consistent with lower stem.

Novel Aspect

This extensive study significantly expands the current insight of mycoheterotrophic plants and represent their metabolic diversity and ultrastructural specifications.

41. Amanda Helms

Impact of Charge Sites on Fragmentation of Peptides and Proteins: Carbamylation and Guanidination

Jennifer S. Brodbelt, Department of Chemistry, University of Texas at Austin

Introduction

The charge sites and charge states of peptides exert considerable impact on the resulting fragmentation patterns generated by MS/MS methods. Charge effects are particularly prominent for both collisional activation methods which depend on proton mobility and for electron-based methods which are influenced by charge density. Here we explore two derivatization methods that modulate the ionizability of lysine residues in opposite ways. Guanidination increases the basicity of lysine residues and increases proton sequestration, thus reducing proton mobility. Carbamylation decreases the basicity of lysine side-chains, thus decreasing the degree of proton sequestration at those residues. In the present study we examine the fragmentation patterns of peptides upon UVPD, HCD and ETD after modification of the lysine residues by guanidination and carbamylation.

Methods

Carbamylation (using 8M urea at pH 8) and guanidination (using o-methylisourea at pH 10.5) reactions were performed based on procedures reported previously. Peptides were cleaned up using C18 SPE spin columns and introduced by direct infusion. After carbamylation or guanidination, proteins were reduced, alkylated, and digested using trypsin. Tryptic peptides were introduced via an UltiMate 3000 RSLC nano liquid chromatograph. All analysis was undertaken using a Velos Pro linear ion trap mass spectrometer equipped with a 193 nm excimer laser for ultraviolet photodissociation (UVPD) using 4-6 laser pulses. CID and UVPD mass spectra were analyzed using Byonic (v3.1.0).

Preliminary Data

Carbamylation and guanidination modify primary amines, particularly the side-chains of lysines and the N-terminal amines. Guanidination converts each primary amine to a highly basic guanidine group resulting in a mass shift of +42 Da and promoting the formation of higher charge states. Carbamylation convert each primary amine to a less basic carbamyl group, resulting in a mass shift of +43 Da and causing a shift to lower charge states. VCYDKSFPISHVR, a peptide containing one lysine and one arginine residue, was used as a benchmark peptide to evaluate the change in charge states upon carbamylation or guanidination. Prior to modification, the charge state distribution of the model peptide ranged from 1+ (<1%) to 2+ (25%) to 3+ (74%). The charge state distribution for the carbamylated peptide was 1+ (1%) to 2+ (77%) to 3+ (22%), and it was 1+ (2%) to 2+ (7%) to 3+ (91%) for the guanidinated peptide. This change in charge state distribution observed for this representative peptide is indicative of the shifts in charge states expected for other peptides. The fact that the 2+ and 3+ charge states were observed for all three forms of the peptide allowed comparisons of the MS/MS behavior by HCD, UVPD, and ETD as a function of charge state and

offered a means to evaluate the impact of the variation in charge sites based on the type of modification. A prominent doubly charged y9 ion observed for the unmodified and guanidinated peptide was significantly decreased for the carbamylated peptide, consistent with the reduction in the basicity of the Lys5 residue. A much greater array of fragment ion types was observed for UVPD of all three forms of the VCYDKSFPISHVR peptide.

Novel Aspect

Impact of charge sites of peptides on UVPD fragmentation based on conversion of lysines to more or less basic residues.

42. Kyle Hess

Proteome-wide mistranslation uncovers proline residues important for protein stability and solubility

Ricard Rodriguez-Mias (University of Washington, Department of Genome Sciences)
Ian Smith (University of Washington, Department of Genome Sciences)
Ariadna Llovet (University of Washington, Department of Genome Sciences)
Judit Villen (University of Washington, Department of Genome Sciences)

Introduction

Modern DNA sequencing has unearthed millions of genetic variants in humans, many of which fall within coding regions and alter the amino acid sequences of proteins. Characterizing the impact of these amino acid substitutions (AAS) on protein structure and function remains a monumental challenge in genome analysis and is essential to unravel the genetic architecture of human traits and diseases. While high-throughput mutagenesis strategies, such as deep mutational scanning, have accelerated variant annotation, these approaches typically assess only a single protein at a time. Here, we present a novel mass spectrometry-based platform for assessing the impact of AAS across hundreds of proteins simultaneously that relies on mistranslation with non-canonical amino acids (ncAAs).

Methods

We screened 29 ncAAs for mistranslation in yeast using mass spectrometry. We then selected one of these ncAAs, azetidine-2-carboxylic acid (azetidine), and stochastically incorporated it in place of proline residues across the yeast proteome. We measured the impact of azetidine substitutions on both protein thermal stability and solubility by using Thermal Proteome Profiling (TPP) and comparing the amount of soluble protein in an NP40-extracted lysate to the amount of protein present in an SDS-extracted lysate. Samples were reduced, alkylated, lysC-digested, and labeled with TMT11plex before being analyzed on an Orbitrap-Fusion-Lumos using an MS3 method. TMT reporter ion intensities were extracted for each peptide and used to generate peptide-level melting curves and solubility ratios across the entire proteome.

Preliminary Data

In total, we identified 20 ncAAs that mistranslate across the yeast proteome, expanding the application of our method to 12 different amino acid residues. The degree of proteome-wide mistranslation varied drastically, with some ncAAs incorporating at the expected canonical amino

acid residue up to 42% of the time (for example, fluorotryptophan incorporating at tryptophan sites). This pilot screen highlights the ability of our method to easily produce mistranslated proteomes and represents a potential strategy for investigating translational fidelity in yeast. Next, we wanted to showcase the ability of our method to quantify the impact of AAS on protein structure and function in a high-throughput, proteome-wide manner. We decided to measure changes in protein thermal stability and solubility by coupling Thermal Proteome Profiling with a yeast proteome containing proline-to-azetidine substitutions. In total, we quantified thermal stability and solubility for over 800 yeast proteins comprised of ~4,300 peptides. We sampled a broad range of melting temperatures and solubility ratios with a high-degree of reproducibility (Thermal stability, R2=0.95; Solubility, R2=0.81), and found moderate correlations between stability, solubility, and protein properties such as size and abundance. Importantly, we were able to measure changes in protein thermal stability and solubility for 662 proline-to-azetidine substitutions occurring across 317 different yeast proteins. We found that the majority of proline positions were robust to replacement with azetidine, with less than 11% of substitutions significantly altering either protein stability or solubility. Surprisingly, the majority azetidine substitutions that significantly impacted protein stability tended to have minimal impacts on protein solubility, and vice-versa. The degree of sensitivity of proline positions to azetidine substitution correlated with specific secondary structures and neighboring amino acids, suggesting a general role for local sequence context when determining a proline's contribution to protein stability or solubility.

Novel Aspect

First method to assess the consequences of amino acid substitutions across several hundred proteins in a single experiment.

43. Nicole Hudson

Proteomic Profiling of the Peritoneal Cavity in Aged Mice: Understanding Host Contributions to Metastatic Success in Ovarian Cancer

Dr. Sharon Stack, University of Notre Dame Department of Chemistry and Biochemistry
Dr. Rebecca Whelan, University of Notre Dame Department of Chemistry and Biochemistry

Introduction

Ovarian cancer is the leading cause of fatality of the gynecological malignancies in US women. It is unique in its mechanisms of metastasis as it spreads primarily into the peritoneal cavity. It has been observed that aged women receive poorer prognosis when diagnosed with ovarian cancer in comparison to younger women, especially in the event that metastasis has occurred. It is hypo–thesized that the proteomic profile of the peritoneal cavity in aged hosts differs from that of younger hosts and helps to achieve higher levels of metastatic success and tumor adhesion in ovarian cancer murine models. Future work would aim to begin clinical trials for human patients.

Methods

1. Establish a proteomic profile of the peritoneal cavity in both tumor-naïve young and aged mice, as well as in both young and aged mice that are tumor-bearing using mass spectrometry, western blotting, and multiplex assays. This will include profiling of secreted proteins, cell surface proteins,

and intracellular proteins for all cohorts, a profiling of the ascites proteome in tumor-bearing mice, and profiling of exosomes secreted by cancerous cells.

2. Analyze changes in the proteomic profiles of young vs. aged mice and explore how these changes could promote metastasis and tumor adhesion via studying individual protein functions, protein-protein interactions, and the proteins' interactions with the tumor cells.

Preliminary Data

Epidemiological studies have shown that older ovarian cancer patients have a two-fold increase in peritoneal metastases compared to younger patients at the time of diagnosis. Ovarian cancer metastasizes via a unique mechanism, where cells move from the primary tumor and circulate through the peritoneal cavity via diffusion in the peritoneal fluid. The circulating cells adhere to secondary sites, such as the omentum and parietal peritoneum, by interacting with mesothelial cells. Mesothelial cells, the peritoneal extracellular matrix, fibroblasts, adipocytes and immune cells, which are all part of the peritoneal cavity microenvironment, all exhibit distinct changes with age. In mouse models, it has been shown that aged mice had greater tumor burden than their younger counterparts when intraperitoneally injected with ovarian tumor cells. When IP injected with syngeneic tumor cell lines, both C57Bl/6 and FVB mice have shown a dramatic difference in disease progression between the young mice (3-6 months) and aged mice (20-23 months), with the aged mice harboring greater tumor burden than their younger counterparts. (Harper, et al. 2018)

Novel Aspect

The peritoneal cavity proteome has not been explored. Understanding this proteome and age-related changes could help develop new cancer therapies.

44. Robin Hurst

Chemical Proteomic Strategy Utilizing a Photoreactive Cleavable Chloroalkane Capture Tag for Target Discovery

Sergiy Levin, Mike Rosenblatt, Keith V. Wood and Rachel Friedman Ohana, Promega Corporation

Introduction

Identifying the targets of bioactive compounds is often the rate limiting step toward understanding their mechanism of action. We developed a novel photoreactive cleavable chloroalkane tag, which can be attached to bioactive compounds to isolate their cellular targets for identification by mass spectrometry. The tag minimally affects compound potency and cell permeability while providing efficient enrichment of relevant targets. Following binding to the tagged-compound in intact cells, UV-induced photo-crosslinking preserves the interactions with intracellular targets. Effective enrichment of those targets is achieved through their covalent capture onto HaloTag coated particles and subsequent selective release by tag cleavage. Using three compounds we demonstrated the capabilities of this approach to identify the cellular targets including those with low affinity and/or low abundance.

Methods

We used three model compounds Vorinostat (SAHA), dasatinib and propranolol to demonstrate the effectiveness of the photoreactive cleavable chloroalkane tag for target discovery. SAHA is a broad

histone deacetylase (HDAC's) inhibitor. Treatment of K-562 cells with SAHA or a derivative having the chloroalkane tag revealed high cellular potency against HDAC class I/IIb with IC50 values of 0.1 μM and 0.4 μM, respectively. Dasatinib is a broad kinase inhibitor targeting the BCR-ABL oncogenic pathway, thereby reducing the downstream activation of STAT5. Dasatinib and a derivative having the chloroalkane tag inhibited the expression of a STAT5 reporter in K-562 cells with IC50 values of 0.4 nM and 4 nM, respectively. Propranolol is a beta blocker targeting the β-adrenergic receptors. Propranolol and a derivative having the chloralkane tag inhibited the expression of a CRE reporter in isoproterenol stimulated PC3 cells with IC50 values of 60 nM and 1 μM, respectively. Together these results demonstrate the minimal influence of the chloroalkane tag on a compound cellular potency and membrane permeability, thus increasing confidence in the biological relevance of the identified targets.

Preliminary Data

We further used the chloroalkane derivatives to selectively enrich the cellular targets for SAHA and dasatinib from K562 cells and propranolol from PC3 cells. Western blot analysis of enrichments performed with and without UV irradiation indicated that photo-crosslinking successfully preserved the intracellular interactions and was crucial for efficient enrichment. Mass spectrometry analysis of the enriched proteins identified for all compounds the expected targets as well as novel "off-targets" regardless of their affinity, abundance or subcellular localization (e.g. cytosolic, nuclear and multi-trans membrane domains targets). These results suggest that this approach can provide reliable identification of cellular targets for bioactive compounds.

Novel Aspect

Novel photoreactive chloroalkane tag enables identification of difficult targets with low affinity and/or low abundance as well as membrane proteins.

45. Kathryn Jacobson

Proteomic analysis of extracellular matrix dynamics during mouse forelimb development

Kathryn R Jacobson; Sarah L Lipp; Alex R. Ocken; Tamara L. Kinzer-Ursem; Sarah Calve
Purdue University, West Lafayette, IN

Introduction

Tissue engineers aim to restore damaged tissues by creating scaffolds that promote cellular adhesion, proliferation and eventual differentiation into functional tissue. It is known that the chemical and mechanical properties of the extracellular matrix (ECM) regulate cellular behavior; however, current scaffolds often mimic the ECM of adult, homeostatic tissue and frequently lead to poor tissue restoration. What is rarely taken into consideration is that the ECM undergoes extensive remodeling during development to facilitate growth. The regulatory roles of these changes are largely unknown due to the lack of knowledge regarding ECM composition and turnover. The objective of this project is to determine protein abundance and dynamics of the ECM as a function of development in the mouse musculoskeletal system.

Methods

To evaluate ECM protein composition during musculoskeletal assembly, forelimbs were collected from embryonic day (E)11.5 through E14.5, postnatal day (P)3, and adult wild type mice (C57Bl6, Jackson Laboratory). Prior to proteomic analysis, ECM proteins were enriched through sequential extraction of cellular components into cytosolic, nuclear, membrane, and cytoskeletal fractions, resulting in an insoluble, ECM-rich pellet. Total protein content in each fraction, including the insoluble pellet, was quantified. Preliminary studies indicated that >99% of core and associated ECM proteins were identified in cytoskeletal and insoluble fractions; therefore, only these fractions of the forelimbs were analyzed using liquid-chromatography tandem mass spectrometry. Relative protein abundance was determined using label-free quantification (LFQ, MaxQuant).

Preliminary Data

LFQ intensities of ECM proteins in the insoluble pellet were >6-fold greater than in the cytoskeletal fraction. Overall, 129 core and associated ECM proteins were identified; 72 were found in both fractions, while 44 and 13 proteins were exclusive to the insoluble or cytoskeletal fractions, respectively. To compare individual protein abundance as a function of forelimb development, LFQ intensity values from the cytoskeletal and insoluble fractions were normalized by the amount of ECM protein in each fraction. Fibril forming collagens, type I and III, were more than 40% of the total ECM content. Proteins involved in skeletogenesis were transiently expressed around E14.5 and P3, including aggrecan, COMP, perlecan, and collagens type II, VI, IX, and X. Proteins associated with development, such as type V collagen, EMILIN1, fibrillin-2, fibronectin, and TGFBI, were abundant in embryos and significantly downregulated at older time points.

Overall, our findings indicate that ECM composition dynamically changes during tissue development. Future studies will focus on quantifying ECM protein turnover in vivo to resolve individual protein function during the development of muscle, tendon, and bone. In addition, we will confirm that these methods are able to detect differences in protein composition during development using models in which the ECM is dysregulated. Ultimately, these studies will reveal important changes in the ECM during development and provide tissue engineers with directed criteria to design scaffolds that promote the assembly of functional tissue.

Novel Aspect

Our normalization method to account for significant increases in protein content between stages of forelimb development.

46. Roland Jones

Decomposition of H+(GlyProAla): A Guided Ion Beam and Computational Study

Peter B. Armentrout (University of Utah, Department of Chemistry)

Introduction

Demand for robust peptide sequencing has increased as a result of the rising number of applications ranging from general protein characterization to biomarker identification. Current sequencing often entails collision-induced peptide dissociation followed by product analysis using

mass spectrometry and sequencing. This technique could be improved in reliability and completeness by incorporating the propensity for different product fragments to form via competitive reaction pathway analysis, which could arise from energetic analyses of different peptide decompositions as demonstrated with H+(GlyProAla) in this work. More specifically, proline-containing peptides often exhibit unique structural and energetic properties as a result of the unique trans/cis isomerization of proline. So, analyzing such peptides provides an excellent starting point for identifying competitive key reaction pathways.

Methods

Our lab utilizes Guided Ion Beam tandem Mass Spectrometry (GIBMS). Briefly, GIBMS enables us to select a precursor ion to undergo decomposition via collision-induced dissociation (CID). Once the precursor ion is selected, it is guided through an exponential retarder to adjust it to well defined kinetic energies. Next, the ion enters a gas cell containing xenon for CID at a defined pressure. The fragments resulting from CID are then analyzed using a quadrupole mass selector and converted to cross-sections as a function of kinetic energy. These measurements are taken across multiple xenon pressures in order to extrapolate the results to single-collision conditions. By mapping product formation as a function of kinetic energy, we can analyze competition among product formation pathways.

Preliminary Data

This work utilizes uses Guided Ion Beam Tandem Mass Spectrometry to collect kinetic energy dependent cross sections of the collision-induced dissociation (CID) of protonated GlyProAla (GPA). Products are named using the “all-explicit” nomenclature system from Siu and co-workers. As the N-terminal peptide identity influences the structure of the b2+ ion formed, GPA forms b2+ ions exclusively in the oxazolone form, whereas the replacement of glycine with other aliphatic amino acids has been shown to also produce diketopiperazine b2+ ions. The b2+ ion structure is of importance as it dictates part of the competition with the formation of other fragment ions. This b2+ oxazolone ion leads to the formation of the a2+ ion through sequential dissociation of CO. The intensities of these two ions are further increased because there is no competing pathway with the formation of a [y1 + 2H]+ ion because proline is the middle peptide and does not have the necessary proton on its nitrogen needed for [y1+ 2H]+ ion formation. Another oxazolone structure is also formed through the loss of water at a lower collision energy. Like the b2+ product, this b3+ product can also undergo CO loss to form an a3+ product. For other product channels, primary loss of CO leads to an unobserved intermediate as further dissociation is thought to only require a minimal amount of additional energy. Unlike in triglycine dissociation, additional loss of ammonia is not observed. Theoretical calculations suggest this is a result of the process being entropically and enthalpically unfavorable compared to the competing formation of the [y2 + 2H]+ from the same initial intermediate. An imine structure (a1+) is also observed from the primary CO loss. Observations of these pathways as a function of energy aim to provide key insight as to what products formed are indicative of proline-containing peptides.

Novel Aspect

Incorporation of competitive reaction pathways into CID-based peptide sequencing algorithms can ultimately lead to more efficient and robust sequencing.

47. Illiyana Kaneva

Quantitative Proteomic Analysis of Cdc14 interactors in Candida albicans Using SILAC-based Mass Spectrometry

Iliyana Kaneva, University of Sheffield, MRC London Institute of Medical Sciences
Ian Sudbery, University of Sheffield
Joseph Longworth, University of Sheffield
Mark Dickman, University of Sheffield
Peter Sudbery, University of Sheffield

Introduction and Methods

Stable isotope labelling by amino acids in cell culture (SILAC) in conjunction with MS analysis is a sensitive and reliable technique for quantifying relative differences in protein abundance and post-translational modifications between cell populations. We have developed SILAC-MS workflow for quantitative proteomics in the fungal pathogen Candida albicans. Arginine metabolism provides important cues for escaping host defenses during pathogenesis, which limits the use of auxotrophs in Candida research. Our strategy, known as native SILAC (nSILAC), eliminates the need for engineering arginine auxotrophs for SILAC experiments and allows the use of ARG4 as selectable marker during strain construction. Additionally, we employed the described nSILAC method to do a proteome-wide screen of interacting partners of the phosphatase Cdc14 in C. albicans.

Preliminary Data

Cells that are auxotrophic for lysine were successfully labelled with both, lysine and arginine stable isotopes. We found that prototrophic C. albicans preferentially uses exogenous arginine and downregulates internal production, which allows is to achieve high incorporation rates of heavy arginine. This makes C. albicans a suitable organism for nSILAC. However, similar to other yeast, C. albicans is able to metabolise heavy arginine to heavy proline, which compromised the accuracy of protein quantification. We developed a computational method to correct isotope ratios for the incorporation of heavy proline. Using nSILAC-MS, we have carried out a Cdc14 interaction screen. Since dephosphorylation reactions are very transient in nature and difficult to capture, we generated a phosphatase-dead mutant of Cdc14 (Cdc14PD), as such mutants are known to form more stable bonds with their substrates. As a result, we identified 126 proteins that interact with Cdc14, of which 80% have not been previously described in the budding and fission yeasts. In this set, 55 proteins are known from previous research to be involved in the cell cycle, regulating the attachment of the mitotic spindle to kinetochores, mitotic exit, cytokinesis, licensing of DNA replication by re-activating pre-replication complexes, and DNA repair. Five Cdc14-interacting proteins with previously unknown functions localised to the Spindle Pole Bodies (SPBs). The results fit well with the established role of Cdc14 in organising mitosis and cell division. Thus, we have greatly increased the number of proteins that physically interact with Cdc14 in C. albicans.

Novel Aspect

This is the first application of SILAC in C. albicans and we have identified over 100 novel Cdc14 interactors.

48. Jess Kelliher

Elucidating the phosphoproteome of a central regulator of intracellular survival during Listeria monocytogenes infection

Daniel A. Pensinger and John-Demian Sauer
Department of Medical Microbiology and Immunology, University of Wisconsin-Madison

Introduction

Listeria monocytogenes is a dangerous human pathogen that is also widely leveraged as a model to study host-pathogen interactions. L. monocytogenes requires access to the cytosol in order to replicate and spread; thus, understanding its adaptations to intracellular growth has the potential to shed light on avenues for antimicrobial intervention. Previous work in our laboratory has established a role for the highly conserved eukaryotic-like Ser/Thr kinase PrkA in cell wall homeostasis, central metabolism, intracellular survival, and ultimately virulence. Excitingly, our lab has identified inhibitors of PrkA and its homologs that potentiate the effects of β-lactam antibiotics. However, the phosphorylation targets of PrkA and its role in cell physiology and during infection remain poorly understood.

Methods

To understand the role of PrkA during infection, we are currently developing a protocol to isolate whole bacteria from macrophages in cell culture, enabling us to elucidate the phosphoproteome of PrkA ex vivo. We are harnessing a Listeria phage cell wall-binding protein conjugated to magnetic beads to quickly and efficiently isolate bacteria from macrophage lysates, allowing us to minimize contamination of our samples with host cell proteins. We will use wild type and ΔprkA strains, plus a strain lacking the cognate phosphatase of PrkA, PrpC (ΔprpC). To process these samples, we will apply MS/MS combined with Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC) or Tandem Mass Tagging (TMT).

Preliminary Data

As a first step to identifying PrkA targets, we used shotgun proteomics to define the phospho-proteome of WT, ΔprkA, and ΔprpC strains grown in rich medium in the presence of sub-inhibitory concentrations of a cell wall-acting antibiotic. Our preliminary data suggest that PrkA phosphorylates ~40 proteins with functions ranging from cell wall synthesis to stress response to central metabolism. These findings are in agreement with our growth phenotypes and suggest that PrkA is a direct regulator of a variety of cellular processes.

Novel Aspect

If successful, our experiments would be the first to explore the phosphoproteome of a pathogen during infection.

49. Roxana Khoshravesh

Hyperspectral Raman Light Sheet Microscope for In Vivo Imaging of Leaf Metabolism

Roxana Khoshravesh: Postdoc, Department of Biology, University of New Mexico

Keith Lidke: Professor, Department of Physics and Astronomy, University of New Mexico
Jerilyn Timlin: Distinguished Member of the Technical Staff, Sandia National Laboratories
David Hanson: Professor, Department of Biology, University of New Mexico

Introduction

Re-engineering photosynthetic pathways such as C4 in energy and food crops will be facilitated by developing advanced technologies that can demonstrate the success of the intended modifications. This is especially true when intermediate steps are needed and the physiological phenotypes are hard to predict or measure. Non-destructive, in vivo, tools are important to assess temporal dynamics and imaging tools will assess spatial dynamics. To date, the majority of the approaches used to monitor metabolite flux, including stable isotope labeling, are destructive or suffer from lack of temporal or spatial resolution which limits the power of in vivo experiments. Currently, we are developing techniques and tools for non-destructive measurement of temporal and special dynamics of primary and secondary metabolites in leaves.

Methods

We are developing a high-resolution Hyperspectral Raman Light Sheet Microscope to measure primary and secondary metabolites of leaves within a gas exchange chamber where the rate of photosynthesis and the environmental conditions are controlled. By feeding 13C, 18O, and 15N labeled metabolite through the leaf petiole or gas-phase pulse-chase labeling, we will 1) analyse the spatiotemporal distribution of the labeled chemicals in cellular and sub-cellular level and 2) monitor metabolite conversion and identify the intermediates carrying 13C, 18O, and 15N by capturing their Raman vibration; 3) validate and compare the obtained data with the standard available methods of metabolite analysis such as LC-MS to identify metabolites and proteins.

Preliminary Data

Currently, we are in the phase of technological and experimental design. The technological design is in progress and simultaneously, we have defined the target plants and pathways. The experiments will be conducted on C3 and C4 biofuel crops and their phylogenetically close C2 relatives. Our results will include the distribution of photorespiratory intermediates after feeding the leaf with 18O, 13C, and 15N. Currently, we are testing our target leaves with the previously developed Raman spectroscopy imaging tools in Sandia National labs to set the criteria for the new design.

Novel Aspect

Our method will provide high-resolution spatiotemporal data on the distribution of the photo-synthetic metabolites in the leaf non-destructively.

50. Lisa Kirkemo

Characterizing and Targeting the Surface Proteome of Hypoxic Pancreatic Cancer

Lisa L. Kirkemo [1] and James A. Wells [1,2]
[1] Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA, USA

[2] Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, USA

Introduction

Low oxygen in tumors, termed hypoxia, leads to poorer patient prognoses. In response to hypoxia, cancer cells activate cellular pathways and gene programs that promote survival, migration, and immune privilege. These hypoxia-specific effects suggest that tumor hypoxia can be leveraged to elucidate cell surface markers for selective targeting of hypoxic tumors. We have utilized cell surface proteomics to identify a novel hypoxia-regulated target in pancreatic cancer, target X. Additionally, we have shown that knockdown of target X decreases the survival of pancreatic cancer under hypoxia and that target X undergoes proteolytic cleavage in vitro. We hypothesize that target X proteolysis leads to altered signaling that enhances survival and proliferation of hypoxic pancreatic cancer cells.

Methods

Panc-1 and Capan-1 pancreatic cancer cell lines were grown in SILAC media containing heavy and light arginine and lysine isotopes under hypoxic (1% O2) and normoxic (20% O2) conditions. Surface proteins were identified using an established biocytin-hydrazide surface glycoprotein enrichment strategy and subsequent tryptic LC-MS/MS. Analysis of hypoxia-induced proteins revealed target X as a marker of hypoxic pancreatic cancer, which was confirmed with western blot. To determine the functional role of target X in the proliferation of hypoxic pancreatic cancer cells, dCas9KRAB containing Panc-1 cells were transduced with sgRNA against target X and proliferation was monitored using CellTiterGlo. Proteolysis of target X was investigated using media immuno-precipitation and subsequent western blotting.

Preliminary Data

To elucidate the effects of hypoxia on the cell surface proteome of pancreatic cancer cells, Panc-1 and Capan-1 cell lines were cultured in SILAC media containing heavy and light arginine and lysine isotopes to allow for quantitative comparison via tryptic LC-MS/MS. We utilized a glycoprotein labeling biocytin-hydrazide strategy for selective cell surface protein isolation to identify novel hypoxic cell surface proteins in pancreatic cancer. Within this dataset, we discovered a novel hypoxia-regulated pancreatic cancer marker, target X. To probe the function of target X we performed knockdown experiments, which showed that the absence of target X decreased pancreatic cancer cell survival and proliferation under hypoxia. These findings suggest target X serves as an essential protein for pancreatic cancer cell adaptation to hypoxic environments. Through the use of phage-display, we isolated antibody clones against the ectodomain of target X. Flow cytometry experiments showed that these clones did not bind to the surface of target X expressing cells. However, through immunoprecipitation of the filtered media with our biotinylated antibody clones, and subsequent western blotting with commercial antibodies, we were able to show that our in-house antibody clones recognized a shed portion of the target X ectodomain in the media. We confirmed these findings through expression of an N-terminally flag-tagged target X, which was retrievable in filtered media, indicating that target X undergoes numerous cleavage events under hypoxia in vitro. Identification and characterization of this cleaved form of target X is critical to understand the role this cleavage event might be playing in downstream signaling, as well

as whether this cleavage event is necessary for imparting a growth advantage to cells expressing target X under hypoxia.

Novel Aspect

Characterization of target X expands our mechanistic knowledge of pancreatic cancer and is leverageable for downstream antibody-based therapeutics and diagnostics.

51.Darcy Knaack

The roles of SR-BI and CD36 in maintenance of macrophage cholesterol homeostasis

Schill, RL (Medical College of Wisconsin, Milwaukee, WI), Chen, Yiliang (Blood Research Institute, Milwaukee, WI), Silverstein, RL (Blood Research Institute, Milwaukee, WI), Sahoo, D (Medical College of Wisconsin, Milwaukee, WI)

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide. Atherosclerosis, a major cause of CVD, is a chronic inflammatory disease characterized by the buildup of cholesterol-rich macrophages within the endothelial wall. Macrophage cholesterol homeostasis is regulated by membrane-bound scavenger receptors that facilitate cholesterol transport between cells and lipoproteins. Two critical macrophage scavenger receptors that mediate the flux of cholesterol are scavenger receptor BI (SR-BI) and cluster of differentiation 36 (CD36), which bind high-density lipoproteins (HDL) and oxidized low-density lipoproteins (oxLDL), respectively. Their individual roles in modulating atherosclerosis have been widely studied, but how they influence each other's functions has yet to be investigated. We hypothesize that SR-BI and CD36 are functional partners that mediate cholesterol homeostasis in macrophages.

Methods

Experiments were performed in thioglycolate-stimulated primary peritoneal macrophages isolated from wild-type (WT), SR-BI knock-out (SR-BI-/-), and/or CD36 knock-out (CD36-/-).

Co-immunoprecipitation assays: WT macrophages were incubated in the presence or absence of 50 μg/mL oxLDL for 20 minutes. CD36 was immunoprecipated by a CD36 antibody. Both CD36 and SR-BI expression were assessed by immunoblot analysis.

Binding and uptake assays: Macrophages were incubated at 4 °C or 37 °C for 90 minutes to measure HDL binding and DiI-labeled cholesterol uptake. Mean fluorescence intensities of DiI were measured by flow cytometry.

Lipid rafts analysis: Macrophages were subjected to discontinuous sucrose-gradient ultracentrifugation. Fractions were separated via SDS-PAGE and immumoblotted for SR-BI, CD36, non-raft markers (i.e. tubulin), and raft marker (i.e. floatillin, caveolin-1).

Preliminary Data

To test our hypothesis, we performed three different sets of experiments to explore the possibility of a partnership between SR-BI and CD36. First, through preliminary co-immunoprecipitation assays, we were able to demonstrate a potential interaction between SR-BI and CD36 in WT peritoneal

macrophages, that was enhanced with oxLDL treatment. Next, we wanted to examine how the cholesterol transport functions of these receptors were affected when one receptor was absent. We were able to show that when CD36 is absent, HDL's ability to bind macrophage receptors was impaired, as was the macrophage's ability to internalize HDL-cholesterol as compared to macrophages from WT or SR-BI-/- mice. Lastly, it is known that SR-BI and CD36 exist in raft-like membrane microdomains. We used a sucrose-gradient fractionation method to examine whether the absence of SR-BI or CD36 impacts plasma membrane localization of the other receptor. Our data suggest that, in WT cells, both SR-BI and CD36 reside within the lipid raft domains, but when one receptor is absent, the other migrates to non-raft domains. Based on these results, there appears to be a cooperative partnership between SR-BI and CD36, suggesting that these two scavenger receptors may partner together to help promote cholesterol homeostasis in macrophages. We are currently investigating how scavenger receptor expression changes in the presence and absence of HDL and oxLDL using quantitative real-time PCR and immunoblot methods. We also plan to utilize mass spectrometry for measuring protein-protein interactions, as well as Parallel Reaction Monitoring (PRM) methods for absolute quantification of SR-BI, CD36, and other transporters and receptors known to participate in cholesterol transport.

Novel Aspect

The potential partnership between cholesterol transport proteins, SR-BI and CD36, could lead to new therapeutic targets for atherosclerosis and CVD.

52. Lisa Kobos

In Vitro Toxicity Assessment of Emitted Materials Collected during the Manufacture of Water Pipe Plastic Linings

Teimouri Sendesi, Seyedeh Mahboobeh; Purdue University
Whelton, Andrew; Purdue University
Howarter, John; Purdue University
Boor, Brandon; Purdue University
Shannahan, Jonathan; Purdue University

Introduction

U.S. water infrastructure is in need of widespread repair due to age-related deterioration. Currently, the cured-in-place (CIPP) procedure is the most common and cost-effective method for water pipe repair. This method involves the on-site manufacture of a new polymer composite plastic liner within the damaged pipe. The procedure's use is expected to expand globally, with the CIPP market exceeding $2.5 billion by 2022, and accounting for 40% of the U.S. pipe rehabilitation market. The CIPP process can release materials into the environment resulting in occupational and public health concerns. A number of indoor and ambient air public health incidents (> 100) have been associated with CIPP worksites, with some prompting formal responses from government agencies.

Methods

To understand hazards associated with CIPP-related emission exposures, an in vitro toxicity assessment was performed utilizing alveolar epithelial and alveolar macrophage cell lines and emission samples collected at 3 worksites utilizing styrene-based resins. Samples were normalized

based on the major emission component, styrene. A styrene-only exposure group was used as a control to determine mixture-related toxicity. A proteomic evaluation was performed to examine protein expression changes induced by exposure to samples or styrene alone. Venn diagrams were produced to determine commonalities and differences in protein identities modified following exposures. LFQ intensity values were utilized to quantify fold changes. The lists of proteins determined to be significantly altered were imported into Ingenuity Pathway Analysis for gene ontology and molecular pathway analysis.

Preliminary Data

Cytotoxicity differences were observed between worksite samples, with the CIPP worksite 4 sample inducing the most cell death. Exposures were also induced differential gene expression of inflammatory and oxidative stress markers that varied based on worksite and compared to styrene-only controls. Our examination of protein expression changes determined styrene-, worksite-, and cell-specific alterations, identifying potential biomarkers of exposure and inducible pathways of toxicity. C10 cells exposed to CIPP condensates and styrene-alone exhibited differential protein expression compared to untreated controls. The CIPP condensate sample collected at worksite 4 was demonstrated to induce the greatest number of altered proteins compared to controls, while worksite 3 induced the least. Exposure of C10 to styrene-only resulted in alterations of 129 proteins with the majority being down-regulated. Of the proteins altered following exposure to all three CIPP condensates, the majority were up-regulated. Alveolar macrophages also demonstrated differential protein expression following CIPP condensate or styrene-only exposures compared to controls. A number of differentially expressed proteins were identified between individual CIPP worksite condensates and RAW cells exposed to only styrene. Specifically, worksite 4 demonstrated the most differences when compared with styrene, whereas the response from worksite 1 was the most similar to styrene-only. Worksite-specific responses were also identified in terms of protein responses. CIPP condensates collected from worksites 3 and 4 were determined to be the most different, whereas worksites 1 and 3 were the most similar. Pathways found to be induced by exposure to CIPP condensates include inflammatory disease and organismal injury. Together, these findings demonstrate risks associated with the CIPP procedure as well as variations between worksites in regard to emissions and toxicity. Future studies are needed to understand short- and long-term health effects. Our findings suggest that the CIPP process requires administrative and engineering control upgrades to protect workers and the public from harm.

Novel Aspect

Our study is the first to utilize mass spectrometry to examine the toxicity of CIPP emissions.

53. Iris Kreft

Proteome profiling of platelet disorders using mass spectrometry

Elise J. Huisman[#], Floris P.J van Alphen[*], Carmen van der Zwaan[*], Mariette Boon-Spijker[*], Karin van Leeuwen[*], Maartje van den Biggelaar[*], Alexander B. Meijer[*]

[#] Department of Pediatric Hematology, Erasmus Medical Centre, Sophia Children's Hospital, Rotterdam, The Netherlands

[*] Department of Plasma Proteins, Sanquin Research, 1066 CX Amsterdam, The Netherlands

Introduction and Methods

Platelet function disorders are characterized with mild or severe bleedings which are poorly understood. Currently, diagnosis of rare platelet disorders involves function tests and DNA analysis. Mutations in genes often lead to absence of the involved proteins or defects in functionality of associated proteins. However, it is unclear whether novel mutations in established critical proteins also affect protein expression. In addition, when no mutations are found a disorder remains unresolved. Mass spectrometric analysis holds great potential to tackle these issues and may reveal unique expression profiles that can be linked to platelet diseases.

Preliminary Data

In the present study we examine the feasibility of mass spectrometry as a diagnostic tool by assessing whether protein profiles can be associated with disorders. We applied shotgun mass spectrometry with label free quantification to investigate platelets disorders in children. This resulted in the identification of 9 platelet disorders.

Novel Aspect

We showed that label free quantification-based proteomics is suitable to link certain protein profiles to known genetic defects.

54. Laura Kremer

Mitochondrial proteome changes in mouse models of mitochondrial disorders

Inge Kühl (Department of Cell Biology, Institute of Integrative Biology of the Cell (I2BC) UMR9198, CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, Gifsur-Yvette, France)
Maria Miranda (Department of Mitochondrial Biology, Max Planck Institute for Biology of Ageing, Cologne, Germany)
Nils-Göran Larsson (Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden)

Introduction

Mitochondria are double-membrane enclosed cellular organelles largely known as the powerhouse of the cell. They transduce energy stored in fats, carbohydrates, and proteins to the cellular energy currency ATP via a process called oxidative phosphorylation (OXPHOS). Impairment of OXPHOS largely disturbs energy supply by mitochondria and gives rise to a plethora of clinical symptoms including cardiomyopathy. However, up to now it is unclear whether the pathophysiological outcomes of defective OXPHOS can be primarily attributed to ATP deficiency or whether additional molecular consequences substantially contribute to mitochondrial disease.

Methods

This project will harvest the comprehensive proteomics datasets generated in the lab previously (Kühl et al., 2017). The data comprises proteomics data derived from ultrapure mitochondrial isolations prepared from the hearts of five heart- and skeletal muscle-specific knockout mouse strains with severe mitochondrial dysfunction as well as controls. Notably, mtDNA gene expression

is disturbed at different levels in the knockout mouse strains ranging from impaired mtDNA replication to translation (Twnk, Tfam, Polrmt, Lrpprc, Mterf4).

Preliminary Data

Previous work from our lab has shown that the regulation of the mouse heart mitochondrial proteome mainly takes place at the protein level (Kühl et al., 2017). The study confirmed previous reports showing a drastic disturbance of the 1C metabolism upon OXPHOS impairment and could show that this was an early event present before detectable OXPHOS deficiency. Intriguingly, a downregulation of key coenzyme Q (ubiquinone, Q) biosynthesis enzymes mirrored by reduced Q levels was also evident in all five knockout mouse strains. This finding is outmost importance as Q supplementation is often considered as a treatment regime for mitochondrial disease patients.

Importantly, the precise mechanisms of the compensatory responses remained elusive so far. I am now aiming to shed further light onto the specifics of the molecular processes as well as the temporal sequence of events by in depth analysis of the rich proteomics resource provided by Kühl et al. For validation of the findings and modulation of the involved pathways I will employ cellular models.

Novel Aspect

Determination of molecular mechanisms and chronology of events contributing to mitochondrial disorders is crucial for advising disease intervention strategies.

55. Rasmus la Cour

Influence of nitrogen supply and biorefinery fractionation on the proteome of green biomasses

Associate professor Henning Jørgensen
Professor Jan K. Schjoerring
University of Copenhagen, Department of Plant and Environmental Science

Introduction

Investigations into the proteome of green biomasses could yield invaluable insight into the effects of growth conditions. Nitrogen is a major plant nutrient and key factor in plant protein synthesis. The nitrogen supply during growth is therefore expected to have great impact on the proteome. Knowledge of the effects of nitrogen supply on the proteome is key in understanding plant growth. In a biorefinery scheme, knowledge of the proteome can improve yields, as well as give a better understanding of how the proteins separate during the fractionation process. Another advantage is that insights into the proteome of biorefinery fractions enables us to identify high value proteins that can potentially be isolated. These compounds will make the production more economically feasible.

Methods

The primary method of this study is the proteomics study performed on fractionated green biomasses. The fractionation was performed on perennial rye grass (lolium perenne) and white clover (trifolium repens) grown together. Samples were taken from different fields that were given different fertilizer treatments. The materials were mechanically fractionated into a pulp fraction and

a juice fraction. The two fractions, as well as the non-fractionated material were analyzed for amino acid composition as well as for the proteome. The amino acid composition was determined by HPLC with single quadrupole MS and the proteome analysis was performed on a Thermo Fusion Orbitrap MS with a nano-LC.

Preliminary Data

We have shown that the nitrogen status strongly affects the amount of harvested biomass as well as the ratio of grass to clover. This is already well-established knowledge. We have shown that the nitrogen supply has very little effect on the crude protein concentration if the plants, except for in very high doses. This led us to believe that the amino acid composition of the protein in the plants was very similar. We found that the content of several amino acids was significantly different depending on the nitrogen supply. Notably, the clover samples showed differences in 11 of the 21 amino acids tested. When the fractionated material was tested, however, the significant differences were lost. Almost no significant differences were observed in the juice or the pulp fraction from either plant. With this in mind, a proteome analysis was performed to investigate if the lack of differences in amino acid composition was a result of a lack of difference in the proteome, or simply a result of limited variation of amino acid composition of different proteins. From the preliminary data, we see that the fractionation step introduces a large variation among biological replicates, diminishing the statistical significance of the differences present in non-fractionated materials. When analyzing the juice fraction, the amount of only a few proteins are different with different nitrogen status, whereas many of the proteins are measured in different amounts in non-fractionated materials. The non-fractionated material will be used for studies of how growth conditions affect the proteome of green biomasses. From preliminary data, upwards of 10 % of proteins in clover and 20 % of proteins in rye grass seem to be affected by nitrogen supply.

Novel Aspect

Proteome analysis on fractionated green biomass has not been performed before.

56. Daniel Lee

Metabolic Perturbations Include Homocysteine Degradation and Polyamine Synthesis in Pathogenesis of Bronchopulmonary Dysplasia

Lauren Zacharias[2], Sang Jun Park[1,3], Kirsten Zborek[1], Eric Sah[1,3], Adam Pajakowski[1], Ralph DeBerardinis[2], Margaret A. Schwarz[1]

[1]Indiana University School of Medicine, South Bend, IN, [2]Children's Medical Center Research Institute at University of Texas–Southwestern, Dallas, TX, [3]University of Notre Dame, South Bend, IN

Introduction

Changes in dynamic metabolic processes during lung development is not well known, particularly during the saccular and alveolar stages. Lungs of infants prematurely born during either stage have underdeveloped alveolar sacs with arrested growth and frequently require oxygen supplementation, which can result in lung disease of prematurity (bronchopulmonary dysplasia, BPD). Oxidation-reduction (redox) processes are important in both lung development and pathogenesis; however, clinical studies attempting to limit BPD pathogenesis targeting redox processes have not been

promising. In this study, metabolomics were utilized to identify alternative metabolic perturbations during the two lung development stages in an established murine model of hyperoxia-induced BPD.

Methods

Postnatal day (PN) 3 mice from pairs of pregnant dam mice were pooled, randomized, and left at normoxia (room air, 21% O2) or exposed to hyperoxia (85% O2) in a tight-sealed, plexiglass chamber until PN 15. Nursing dams were rotated every 24 hours to limit oxygen toxicity. Lungs were isolated from mice on PN 1, 3, 5, 7, 10, and 15 within two minutes then snap frozen in liquid nitrogen. Following methanol extraction, metabolites were detected by liquid chromatography-mass spectrometry. Statistics including partial least squares–discriminant analysis (PLS-DA) and Variable Importance in Projection (VIP), i.e. a quantitative measure of discriminating metabolites, were performed using Python 2.7/3.0 and R.

Preliminary Data

A series of analysis were performed to validate, identify, and characterize both significant metabolites and their involved pathways. Samples could be separated by both time and oxygen-level using PLS-DA. Enrichment analysis of metabolites with VIP ≥ 1 indicated that both the methionine-homocysteine degradation cycle ($p \leq 1e\text{-}4$) and downstream of it, polyamine synthesis was perturbed ($p \leq 1e\text{-}4$). Components of both the arginine and glutathione metabolism pathways were also significantly enriched ($p \leq 1e\text{-}4$). Metabolites specifically found in the prior enrichment analysis (e.g., cystathione in glutathione metabolism, spermine, spermidine in polyamine synthesis) were significantly elevated in hyperoxic lung tissues on PN 5, 7, and 10 (one-way ANOVA, $p \leq 1e\text{-}2$). Metabolites with a fold change of ≥ 1.3 from only day 5 lung tissues were significantly enriched for methylhistidine metabolism ($p \leq 1e\text{-}5$).

Novel Aspect

Which and how metabolic networks change over lung development and pathogenesis of broncho–pulmonary dysplasia is not yet known.

57. Jung-Youn Lee

A specific transmembrane domain-mediated dimerization is required for the receptor-like, plasmodesmata-located protein to regulate cell-to-cell movement in plants

Xu Wang (University of Delaware)
Cecilia N. Arighi (University of Delaware)

Introduction

Numerous cell surface receptors and receptor-like proteins (RLPs) undergo activation or deactivation via a transmembrane domain (TMD) serving as a dimerization interface while anchoring the protein at the membrane. A large number of receptors and RLPs are expressed in plants, and only a subset of those are known to localize to intercellular junctions where plasmodesmata (PD), membrane-lined intercellular nanochannels, are present. Those PD-localized RLPs include the PD regulator and defense protein Arabidopsis thaliana plasmodesmata-located protein (PDLP) 5.

Although PDLP5 plays a vital role in plant immunity by regulating molecular movements between cells across PD, little is known about the mechanism by which the protein is activated.

Methods

We used experimental approaches that combine domain/motif mutagenesis, fluorescent imaging and microscopic techniques in conjunction with viral movement assays and computational modeling for TMD dimerization.

Preliminary Data

We found that the TMD of PDLP5 facilitates specific homomeric and heteromeric interactions and that this native TMD-based dimerization is required for the protein's PD-regulating activity. We also present a model describing how PDLP5 activation might be achieved through a specific TMD-based dimerization via the conserved Ax3G motif.

Novel Aspect

An evolutionarily conserved, novel TMD-based dimerization motif is essential for regulating cell-to-cell movement by the receptor-like protein PDLP5

58. Jianhua Li

Functional proteomics analysis of epiblast self-organization

Jianhua Li[1], Rui Fan[1], Hannes CA Drexler[2], Ivan Bedzhov[1]

[1] Embryonic Self-Organization research group, Max Planck Institute for Molecular Biomedicine

[2] Bioanalytical Mass Spectrometry, Max Planck Institute for Molecular Biomedicine

Introduction

The life of a new organism starts with fertilization. After several rounds of cell divisions, the first cell lineages emerge in the developing embryo. In mice, the embryonic lineage (epiblast) is specified at around day 4 of embryogenesis when the embryo reaches blastocyst stage. In the next 24h the blastocyst initiates implantation and transforms into an egg cylinder. At the same time the epiblast (EPI) undergoes a dramatic transformation from unorganized ball of cells into a polarized epithelium with central cavity. The mechanism of EPI morphogenesis is poorly investigated. Recently, it was suggested that polarization cues from the extracellular matrix (ECM) drive the establishment of apical-basal epithelial polarity in the peri-implantation EPI. As a result, the EPI self-organizes into rosette-like structure.

Preliminary Data

Interestingly, this epiblast self-organization process can be recapitulated in vitro, by culturing embryonic stem (ES) cells embedded into ECM hydrogel (matrigel). Within 48h of 3D culture, ES cell clumps to form polarized rosette-like structure with central lumen, mimicking the EPI development. This method overcomes the limitation of the low cells number of the mouse embryo, providing virtually unlimited material for downstream functional proteome analysis. In addition to whole cell lysate proteome, I performed further nuclear, cytoplasmic and membrane fractionation, the proteomes are investigated at subcellular resolution. Two channels SILAC approach was employed to label naiive state and primed state.

Methods

The ES cell culture condition using SILAC media and incorporation of labeled amino acids were optimized. The cells cultured with Gsk3b and Mek inhibitors plus Lif (2i/Lif) were maintained as pre-implantation state of the epiblast. And cells cultured in the presence of Fgf2 and Activin (FA) resembled the post-implantation state. Proper development of cells was validated by polarity and pluripotency markers using western blot.

Subcellular proteome fractions from nuclear, cytoplasmic and membrane were extracted. Whole cell lysate proteome was employed as control. Individual proteome analysis identified differentially expressed factors; integrated proteome analysis of all four fractions indicates, that subsets of the proteome changed their subcellular distribution particularly between nuclear and cytoplasm fractions before and after differentiation.

Preliminary Data

Short list of candidate factors was selected for functional analysis to examine their potential roles in EPI self-organization.

Novel Aspect

3D culture, functional proteomics analysis, epiblast self-organization, subcellular resolution, network analysis, PTMs.

59. Dan Lim

Identifying redox modifications of the mitotic kinase Aurora A

Marian Kalocsay (Harvard Program in Therapeutic Science, Harvard Medical School)
Vladimir Joukov (N.N. Petrov National Medical Research Center of Oncology, Saint-Petersburg, Russian Federation)
Johannes Walter (Howard Hughes Medical Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School)
Steven P. Gygi (Department of Cell Biology, Harvard Medical School)
Michael B. Yaffe (Koch Institute for Integrative Cancer Research, Center for Precision Cancer Medicine, Massachusetts Institute of Technology)

Introduction

Redox modifications of protein cysteine thiols play important roles in regulating diverse physiological processes. An increasing number of protein kinases are recognized to contain redox-sensitive cysteine residues that regulate their activities. Aurora A is a key cell cycle regulatory kinase and cancer drug target. A cysteine residue within the activation loop of Aurora A is conserved in many other AGC and CAMK family kinases, and evidence has been emerging that this is an important site of redox-dependent regulation of these kinases. Redox modifications of the activation loop cysteine residue in Aurora A may provide a mechanistic link that allows intracellular redox changes to regulate cell cycle progression.

Methods

Strep-Tag II tagged constructs of Aurora A will be expressed in mammalian cell lines and affinity purified under non-reducing and reducing conditions for proteomic analysis to identify disulfide-

dependent protein interaction partners. Additionally, LC-MS/MS analysis of tryptic peptides of affinity-purified Aurora A will be used to identify redox post-translational modifications. Analysis of Aurora A purified from asynchronous and synchronized cells will allow identification of cell cycle-dependent redox modifications.

Preliminary Data

As proof of concept, we have incubated recombinant Strep-Tag II tagged Aurora A kinase domain in Xenopus egg extract and identified a novel Aurora A interactor that is bound in a disulfide-dependent manner under oxidizing conditions. Creation of mammalian cell lines expressing suitable constructs of Aurora A is in progress.

Novel Aspect

Identification of redox modifications of Aurora A will uncover important regulatory mechanisms and may lead to novel anti-cancer therapeutics.

60. Iasmim Lopes de Lima

Plasma Protein Changes Analysis in Colorectal Adenocarcinoma by Label-Free Quantitative Proteomics

Iasmim L. de Lima[1], Camila R. da Cruz [2], Sandra M. N. Scapin[3], José M. Granjeiro [3], Roberto A. da Silva[1,5], José Luiz de Lima Filho [1,4]

[1] Keizo Asami Immunopathology Laboratory—LIKA, Federal University of Pernambuco — UFPE, Recife, PE - Brazil.

[2] Surgery Department, Clinical Hospital — HC, Federal University of Pernambuco — UFPE, Recife, PE - Brazil.

[3] Metrology Applied to Life Sciences, National Institute of Metrology, Quality and Technology — INMETRO, Duque de Caxias, RJ, Brazil.

[4] Biochemistry Department, Federal University of Pernambuco — UFPE, Vitória Campus, Vitória, PE, Brazil.

[5] Biochemistry Department, Federal University of Pernambuco — UFPE, Recife, PE - Brazil.

Introduction

Colorectal cancer (CRC) is a very heterogeneous disease and is the third most frequent cancer with 1.8 million new cases in 2018. Also, it is the second leading cause of cancer deaths in the world with approximately 0,8 million deaths expected in the last year. In Brazil, 35.360 new cases are expected for the biennium of 2018-2019. Molecular markers are urgently required to respond to the increasing incidence of this type of cancer, improving the patient's clinical outcome and increasing the survival time. The evaluation of changes in plasma proteins expression in colorectal cancer is valuable to the search for potential proteins for the composition of signatures that can be used in various clinical contexts.

Methods

Plasma samples were collected from ten healthy donors and ten colorectal adenocarcinoma patients without neoadjuvant therapy before surgical resection. IgG and Albumin SpinTrap Kit (Ge Healthcare) was used before precipitation and protein trypsinization. Then, the tryptic digestion

products were separated by nanoACQUITY UPLC system (Waters) at a flow rate of 450 nL/min. A Synapt HDMS mass spectrometer (Synapt G2 Si, Waters) was used for data-independent analysis (MSE) of the tryptic peptides. All analyses were performed in the "V" mode, using nano-electrospray ionization in the positive-ion mode (nanoESI+). The data obtained were processed by ProteinLynxGlobalServer v. 2.4 software (PLGS) and differentially expressed proteins were identified by PLGS ExpressionE software (Waters, UK).

Preliminary Data

We identified 98 human plasma proteins with score ≥ 100 and confidence interval ≥ 99%. The analysis of the differential expression of proteins revealed the presence of 79 up-regulated proteins and 11 down-regulated in the colorectal patient's plasma. According to DAVID functional analysis, the biological process, molecular function and cellular component terms more overrepresented were: negative regulation of endopeptidase activity (22, p= 6,7e-29), serine-type endopeptidase inhibitor activity (15, p= 9,6e-19), and extracellular region (64, p= 5,1e-57). The most enriched signaling pathways by Reactome were related to Immune System (36, 2.00e-07), Innate Immune System (34, p= 1.01e-13) and Hemostasis (34, p=1.11e-16).

Novel Aspect

We found potential proteins that may be promising for the composition of a panel of biomarkers for colorectal cancer.

61.Sergio Madera

Development of a Mass Spectrometry Assay to Analyze the Ovarian Cancer Biomarker CA125

Naviya Schuster-Little (University of Notre Dame)
Roberta Fritz-Klaus (University of Wisconsin)
Manish Patankar (University of Wisconsin)
Mark Etzel (University of Wisconsin)
Rebecca Whelan (University of Notre Dame)

Introduction

We want to use analytical methods to investigate the primary amino acid sequence of the ovarian cancer biomarker CA125. CA125 is a large, 3-5 MDa protein that is used as a biomarker for ovarian cancer. It is composed of a core domain that is repeated more than 60 times. In this study, we aim to develop a workflow that enables us to take purified CA125, digest it with an enzyme, analyze it using mass spectrometry, and identify peptides that correspond to CA125. We will take CA125 that has been purified from ascites fluid and map the CA125 peptides to the parent CA125 sequence and determine if there are variations between peptides identified.

Methods

Sample preparation begins with roughly 50 µg CA125 that has been purified from ascites fluid or commercially available CA125. The protein is denatured in-solution with SDS, disulfide bonds are reduced with TCEP, and free thiol groups are alkylated with IAA. We will include DCA as a passivating agent to prevent CA125 from sticking to the tube. Following alkylation, the protein is loaded onto a

suspension trap and residual reagents are washed away. The protein is digested overnight using trypsin. The next morning peptides are eluted. Eluted peptides are concentrated and desalted using C18 ZipTips. Samples are dried and analyzed using nano liquid chromatography-mass spectrometry and capillary zone electrophoresis-mass spectrometry. We will analyze the data collected using Proteome Discoverer.

Preliminary Data

Our lab is currently optimizing digestion conditions using a standard protein, bovine serum albumin (BSA). We have also been successful with digesting and detecting peptides from recombinant version of CA125. However, we want to increase our sequence coverage before we begin to digest CA125 from ascites fluid samples.

Novel Aspect

We will digest CA125 from ascites fluid using a suspension trap and then compare the peptide profiles from different patients.

62. Morgan Mann

Isoform Specific Protein Interaction Mapping of BRD4-mediated Airway Inflammation via Immunoprecipitation Mass Spectrometry

Yanlong Zhu, University of Wisconsin
Allan Brasier, University of Wisconsin

Introduction

Every year, 2.1 Million American children require attention for Respiratory Syncytial Virus (RSV) infection. RSV has been shown to require Bromodomain Containing Protein 4 (BRD4) to induce airway inflammation via transcriptional activation of interleukin 6 (IL6), and inhibition of BRD4 has been proposed as a therapeutic treatment. However, several isoforms of BRD4 exist within the cell, and the contribution of each to RSV inflammatory pathways is unknown. To address this issue, we have applied Immunoprecipitation coupled to Label-Free Shotgun Proteomics to identify changes in BRD4 protein interaction partners specific to major BRD4 isoforms and relative to induction of inflammatory pathways using the Toll-like Receptor 3 ligand PolyIC.

Methods

Human Small Airway Epithelial Cells were cultured to confluency and treated with PolyIC or a vehicle control. After four hours, the cells were lysed in a nondenaturing detergent, and BRD4 was immunoprecipitated using both Long and Short-form specific antibodies and purified using Protein G magnetic beads. A nonspecific antibody was used as a control. Protein was eluted from the beads using a denaturing, photocleavable detergent, and digested with trypsin in-solution. After surfactant photocleavage, the samples were vacuum concentrated and loaded onto a Waters nanoACQUITY trap column for online desalting and separation prior to MS/MS using a Bruker Impact II Mass Spectrometer.

Preliminary Data

Using a similar workflow, our group previously immunoprecipitated the long isoform of BRD4. We found that its interactome was significantly enriched in proteins belonging to several known protein

complexes, including the AP-2 adaptor complex, the SWI/SNF complex, and the DNA-directed RNA Polymerase II complex. These protein complexes are consistent with proposed roles of BRD4 in cancer, HIV, and inflammatory pathways. In particular, the recruitment of RNA Polymerase II is consistent with currently proposed mechanisms for BRD4-mediated activation of IL6.

Novel Aspect

Identification of BRD4 interaction partners in an isoform specific and disease relevant manner.

63. Christina Mark

Elucidating HAPLN1 Regulation and Processing in Multiple Myeloma Disease Progression

Natalie S. Callander (Department of Medicine, University of Wisconsin Carbone Cancer Center)
Mailee Huynh (Cancer Biology Program, McArdle Laboratory of Cancer Research, Department of Oncology)
Peiman Hematti (Department of Medicine, University of Wisconsin Carbone Cancer Center)
Fotis Asimakopolous (Department of Medicine, University of Wisconsin Carbone Cancer Center)
Shigeki Miyamoto (McArdle Laboratory of Cancer Research, Department of Oncology, University of Wisconsin Carbone Cancer Center)

Introduction

Dysregulation of nuclear factor-kappa B (NF-κB) signaling involved in immune and cell survival regulation contributes to multiple myeloma (MM) progression and drug resistance. One of the cell types that influence NF-kB activity in MM cells is bone marrow stromal cells (BMSCs). We recently identified that hyaluranon and proteoglycan link protein 1 (HAPLN1), an extracellular matrix protein, is secreted from patient-derived BMSCs and capable of activating NF-κB and causing drug resistance in MM cells. Interestingly, proteolytically processed forms but not the full-length HAPLN1 cause NF-κB activation and drug resistance. However, HAPLN1 proteolytic processing mechanisms critical for NF-κB activation and drug resistance in MM cells remain unknown. As such, HAPLN1 has never been described as an extracellular oncogenic signaling factor.

Methods

BMSCs were derived from healthy donors or BM aspirates obtained from patients across MM premalignant and disease progression stages by culturing and passaging 2-4 times to eliminate contaminating cells. BMSCs were co-cultured with RPMI8226 MM cell line for 24 hours, and RPMI8226 cell lysate was evaluated for NF-κB activity using an electrophoretic mobility shift assay (EMSA). Conditioned media from these co-cultures were evaluated for HAPLN1 secretion by western blot and MMP-2 activity by gelatin zymography analyses. BMSC were evaluated for HAPLN1 and MMP2 mRNA expression by qRT-PCR. RNA interference was implemented to evaluate the requirement of HAPLN1 in inducing NF-κB activity. MMP chemical inhibitor and activator were also used to test the role of MMP in NF-κB activation.

Preliminary Data

Among the proteases capable of cleaving HAPLN1, qRT-PCR analysis found that MMP-2 and ADAMTS-1 are the predominant enzymes expressed in MM patients BMSCs. Zymography assays

showed that MMP-2 is the only active enzyme detected in BMSCs conditioned media. A panel of MM BMSCs from patients of varying disease stages variably secreted HAPLN1 compared to BMSCs derived from normal donor aspirates. Through co-culture, MM BMSCs induced greater NF-κB activity in RPMI8226 cells than non-MM BMSCs. These MM BMSCs secreted greater quantities of active MMP-2 than non-MM BMSCs both alone and in co-culture with RPMI8226 cells. A significant increase and correlation between MMP-2 and NF-κB-inducing activities was found in BMSCs across disease stages. Moreover, addition of MMP inhibitor, Batimastat, completely prevented BMSC's ability to cause NF-κB activation in MM cells, while a MMP activator, APMA (4-aminophenylmercuric acetate), increased NF-κB activation. These data suggest a strong correlation among disease progression, MMP2, and NF-κB-inducing-inducing activities in MM BMSCs, and inhibitor data suggest a causal relationship.

Novel Aspect

Proteolytic processing of HAPLN1 may represent a novel disease biomarker or therapeutic target.

64. Julia Martien

Systems-level analysis of environmental perturbation in Zymomonas mobilis: Applications for biofuel production

Julia I. Martien[1,2], Alexander S. Hebert[1,3], David M. Stevenson[1,2], Matthew R. Regner[1,4], Daven B. Khana[2], Joshua J. Coon[1,5,6,7] Daniel Amador-Noguez [1,2]

[1]DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, Wisconsin, USA

[2]Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin, USA

[3]Genome Center of Wisconsin, Madison, Wisconsin, USA

[4]Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA

[5]Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA

[6]Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA

[7]Morgridge Institute for Research, Madison, Wisconsin, USA

Introduction

Zymomonas mobilis is an aerotolerant anaerobe and prolific ethanologen with attractive characteristics for industrial bioproduct generation. However, there is currently insufficient knowledge of the impact that environmental factors have on flux through industrially relevant biosynthetic pathways. Here, we examine the effect environmental perturbation on metabolism and gene expression in Z. mobilis by combining targeted metabolomics, mRNA sequencing, and shotgun proteomics.

Methods

First, we leveraged oxygen exposure as a means to perturb central carbon metabolism, allowing us to observe the formation and resolution of a metabolic bottleneck in the methyl erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis. We determined that this bottleneck was caused by oxidative damage to the iron-sulfur cofactors of the final two enzymes in the MEP pathway. The metabolic bottleneck was resolved with minimal changes in expression of MEP pathway enzymes. Instead, it was associated with pronounced upregulation of enzymes related to

iron-sulfur cluster maintenance and biogenesis (i.e., flavodoxin reductase and the suf operon). Our multi-omics analysis of oxygen exposure thereby enabled us to identify key auxiliary enzymes whose expression correlates with increased production of isoprenoid precursors, which we propose as potential targets for future metabolic engineering. Next, we plan to examine the shift from ammonium assimilation to nitrogen fixation. It has been shown by Kremer et al. that Z. mobilis is capable of fixing N2 as a sole nitrogen source. Kremer et al. also showed that the specific rate of ethanol production by Z. mobilis increases during nitrogen fixation despite the increased need for energy and reducing power imposed by nitrogenase activity.

Preliminary Data

Our preliminary data indicates that nitrogen fixation dramatically alters central metabolism. We propose that a systems-level analysis of metabolism and gene expression during the shift to nitrogen fixation will reveal strategies to increase the specific rate of biofuel production by Z. mobilis.

Novel Aspect

This study is the first to report system-level metabolite, protein, and mRNA abundance with high time resolution in Z. mobilis.

65. Patricia Martínez-Botía

Dissecting the platelet proteome, reactome and secretome under inflammation: potential biomarkers and therapeutic applications

Patricia Martínez-Botía (Instituto de Investigación Sanitaria del Principado de Asturias (ISPA) and Dept. of Medicine, University of Oviedo, Spain)
Laura Gutiérrez (Instituto de Investigación Sanitaria del Principado de Asturias (ISPA) and Dept. of Medicine, University of Oviedo, Spain)

Introduction

There is emerging evidence that platelets play other roles than mere hemostasis. For example, platelets have inflammatory functions and influence immune responses. Furthermore, recent studies from our laboratory have shown that, under pathological conditions (i.e. chronic inflammation), megakaryocytes change their transcriptional program and produce fine-tuned platelets. These platelets “of inflammation” have a different aggregation response profile compared to healthy platelets, and we think that they may differ substantially at the proteomic level, which will largely condition their reactome and secretome in patients with different underlying pathologies. In this work we aim to dissect the platelet proteome in pathologies with underlying inflammation, and to determine whether this information could be used to define novel biomarkers or to generate novel platelet-derived therapies.

Methods

We are recruiting patients from five different cohorts (rheumatoid arthritis, major depressive disorder, dry eye condition, atopic dermatitis and diabetes mellitus type 1), all of them characterized by subjacent inflammation, and matched healthy controls. We will characterize their platelet proteome, besides the platelet phospho-proteome (i.e. reactome), and releasate (secretome) upon

activation with different agonists. Blood samples will be collected and the platelet rich plasma will be separated by differential centrifugation. Washed platelets will be used to make extracts before (platelet proteome) or after stimulation (platelet reactome). The supernatant, after stimulation and high-speed centrifugation, will be collected as the platelet secretome. All samples will be processed prior mass spectrometry analysis (in-solution digestion) and data will be analyzed with label-free quantification algorithms.

Preliminary Data

These results will yield a number of differentially expressed proteins in the patient cohort platelets proteome, reactome and secretome, in a qualitative and quantitative manner, and this knowledge will generate not only potential biomarkers, but also additional targets for the manipulation of these platelets, thinking on developing platelet-based therapies. We will be able to see how platelets differ in health and disease, and we will be able to correlate their reactome and secretome (upon stimulation with different agonists) with their receptor profiling and aggregation capacities tested in vitro. Combining MS data with surface marker expression, we will be able to evaluate whether a defective response to an agonist is due to receptor internalization (lower on the surface, but in normal amounts in MS) or to receptor shedding (incomplete peptide coverage by MS), since this process should result on a higher proportion of peptides corresponding to the intracellular domain of the receptor compared to the extracellular.

All in all, we expect to identify proteomic changes on platelets in different pathologies with underlying inflammation. We will also identify potential platelet molecular fingerprints and qualitative differences among those pathologies and the control group. With the obtained data, we will be able to analyze whether the changes in the proteomic profile are due to the subjacent inflammation and inherent in the produced platelets, or whether they are acquired in the circulation (as we can correlate platelet MS data with mother cell megakaryocytes MS data). A potential clinical application of this analysis is the development of ELISA-based diagnostic approaches using targets identified differentially in pathological conditions. Furthermore, proteomic data obtained from platelets will shed light on how megakaryopoesis and platelet production sense inflammation and how we can manipulate platelets in our advantage, for example in the development of platelet-based therapies as immunomodulators or anti-tumoral agents.

Novel Aspect

Platelets are not fixed entities. Platelet qualitative and functional differences may be characteristic to certain pathologies or physiological demands.

66. Conor McCabe

Proteomic Analysis of the Impact of Circadian Rhythm Disruption on Adipose and Muscle Tissue Mobilization in Periparturient Dairy Cows

Dr. Aridany Suarez-Trujillo[*], Dr. Jacquelyn Boerman[^], Dr. Theresa Casey[*]
[^]Purdue University, Department of Animal Sciences, West Lafayette, IN
[*]Purdue University, Department of Biochemistry, West Lafayette, IN

Introduction
The modern-day dairy cow produces in excess of 10 gallons of milk per day. The three weeks before and after calving, are the predominant period where cattle mobilize muscle and adipose tissue to match the large energy and amino acid demands to support the onset of lactation. The previous experiment showed, cows with a prepartum disrupted circadian rhythm produced 5.0 lbs. more milk per day from calving to 60 d in milk. While there were no differences in insulin or daily intake but a decrease in blood glucose concentration, the major precursor for milk lactose synthesis. With contrasting results, a shotgun proteomics analysis of the tissue is necessary to gain an understanding of proteins controlling tissue mobilization and energy balance.

Methods
Twelve multiparous dairy cows were blocked by previous milk production, health incidences and lactation number and divided into two treatments a control (CON) and phase shifted (PS) group. The CON animals were exposed to 16 h of light to 8 h of dark, while the PS had the 16 hours of daily light shifted forward six hours every three days. Biopsies of adipose tissue were taken from the subcutaneous fat over the intercostal space of the ribs and muscle biopsies were taken from the longissimus dorsi at 21 days before calving (BEC) and 21 days postpartum (PP). The ~0.5 g samples were snap frozen and stored in -80°C until analysis.

Preliminary Data
Adipose and liver biopsies are still being collected. Current plans are to measure proteins using gel-free, label-free shotgun LC-MS/MS proteomics, in the Q Exactive Orbitrap HF mass spectrometer coupled with the Dionex UltiMate 3000 RSLC Nano System. Although fractionation of cellular components (e.g. total, cytosolic and nuclear) may be considered as a means for greater understanding of pathways and systems affected by treatment and physiological state. Data will be analyzed using MaxQuant software (v. 1.5.3.28) against UniProt Bos taurus protein database. Downstream analysis will be done using Inferno, DAVID and Ingenuity Pathway Analysis. Comparisons will be made between treatment groups as well as between prepartum and postpartum samples. Assuming similar production responses to the previous study, the results will increase understanding of why circadian disruptions during the prepartum yielded greater milk production.

Novel Aspect
Quantify the adipose and muscle proteome of lactating animals undergoing circadian rhythm disruptions during the prepartum and the resulting postpartum.

67. Jake Melby

Single Muscle Fiber Proteomics Enabled by High Sensitivity Top-Down Mass Spectrometry

Yutong Jin, Yanlong Zhu, Ziqing Lin, Gary Diffee, Ying Ge
University of Wisconsin-Madison

Introduction

Single muscle fibers (SMFs) are individual contractile units within muscle tissue that can be classified into fast- and slow-twitch fibers. Post-translational modifications (PTMs) and isoforms of the myofilament proteins which make up these fibers have profound effects on fiber functions. To best understand how PTMs and isoforms affect fiber function, it is important to analyze SMFs. However, conventional methods for myofilament protein analysis are based on the analysis of muscle tissues containing mixed fast- and slow-twitch fibers, which convolutes the relationship between isoforms/PTMs and specific fiber types. Herein, we developed a highly sensitive top-down mass spectrometry (MS) approach for analysis of SMFs, which enables a better understanding of fiber function.

Methods

Skeletal muscle fibers were dissected from hind legs of rat and mouse. Contractile properties of the different muscle fibers were measured on SMFs in muscle relaxation solution to confirm muscle fiber types. After contractile measurements, muscle fibers were then stored at -80 °C prior to top-down proteomics analysis.

Myofilament proteins were extract from 1-10 SMFs using either 1% TFA or 0.1% anionic acid labile surfactant (AALS) solution. Proteins were separated by reversed-phase chromatography using a 75 µm I.D. capillary column in nano-LC and ionized by an online nano-ESI source interfaced with Bruker Impact II quadrupole time of flight (Q-TOF) mass spectrometer for highly sensitive LC-MS analysis. Bruker DataAnalysis software was used to process LC-MS data.

Preliminary Data

Contractile properties of 10 SMFs from rat gastrocnemius (GM) and soleus (SOL) muscles were measured. The measurements showed that the maximum shortening velocity is around 1.9 fl/s for GM and 0.7 fl/s for SOL, which indicated that GM muscle fibers are fast-twitch fibers while SOL muscle fibers are slow-twitch fibers. After performing contractile measurements, sarcomeric proteins, including tropomyosin (Tpm), myosin light chain 1, 2 and 3 (MLC1, MLC2, and MLC3) and troponin complexes I, T, and C (TnI, TnT and TnC), were successfully isolated from the same 10 SMFs and analyzed by top-down MS using the described method above. Most proteins detected in GM fibers were fast-twitch isoforms, whereas most proteins detected in SOL fibers were slow-twitch isoforms. For example, MLCs detected in GM fibers are all in their fast-twitch isoforms, whereas in SOL fibers, MLCs are all in their slow-twitch isoforms, which agrees with their contractile properties.

Next, we extracted and analyzed myofilament proteins from 1 SMF. The highly sensitive top-down MS platform involving nano-LC separation and nano-ESI ionization enabled detection of myofilament proteins extracted from 1 SMF with different isoforms and PTMs, such αTpm, βTpm, MLC2, and phosphorylated MLC2. The protein isoforms detected in the single fiber match well with their fiber types.

Tandem MS (MS/MS) analysis was also carried out on proteins extracted from 7 SMFs for protein identification. Protein sequences of most myofilament proteins, such as TnT and MLC2, were confidently identified with characterization of N-terminal acetylation and tri-methylation.

The development of high-sensitivity top-down MS strategy enabled single muscle fiber proteomics and provided significant insights into the relationship between protein isoforms/PTMs and muscle

fiber contractile properties. We anticipate further increase in protein extraction efficiency and detection sensitivity for analysis of lower-abundance proteins from SMF.

Novel Aspect

Development of a highly sensitive and novel top-down MS method for analysis of single muscle fiber proteomics.

68. Giselle Melendez

Cellular Senescence: A Novel Mechanism of Doxorubicin-induced Cardiotoxicity

Giselle C. Melendez, MD, Assistant Professor
Departments of Internal Medicine, Section on Cardiovascular Medicine and Pathology, Section on Comparative Medicine, Cardiovascular Sciences Center, Comprehensive Cancer Center

Introduction

LV dysfunction in patients undergoing anthracycline-based chemotherapy (Anth-bC) limits the use of this effective anticancer drug. Traditionally, cardiotoxic effects of Anth-bC are attributed to cardiomyocyte injury due to DNA damage and cell death. Recent evidence from our group demonstrates that cardiac fibrosis also contributes to LV dysfunction following Anth-bC and anthracyclines have a direct effect on cardiac fibroblasts to produce excess collagen. A mechanism by which Anth-bC may promote cardiac fibroblast activation is a stress response known as cellular senescence, a permanent arrest of cell proliferation accompanied by the development of a senescence-associated secretory phenotype (SASP). In this study, we sought to determine the proteomic constituents and protein networks of cardiac fibroblast secretome induced by Anth-bC.

Methods

We will use mass spectrometry proteomics to determine the individual proteins and protein networks that constitute the secretome of isolated adult rat cardiac fibroblasts exposed to Doxorubicin (Dox, a frequently used anthracycline). Subsequently, we will use dual chamber culture techniques to expose healthy cells to the SASP supernatant of Dox-treated senescent fibroblasts and assess its effects on extracellular matrix (ECM) genes, release of ECM proteins and collagen production, and myofibroblast pheno-conversion and migration. Furthermore, we will assess whether senescence and SASP features found in vitro can be replicated in cardiac tissue of rats treated with Dox and associate these features with the establishment and progression of myocardial fibrosis and LV dysfunction.

Preliminary Data

We have demonstrated that Dox induces interstitial cardiac fibrosis by evaluating the collagen volume fraction (CVF) in the LV of female Sprague-Dawley rats receiving cyclic doses of Dox (1.5 mg/kg) every week. Acutely (24-48 hours after the first dose), no CVF changes were seen. After 2 weeks of treatment (cumulative dose of 3 mg/kg) we observed a non-significant tendency for LV interstitial collagen to be increased ($p=0.08$). After receiving a cumulative dose of 9 mg/kg, Dox induced a significant increase of cardiac fibrosis ($p=0.03$). In order to assess whether Dox has a direct effect on cardiac fibroblasts to produce extracellular matrix (ECM), independent of

myocellular injury, we isolated primary adult rat cardiac fibroblast and exposed to increasing concentrations of Dox (0, 1, 3 and 10 μM). After 24 hours of incubation, hydroxyproline levels were measured in the supernatant to assess collagen production; we found a dose-dependent increase in secretion of hydroxyproline ($p<0.001$ vs. control). These experiments demonstrated for the first time, that Dox promotes collagen synthesis and secretion by cardiac fibroblasts, independent of cell proliferation. This novel finding represents a paradigm shift in the understanding of Anth-bC cardiotoxicity which was deeply focused on cardiomyocyte damage. To support the hypothesis that cellular senescence regulates collagen synthesis by cardiac fibroblasts exposed to Dox, cells were stained with senescence-associated-β-galactosidase (SA-β-gal) stain, a universal marker of the senescence state. Cardiac fibroblasts treated with Dox exhibit a higher percent of blue cells compared to untreated cells($p<0.0001$). In the proteomics analysis of the SASP we expect to find pro-fibrotic proteins and networks linked to extracellular matrix remodeling and collagen production.

Novel Aspect

Employing comprehensive proteomics to examine the cardiac fibroblast secretome establishes a plausible framework to understand myocardial fibrosis induced by anthracyclines.

69. Mariel Mendoza

TRIM28 as a candidate mutant p53 interacting partner in cancer cells

Mariel Mendoza, Katherine Alexander, Enrique Lin Shiao, Charly Ryan Good, Benjamin A. Garcia, Shelley L. Berger
University of Pennsylvania, Philadelphia

Introduction

p53 is a transcription factor that is mutated in over 50% of cancers. Missense mutations in the DNA binding domain of p53 can result in a gain-of-function (GOF) phenotype, leading to increased cell proliferation and tumor formation. Our lab previously showed that prevalent mutant p53 (mtp53) forms modify chromatin through their interaction with ETS2 and activation of non-canonical transcriptional targets (MOZ, MLL1, and MLL2). Aside from ETS2, other mtp53 partners that have been identified, including Sp1, NF-Y, and PML. However, whether specific proteins are critical for the stability and the GOF effect of mtp53 remains to be seen.

Methods

We developed a quantitative mass spectrometry-based strategy, combined with molecular biology approaches, to identify and validate novel mtp53 binding partners from cancer cell lines with varying GOF p53 mutations. We performed immunoprecipitation coupled to mass spectrometry (IP-MS) experiments to enrich for nuclear p53 in different GOF cell lines. To assess the role of the candidate interactors in regulating mtp53 stability, we used short hairpin RNAs (shRNAs) to knock down the candidate protein in GOF cell lines and measure cell growth and other cancer-like phenotypes using proliferation assays.

Preliminary Data

We performed IP-MS on 4 different cell lines, two of which (PANC1 and MDA468) have R273H mutations, and the other two cell lines (HUPT3 and VU1365) have R282W mutations. We identified

178 proteins that are enriched in all 4 IP experiments. In the list of enriched proteins, we identified known interactors of mutant p53, such as components of the SWI/SNF complex. From these protein lists, we followed up on TRIM28, which is a nuclear corepressor protein, as it was also identified as a gene essential for growth in cancer cells containing GOF p53 mutations. TRIM28 has been shown to interact with MDM2 to promote wild-type (WT) p53 ubiquitylation and degradation; however, whether it interacts with mtp53 in maintaining its stability has not been determined. We used shRNAs to knock down TRIM28 in cancer cells containing either WT or mtp53 and measured cell proliferation and viability. We found that TRIM28 knockdown slows down growth in both WT and mutant cells; however, the WT cells showed higher percentage viability than the mutant cells. These results demonstrate that the WT cells recovered from the TRIM28 knockdown, whereas the mutant cells are unable to do so, illustrating a phenotype specific to p53 GOF.

Novel Aspect

Characterizing novel mtp53 interactors will shed light into molecular mechanisms underlying cancer and provide new therapeutic targets to destabilize mtp53.

70. Oana Madalina Mereuta

Protocol optimization for proteomic analysis of formalin fixed paraffin embedded clot analogs

Rosanna Rossi, National University of Ireland Galway-Physiology Department, CÚRAM–Centre for Research in Medical Devices, Galway, Ireland
Sean Fitzgerald, National University of Ireland Galway-Physiology Department, CÚRAM–Centre for Research in Medical Devices, Galway, Ireland
Andrew Douglas, National University of Ireland Galway-Physiology Department, CÚRAM–Centre for Research in Medical Devices, Galway, Ireland
Abhay Pandit, National University of Ireland Galway, CÚRAM–Centre for Research in Medical Devices, Galway, Ireland
Karen Doyle, National University of Ireland Galway-Physiology Department, CÚRAM–Centre for Research in Medical Devices, Galway, Ireland

Introduction

The specific molecular composition of acute ischemic stroke clots retrieved by mechanical thrombectomy is largely unknown. In particular, in-depth proteomic analysis of the formalin fixed paraffin embedded (FFPE) thrombotic material by mass spectrometry may impact stroke management. This will allow the characterization of the peptide composition of clots as and the identification of a proteomic profile specific for the environment where the thrombus has originated. In this context, clot analogs may be used to increase our knowledge on molecular mechanisms of thrombus formation and may facilitate the development of a shotgun proteomic approach for detection of potential stroke biomarkers. This pilot study is focused on the potential of mass spectrometry to characterize the composition of FFPE clot analogs.

Methods

Different amounts of fresh whole blood constituents are mixed with calcium chloride to create three types of clot analogs: red blood cells-rich, fibrin-rich and mixed. Each clot is washed with phosphate

buffered saline, subsequently formalin-fixed and embedded in paraffin. Briefly, 10-µm scrolls are cut and deparaffinized. Tissue is lysed, sonicated and formalin cross-links are removed by heat treatment. The protein content is quantified, and samples are further denaturated, alkylated, digested with trypsin and desalted. The samples are injected on a Thermo Scientific Q Exactive mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. The mass spectrometer is operated in a Data Dependent Analysis (DDA) and label-free quantification of DDA files is performed with MaxQuant software.

Preliminary Data

We demonstrate here the feasibility of proteomic investigation of FFPE clot analogs by mass spectrometry. Therefore, we describe an optimized protocol for robust and reproducible sample preparation based on removal of paraffin followed by a combined physical (=heat/sonication) and chemical treatment of fixed material resulting in high depth protein identification. Moreover, since only a small portion of FFPE material is used for this analysis, the rest of the material remains available for histological staining and immunohistochemistry. These methods have been extensively used in the last few years to characterize the histopathological features of FFPE clots showing a wide variability in clot composition. The mass spectrometry-based shotgun proteomics may overcome the heterogeneity of thrombus and may provide new insights into stroke pathology.

Novel Aspect

Mass spectrometry-based proteomic profiling of FFPE clots may open new perspectives in stroke biomarker and drug target discovery.

71.Kayla Mills

Shotgun proteome analysis of seminal plasma proteins differentiate boars by reproductive performance

K.R. Stewart (Purdue University), T. Casey (Purdue University), U.K. Aryal (Purdue University), A.M. Minton (The Maschhoffs)

Introduction

Modern (commercial or terminal) boars are selected for improved growth rate, feed efficiency and carcass characteristics. Performance traits are typically inversely related to fertility traits; hence the great variation in reproductive performance among boars. Seminal plasma proteins are essential for normal sperm function and transport. Proteomics analysis using two-dimensional (2D) gel electrophoresis support that seminal plasma protein profiles reflect differences in reproductive performance. However, the identification of potential fertility biomarkers has been limited by the lack of robustness of the 2D gel approach. The objective of this study was to use shotgun LC-MS/MS proteome analysis to investigate whether differences in boar fertility phenotype can be differ–entiated by seminal plasma protein expression.

Methods

Following 50 single sire matings, commercial boars were categorized into one of four phenotypes: high farrowing rate and total born (HH; n=9), high farrowing rate with low total born (HL; n=10), low farrowing rate and total born (LL; n=9), and low farrowing rate with high total born (LH; n=4). Semen

from the first acceptable ejaculate (>75% motile and normal) from boars in the stud was shipped overnight to Purdue University where seminal plasma was extracted and stored in -20°C until fertility data was available. Samples were thawed on ice, proteins precipitated with acetone, reconstituted with 8M urea, reduced, and digested with trypsin and Lys-C mix. Dried peptides were re-suspended in 3% acetonitrile and 0.1% formic acid and analyzed using gel-free, label-free shotgun LC-MS/MS in the Q Exactive Orbitrap HF mass spectrometer coupled with the Dionex UltiMate 3000 RSLC Nano System. Data were analyzed using MaxQuant software (v. 1.5.3.28) against UniProt sus scrofa protein database. LFQ intensities were used for protein relative abundance measurement and downstream analysis.

Preliminary Data

There were 523 proteins measured in at least one sample across all animals (n=32). Among those, 318 were considered high confidence proteins, with 227 expressed commonly among the phenotype groups. Functional annotation analysis of commonly expressed proteins in DAVID 6.8 found enrichment of proteins in secretory, glycoprotein, glycan degradation, lysosome, adhesion proteins, and transferrin categories. There were 103 proteins at least 2-fold different in mean LFQ between LL and the HH group. Among the 48 proteins 2-fold more abundant in LL versus HH were multiple proteins associated with inflammation including heat shock protein A2 (HSPA2), alpha-2-macroglobulin (A2M), S100 calcium binding protein A12(S100A12), and microseminoprotein beta(MSMB), with HSPA2 and A2M linked to infertility in humans. Lactotransferrin (LTF), defensin beta 1 (DFB1), complement factor I (CFI) and complement C3 (C3) were 2-fold or more abundant in HH versus LL group, all of which have antimicrobial functions. These findings support that seminal plasma protein profiles are distinct between boars with different fertility and presence of inflammatory biomarkers may be predictive of reproductive performance.

Novel Aspect

Shotgun proteomics provides a more robust analysis than two-dimensional gels of the past and allows for more accurate biomarker discovery.

72. Reid Milstead

Identifying disinfection byproducts in groundwater using ultrahigh-resolution mass spectrometry

Christina Remucal (University of Wisconsin – Madison, Dept. of Civil and Environmental Engineering

Introduction

Dissolved organic matter (DOM) is a heterogeneous mixture of organic molecules found in natural waters that is primarily composed of carbon, oxygen, hydrogen, and to lesser extents nitrogen, sulfur, and phosphorous. DOM interacts with chlorine-based disinfection agents during water treatment processes to form carcinogenic disinfection byproducts (DBPs) such as chloroform. The molecular composition of DOM plays a critical role in the formation of these DBPs. Despite both the importance of groundwater as a source of drinking water and the universal usage of chlorine-based disinfection agents in water treatment throughout Wisconsin, the composition of groundwater DOM is not well understood. This work fills this gap by investigating the molecular composition and DBP formation potential of groundwater DOM via ultrahigh-resolution mass spectrometry.

Methods

Water samples from 20 drinking water utilities across Wisconsin were collected. Portions of each sample were chlorinated with residuals of 1 mg-Cl2/L after 24 hours. The DOM was then isolated via solid phase extraction. All extracts were diluted and injected into a Bruker SolariX XR 12T Fourier – transform ion cyclotron resonance mass spectrometer (FT-ICR MS) via electrospray ionization in negative mode. The m/z values were exported and calibrated using chemical formulas known to be present in natural waters. Chemical formulas of DOM molecules and DBP precursors were assigned to m/z values using a custom R script developed in the Remucal laboratory. Regulated DBPs were quantified via gas chromatography – electron capture detector (GC-ECD).

Preliminary Data

The molecular compositions of both surface water and groundwater samples were characterized before and after reaction with free available chlorine. The DOM extracts produced highly complex mass spectra with as many as 25-thousand unique features. These spectra are characterized by intense signals at odd m/z values, which are indicative of CHO compounds, and less intense signals at even m/z values, which are indicative of nitrogen-containing compounds and 13C isotopologues. Due to the complex nature of DOM, there are generally 10–20 unique features at each nominal mass, highlighting the need for ultrahigh-resolution mass spectrometry.

Samples of both groundwater DOM and surface water DOM had an average of approximately 1800 unique formula assignments. In all cases formulas composed of carbon, hydrogen, and oxygen were dominant and represented greater than 65% of assigned formulas. Chlorinated DOM samples were composed of up to nearly 10% chlorinated formulas, indicative of both the successful chlorination of the DOM samples and the need to better understand DBP formation potential. Formulas containing nitrogen made up approximately 20% of assigned formulas in all cases, while formulas containing sulfur and phosphorous accounted for relatively small percentages of the total formulas.

Van Krevelen diagrams, which plot H:C ratios against O:C ratios for each assigned chemical formula, were used to help visualize the chemical makeup of the DOM found in each of the water samples. Groundwater samples tend to be shifted more toward larger values on the H:C axis and smaller values on the O:C axis, indicating that groundwater DOM samples are generally both more saturated and less oxidized compared to surface water DOM samples.

Novel Aspect

Novel halogenated disinfection byproducts will be identified using ultrahigh-resolution mass spectrometry.

73. Stanford Mitchell

Top-down Proteomics for Deciphering Hypertrophic Cardiomyopathy in A Patient-Specific Engineered Cardiac Tissue Disease Model

Willem J. de Lange (Department of Pediatrics, UW-Madison)
Jianhua Zhang (Department of Medicine, UW-Madison)
Gina Kim (Department of Medicine, UW-Madison)
Trisha Tucholski (Department of Chemistry, UW-Madison)

Timothy J. Kamp (Department of Medicine, UW-Madison)
J. Carter Ralphe (Department of Pediatrics, UW-Madison)
Ying Ge (Department of Chemistry and Department of Cell and Regenerative Biology, UW-Madison)

Introduction

Hypertrophic cardiomyopathy (HCM) is the most common inherited cardiovascular disease with over 1400 HCM-causing mutations documented in about 18 protein-encoding genes comprising the cardiac sarcomere, the basic contractile unit of heart muscle. Despite its prevalence, the underlying disease mechanisms remain poorly understood. Recent advances in human induced pluripotent stem cells (hiPSCs) and directed differentiation into cardiomyocytes (CMs) and cardiac fibroblasts (CFs) have provided unprecedented opportunities for modeling cardiovascular diseases. Moreover, engineered cardiac tissues (ECTs) derived from hiPSC-CMs and hiPSC-CFs offer excellent disease models which recapitulate patient phenotypes in vitro. Herein, we employ an integrated top-down proteomics platform to comprehensively assess the sarcomeric proteome of an HCM ECT disease model, which allows for in-depth understanding of molecular mechanisms underlying HCM.

Methods

hiPSCs were derived from a family cohort comprised of individuals with HCM and their healthy relatives. The hiPSCs were differentiated into CMs (Lian et al. Nat Protocols 2013) and CFs and their respective purities were evaluated by flow cytometry. ECTs were generated by seeding hiPSC-CMs and hiPSC-CFs into fibrin-based extracellular matrices and were maintained in culture. ECTs were harvested for physiological assessments and proteomics analysis. For top-down proteomics, intact cardiac sarcomeric proteins were extracted for top-down liquid chromatography-mass spectrometry (LC/MS) analysis. The proteins were separated using reversed-phase chromatography and subsequently analyzed using a Bruker Impact II quadrupole time-of-flight mass spectrometer. Data was analyzed using Bruker DataAnalysis (v. 3.2) and in-house developed Mash Explorer.

Preliminary Data

We have established a robust top-down LC/MS platform for simultaneous quantification of sarcomeric protein isoforms and post-translational modifications (PTMs) and applied to hiPSC-CM/hiPSC-CF derived ECTs for assessing the sarcomeric proteome in a functionally relevant patient-specific disease model of HCM.

First, we have assessed maturation of ECTs in comparison to monolayer cell culture using physiological assessments and a panel of sarcomeric protein maturation markers previously determined using top-down proteomics. We found hiPSC-CMs cultured in 2D monolayer are less mature than those maintained in 3D hiPSC-CM ECT culture. Furthermore, we observe improved CM maturation in hiPSC-CM/hiPSC-CF-derived ECTs compared to ECTs lacking hiPSC-CFs. We reveal prolonged culture of hiPSC-CM/hiPSC-CF derived ECTs enhances hiPSC-CM maturation.

Subsequently, we have identified cTnI and mono-phosphorylated cTnI, the predominant isoform in adult ventricular CMs, in hiPSC-CM/hiPSC-CF derived ECTs as early as 55 days post-hiPSC differentiation. Further, we have observed decreased phosphorylation of ÔÅ°-Tpm in hiPSC-CM/hiPSC-CF derived ECTs relative to 2D culture. Moreover, we have linked these proteomics findings with physiological assessments supporting improved maturation.

Importantly, molecular alterations at the PTM-level in sarcomeric proteins have been shown to exist in cardiac tissue from human HCM hearts compared to non-diseased tissue. Concurrently, hiPSC-CM/hiPSC-CF derived ECTs from patients with HCM have been shown to display HCM-specific phenotypes with respect to abnormal calcium handling, cellular morphology, and other functional metrics. We expect to observe differential expression of contractile protein PTMs between disease and control hiPSC-CM/hiPSC-CF derived ECTs. Elucidating the molecular alterations of contractile proteins at the PTM level in the hiPSC-CM/hiPSC-CF derived ECT model of HCM will provide critical insight into the underlying mechanisms of this poorly understood disease.

Novel Aspect

The first top-down proteomics study for understanding a genetically inherited cardiovascular disease.

74. Dmytro Morderer

Characterization of Ribonucleoprotein Assembly Defects in a Cellular Model of Spinal Muscular Atrophy.

Eneko Villanueva, Cambridge Centre for Proteomics, Department of Biochemistry, University of Cambridge, Cambridge, UK; Kathryn S. Lilley, Cambridge Centre for Proteomics, Department of Biochemistry, University of Cambridge, Cambridge, UK; Wilfried Rossoll, Department of Neuro–science, Mayo Clinic, Jacksonville, Florida, USA

Introduction

Spinal Muscular Atrophy (SMA) is a genetic disease caused by mutations in the human SMN1 gene, causing insufficient production of functional SMN protein. Although SMN is ubiquitously expressed, its deficiency mainly affects lower motor neurons leading to progressive denervation, muscle weakness, paralysis and eventual death caused by respiratory failure. While SMN has a canonical function in assembly of spliceosomal snRNA-containing ribonucleoprotein complexes (snRNPs), studies from our lab and others demonstrate that SMN has a more general role in the formation of various classes of ribonucleoprotein (RNP) complexes. In this study, we are employing a novel method for the purification of RNPs to characterize global changes in the RNA-bound proteome resulting from SMN protein deficiency as a model of SMA pathology.

Methods

Cell lines containing genetic construct for doxycycline-inducible expression of anti-Smn shRNA are used as in vitro models of SMA. Control cell lines stably express SMN constructs that are resistant to shRNA-mediated knock-down. UV-crosslinked RNA-protein complexes are isolated from these cells by Orthogonal Organic Phase Separation (OOPS) technique developed in the Lilley lab (Cambridge University). This method is based on the property of RNA-protein complexes to accumulate in the interphase between organic and aqueous fractions upon lysate solubilization in TRIzol:chloroform.

Preliminary Data

We have optimized the conditions for isolation of RNA-protein complexes to get the highest ratio between protein-bound RNA isolated from the interphase and free RNA isolated from the aqueous

phase of TRIzol:chloroform-solubilized cells. Lists of RNA-binding proteins that are significantly changed in ribonucleoprotein fraction upon SMN depletion will be presented.

Novel Aspect

Here we are applying a novel method for the isolation of RNP-complexes to study disease-related changes in RNP assembly.

75. Umarah Mubeen

Label-free quantification of phosphorylation changes in Chlamydomonas reinhardtii upon TORC1 inhibition

Umarah Mubeen, Krzysztof Bajdzienko, Patrick Giavalisco
Max Planck Institute of Molecular Plant Physiology
Max Planck Institute for Biology of Ageing

Introduction

Reversible post-translational modifications (PTMs) of proteins play a crucial role in transmitting stimuli across different signaling cascades. Phosphorylation is known as one of the most abundant PTMs, vital for rapidly transferring environmental signals throughout intracellular protein networks. Due to its significance in understanding cellular signaling, numerous efforts have been made to capture the subtle and rapid response with minimum technical bias in different organisms. Recently, we demonstrated that TORC1 inhibition in Chlamydomonas enhances de novo amino acid biosynthesis immediately (5 min) after rapamycin treatment. The rapid transformation in the metabolism was hypothesized to occur due to changes in the post-translational state of the key metabolic enzymes.

Methods

Hereby, we devised a rapid sample harvesting strategy to evaluate the immediate (within 1 min) response of TORC1 inhibition in Chlamydomonas. For this purpose, we not only had to make sure that sufficient cells were treated and harvested efficiently, we also had to adopt a previously described high-throughput phosphopeptides enrichment method for the efficient use in our photosynthetically-growing model species.

Preliminary Data

Using label free quantification of the phosphosites we could identify 5777 phosphosites, ~600 of which were differentially expressed after inhibition of TORC1 by rapamycin. Interestingly, we not only validated several known substrates of the TORC1 but also detected significant changes in some novel putative targets of the TORC1 signaling pathway, including the enzymes of central carbon and nitrogen metabolism.

Novel Aspect

The instant shifts in the phosphorylation state of the key enzymes regulating carbon and nitrogen metabolism mark an evidence for involvement of TOR signaling pathway in direct regulation of central metabolism.

76. Carina Müller

Quantitative proteomics approach for identification of dopamine transporter interaction proteins using validated DAT-knockout rats

Müller, C. [1], Sialana, F. J. [1], Wackerlig, J. [1], Urban, E.[1], Lubec, G. [2], Langer, T. [1]
[1] Department of Pharmaceutical Chemistry, University of Vienna, AT
[2]Paracelsus Medical Private University, Salzburg, AT

Introduction

Dysregulation of the dopaminergic system is linked to a variety of neuropsychiatric disorders. One of the key regulators of dopaminergic homeostasis in the brain is the dopamine transporter (DAT) conducting the re-uptake of dopamine after neurotransmission. The activity of DAT is regulated by a variety of interacting proteins. Tests have shown that inhibition of DAT can improve cognitive impairment. Therefore, the assumption is high that targeting interacting proteins of DAT could also lead to an improvement of cognition.

Methods

Thus, the present study will use an ex vivo mass-spectrometry-based proteomic approach to detect proteomic changes in abundance in DAT knockout (KO) and wild-type (Wt) rat brains for the identification and characterization of interacting proteins involved in function and regulation of DAT.

Striatum-samples of validated DAT-KO and Wt rat brains were homogenized and sub-fractionated. The resulting synaptosomes were lysed and immunoprecipitated with a validated monoclonal DAT-antibody, digested with trypsin and investigated by nanoLC-MS/MS (Orbitrap Velos, Thermo Fisher Scientific) using label-free-quantification. Quantitative data analysis was done using MASCOT via the Proteome Discoverer 2.2 platform (Thermo Fisher Scientific).

Proteomics approach allows identification of the pulled-down DAT and its interacting proteins in the Wt samples when compared to KO-control.

Preliminary Data

Out of a total of 608 identified proteins, 132 were identified with at least one peptide unique to the protein. From these 132 proteins DAT was unambiguously identified in the Wt samples in comparison to the KO sample, confirming the functionality of the immunoprecipitation with the monoclonal DAT antibody.

All unambiguously-identified proteins were subjected to further statistical analyses. For evaluation only the most abundant proteins with a two-fold-change or higher were considered. The most significant identified proteins are selected and tested by western blot and co-immunoprecipitation on a physical interaction with DAT.

Novel Aspect

This work can improve understanding the dopaminergic neurotransmission and can identify potential target proteins for treatment of cognitive impairments.

77. Snow Naing

Quantitative Proteomics in Xenopus tropicalis brains to study the functions of Autism Spectrum Disorder (ASD) risk genes

Snow Naing[1,2,4], Cameron Exner[3], Jeff Mandell[3], Jeremy Willsey[3], Nevan Krogan[1, 2], Matthew W. State[3], Jiashun Zheng[4], Helen Willsey[3], Ruth Huttenhain[1, 2]
[1] Department of Molecular & Cellular Pharmacology, University of California, San Francisco, CA; [2] J. David Gladstone Institutes, San Francisco, CA;
[3] Department of Psychiatry, University of California, San Francisco, CA;
[4] Department of Biophysics and Biochemistry, University of California, San Francisco, CA

Introduction
Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder with a complex genetic architecture. Advances in genomics technology have led to the identification of over 65 ASD risk genes, and yet, an understanding of the underlying pathways is lacking. Studying the brain proteome of an in vivo model with disrupted ASD risk genes can shed light on the molecular networks involved in the neuropathology of ASD.

Methods
We leverage the diploid vertebrate tetrapod, Xenopus tropicalis (frog) because their brain development is comparable to that of humans and generating many genetic mutants in parallel is routine. However, it is not known how much of the X. tropicalis tadpole brain proteome overlaps with that of humans. Mass spectroscopy (MS) based proteomics is an excellent method to map and compare complex proteomes. One limitation for X. tropicalis tadpole brain is the absence of a high-quality protein reference database, a prerequisite for MS-based proteomics. To address this issue, we assembled a custom reference proteome from RNA sequencing data of X. tropicalis tadpole whole brain and then assigned protein names from a curated human reference database using reciprocal BLAST methodology.

Preliminary Data
The generated database consists of 32,068 proteins, out of which 24,524 proteins (76.5%) were assigned human gene symbols. Using this custom reference protein database for MS-based proteomics, we identified over 2,500 proteins in X. tropicalis brain samples.

Novel Aspect
With this custom protein database, we are now poised to study the differential proteome changes in X. tropicalis brains.

78. Lorena Ndreu

Monitoring the human serum albumin adductome

Lorena Ndreu [1], Alister James Cumming [2], Johan Eriksson [1], Margareta Törnqvist [1], Isabella Karlsson [1]
[1.]Department of Environmental Science and Analytical Chemistry, Stockholm University, SE-10691 Stockholm, Sweden.
[2.]Department of Biochemistry and Biophysics, Stockholm University, SE-10691 Stockholm, Sweden.

Introduction

Contact allergy is a chronic condition affecting approximately 20% of the general population. It is caused by electrophilic compounds (haptens) that modify skin proteins by forming covalent bonds with their nucleophilic moieties, thereby triggering the immune system. The clinical manifestation of contact allergy is allergic contact dermatitis (ACD), i.e. skin inflammation. Contact allergy is the most common form of immunotoxicity in humans; thus, it is important to develop methods to evaluate our exposure to these skin-permeable compounds. Albumin is the most commonly used protein in studies of hapten reactivity due to high concentrations in skin, its function as a carrier protein, as well as the relatively long half-life. Further, correlations between environmental exposure and levels of albumin-adducts have been shown.

Methods

Three UPLC/ESI-MS/MS based methods have been combined to evaluate different nucleophilic sites of albumin with different characteristics, since certain compounds react with certain nucleophiles rather than others. These methods will then be applied to investigate the levels of hapten-albumin adducts in the general population (300 samples), as well as in contact allergy patients. The first method focuses on the analysis of haptens bound to the N-terminal aspartic acid of albumin by utilization of a modified FIRE LC/MS/MS based method, the second method focuses on the analysis of haptens bound to Cys34 of albumin after enrichment of the Cys34 peptide-adducts, and the third method focuses on haptens bound to other amino acids, by peptide analysis.

Preliminary Data

Adducts specific to the N-terminal aspartic acid of human serum albumin could be detected by implementation of the modified FIRE procedure. The developed method exhibits low limits of detections that may enable investigation of background levels of adducts. It was shown however that a limitation of the method is that its suitability depends on the nature of the electrophile, it can for example not be used for analysis of aldehyde-adducts that react via Schiff base formation. As one of the most commonly used method to detect HSA adducts is to analyze electrophiles bound to Cys34, this approach was also explored. We found that Cys34 is a good nucleophilic site for monitoring of certain haptens, such as those reacting via Michael addition. However not all haptens form stable adducts with Cys34. Finally, a proteomic approach was also used to identify other peptides that are commonly modified by haptens. Preliminary data on the attempt to identify other potential sites of adduction of albumin, by enzymatic digestion of incubated albumin with different electrophiles and UPLC/ESI-MS/MS analysis, revealed four adducted peptides other than the N-terminal aspartic acid (DAHKSEVAHR). Peptides AFKAWAVAR, KQTALVELVK, LAKTYETTLEK and KVPQVSTPTLVEVSR were found to be modified. Combination of the three methods provides a better coverage of the human serum albumin exposome, enabling for more effective monitoring of exposure to reactive chemicals, such as contact allergens.

Novel Aspect

Improved coverage of contact allergen adducts with albumin obtained by combining three different methods that monitor different nucleophilic sites.

79. Francisco Olea

Construction of a signaling network phosphoproteome generated by M6P/IGF2R in a trophoblast cell model

Francisco Olea*, Juan José Castro*, Adriana Umana-Pérez*
*Hormone Research Group, Chemistry Department, Universidad Nacional de Colombia
faoleas@unal.edu.co, jujcastroba@unal.edu.co, yaumanap@unal.edu.co

Introduction

Pregnancy is a complex and regulated process, in which the placenta plays a fundamental role. This organ is formed by trophoblastic cells, which differ in two main routes: villous (cyto)trophoblast (VT) and extravillous cytotrophoblast (EVCT). The cells of the trophoblast perform diverse functions, such as the implantation and adaptation of the blastocyst to the uterine environment, and the remodeling of the spiral arteries, which will guarantee the proper nutrition of the developing embryo. However, the sequence of events and the regulatory mechanisms of the human trophoblast are not well understood. IGF-II has a fundamental role in growth, influencing the division and differentiation in embryonic and fetal tissues. It is clear that M6P/IGF2R lacks intrinsic catalytic activity, but induction of migration in trophoblastic cells it has been described. Thus, information of the IGF system signaling on the trophoblast has not been extensively explored.

Methods

Our purpose is to identify the signaling networks dependent of Insulin-like growth factor II (IGF-II) stimulus through Mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) action, and determine those components that show relevant similarities and differences between two cellular models of cytotrophoblast of different lineages: EVCT (HTR-8/SVneo cell line) and VT (BeWo cell line). The experimental design will include Tandem MS, for the quantitative determination of the proteomes and phosphoproteins of each cell line obtained both, in basal conditions, and before the stimulation of the M6P/IGF2R through high affinity ligand [Leu27]IGF-II. The proteins and phosphoproteins identified in the MS/MS will be analyzed by means of different platforms and statistical software, to construct a model of the M6P/IGF2R pathway in these two types of trophoblast cells.

Preliminary Data

Other researchers have previously shown in HTR-8/Svneo cell line, that the stimulation with IGF-II acts on M6P/IGF2R and induces the stimulation of inhibitory G proteins and the reduction of cAMP, but with the concomitant increase in migration and phosphorylation of MAPK. Also, it has been shown that M6P/IGF2R stimulates the survival signal and lysosomal degradation in the BeWo cell line, but when the expression of this receptor is reduced, apoptosis increases.

Our group (Hormone Research Group) has previously shown in the HTR-8/SVneo cell line that stimulus of M6P/IGF2R through IGF-II ligand correlates with the increase in the expression and secretion of human chorionic gonadotropin (hCG) during trophoblastic differentiation. Also, we have some western blotting results that show an increase in ERK Ω activation in response to [Leu27]IGF-II stimulus through M6P/IGF2R signaling.

Finally, M6P/IGF2R could induce a signal when recruiting sphingosine kinase (SK), which promotes the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P), activating G protein-coupled receptors. Thus, in an experiment performed on the HTR-8/SVneo cell line, we stimulated the M6P/IGF2R receptor, and (in results not yet published) by mass spectrometry we found some proteins related to the sphingosine 1-phosphate pathway. Among them, sphingomyelin phosphodiesterase 3 (SMPD3), which catalyzes the hydrolysis of sphingomyelin to form ceramide and phosphatidylcholine. Ceramide mediates many cellular functions, such as apoptosis and arrest in cell growth, and is a substrate for the production of sphingosine. These results link together IGF-II and M6P/IGF2R receptor in the regulation of sphingolipid metabolism, and in the subsequent activation of the G protein signaling pathway, which results in the mediation of important biologic effects such as migration and invasion of trophoblast cells.

Novel Aspect

Generation of new knowledge about the biology of trophoblastic cells, especially the signaling exerted by M6P/IGF2R receptor.

80. Carleena Ortega

Non-HDL and Non-fasting Lipid Contributions to Foam Cell Formation

Joshua Dubland, Teddy Chan, Sima Allahverdian, Gordon Francis
Centre for Heart Lung Innovation, St. Paul's Hospital, University of British Columbia

Introduction

Atherosclerosis, which can lead to ischemic heart disease (IHD) and stroke, is the accumulation of plaque on arteries. It is driven by increased amounts of lipids and lipoprotein that contribute to the formation of foam cells or cholesterol-laden cells from human aortic smooth muscle cells (SMCs) and human monocyte-derived macrophages (MDMs). Low-density lipoprotein (LDL) cholesterol levels of fasted individuals have been the standard for predicting IHD risk but recent studies suggest non-fasting lipids and non-high-density lipoprotein (non-HDL) cholesterol enhance risk prediction. Here we study the capacity of fasting and non-fasting lipoproteins from the LDL and non-HDL fractions to generate SMC and MDM foam cells in vitro.

Methods

Cultured cells were grown in media with 10% fetal bovine serum then switched to 10% lipoprotein-deficient serum for 24 hours to upregulate LDL receptors. Cells were then incubated with 100 µg/ml of protein or 95 µg/mL of cholesterol obtained from either fasting or non-fasting plasma and in both native and aggregated (to mimic lipoproteins in arterial intima) forms for 24 hours. Aggregation was achieved by initially concentrating samples to 500 µg/mL, vortexing at maximum speed for a minute, then diluting to 100 µg/mL. Cells were assessed for lipid accumulation via fluorescence microscopy of the neutral lipid dye BODIPY and quantified for total, free, and esterified cholesterol content via mass spectrometry. The t-test and Mann-Whitney test were used for statistical analysis.

Preliminary Data

Loading both SMCs and MDM with the same protein concentration of lipoproteins had different trends between the BODIPY fluorescence intensity quantification and the total cholesterol content

measured via mass spectrometry. Hence, we decided to normalize lipoproteins based on the cholesterol content. For HASMCs, normalizing loading of cells to the cholesterol content of lipoprotein provided increased lipid accumulation in non-fasting fractions compared with fasting fractions and in LDL fractions in contrast to the non-HDL fraction when measured via fluorescence microscopy. However, we found the opposite trend for HMDM where increased lipid accumulation is observed with the non-HDL fractions when compared to LDL, and also in the fasting lipoproteins when compared to their non-fasting counterparts. Generally, lipid accumulation and total cholesterol content are higher for MDM than SMC. Aggregating lipoproteins provided higher lipid accumulation for SMCs loaded with non-fasting lipoproteins but had higher lipid accumulation for MDMs from almost all fractions compared to non-aggregated lipoproteins. We will continue to refine or alter the methods and techniques used to decrease variability between experiments, quantify the cholesterol content of the MDM and SMC loaded with lipoproteins normalized to cholesterol using mass spectrometry and perform the experiments more times to accumulate more precise data for interpretation.

Novel Aspect

Atherosclerosis in vitro studies use LDL but by using non-HDL we can study the contributions of the other atherogenic lipoproteins.

81.Nancy Paiva

Analysis of nutritional components in edible parts of eastern redbud (Cercis canadensis L.)

Dr. Nancy L. Paiva (PI), Hunter, Laura J., Lily Chandler, and numerous undergraduate researchers past and present. Department of Chemistry, Computer and Physical Sciences, Southeastern Oklahoma State University, Durant, OK.

Introduction

Redbud trees are native across much of the continental U.S., with Cercis canadensis L. being the most common species. Redbuds are small leguminous trees, which are among the first to bloom in the spring and also produce large numbers of multi-seeded pods in late summer. In the early 1900's, Native Americans were reported to consume redbud flowers raw or boiled and consume seeds after roasting in the seedpods. In addition to providing vitamins and other essential trace nutrients at a time of year when few vegetables are available, the pigments in the pink/red flowers and other phenolic components might also have nutritional value. Little phytochemical analysis or genomic information has been published for this ancient, non-model plant species.

Methods

Extraction and HPLC analysis of anthocyanin content was accomplished using reverse-phase (C18) stationary phase columns and an acidified 1% phosphoric acid-acetonitrile gradient (Xie DY, Jackson LA, Cooper JD, Ferreira D, and Paiva NL (2004) Molecular and Biochemical Analysis of Two cDNA Clones Encoding Dihydroflavonol-4-Reductase from Medicago truncatula. 134:979-994). The vanillin/HCl stain for condensed tannins in green redbud seeds involved treating sliced immature seeds with 1% (w/v) vanillin in 6 M HCl or a control solution of 6 M HCl only (Xie DY, Sharma SB, Paiva NL, Ferreira D, Dixon RA (2003) Role of anthocyanidin reductase, encoded by BANYULS in plant

flavonoid biosynthesis. Science 299:396 – 399). Red color in vanillin/HCl but not HCL alone indicates the presence of condensed tannins.

Preliminary Data

Preliminary characterization of acidic methanol flower extracts using HPLC, LC-MS and other techniques revealed a high content of anthocyanins, which are known to be beneficial antioxidants in human diets. Green developing seeds exhibited intense red staining of the outer layers with a vanillin/HCl reagent, but no red staining in HCl alone, indicating high concentrations of condensed tannins (proanthocyanidins), another class of beneficial phenolic antioxidants. GC-MS analysis of FAME derivatives of chloroform/methanol lipid extracts from two ages of green seeds revealed the presence of the essential fatty acids linoleic and alpha-linolenic acid, although alpha-linolenic acid was absent from mature dried seeds. Oleic and palmitic acids were also abundant. Amino acid analysis is planned, to determine the quality of the seed protein. The redbud plant parts clearly offered highly beneficial nutrients to early inhabitants of North America.

In very preliminary experiments, raw, unheated crushed green seeds inhibited development of fruitfly larvae when mixed with standard basal insect diet, while seed pulp boiled for 20 minutes had no effect. The nature of the toxic seed component(s) was not determined, but these results are consistent with the presence of a toxic protein, and many types are found in many uncooked legume seeds. Roasting seeds by indigenous people may have denatured a toxic protein before consumption.

Future plans include preparing a cDNA library from early-stage developing seeds, to examine expression of known genes and to compare the sequences of Cercis genes and proteins to those of other legumes. For example, anthocyanin reductase (ANR, essential to condensed tannins accumulation described above) was first characterized in model plants Medicago truncatula and Arabidopsis thaliana, but no sequences from an ancient, non-nodulating legume have been reported for comparison. Proteomics approaches may yield the sequences of the seed storage proteins (indirectly indicating essential amino acid content) and other proteins.

Novel Aspect

Seeking protein amino acid sequence and metabolite data on food source (non-model plant) of indigenous peoples, involving primarily undergraduate researchers.

82. Dan Pensinger

A novel bacterial uridyl-transferase is essential for bacterial cell wall homeostasis and virulence

Daniel A. Pensinger[1], Adam Schaenzer[1], Hans Smith-[1], Daniel Amador-Noguez[2], John-Demian Sauer[1]
[1] Department of Medical Microbiology and Immunology and [2] Department of Bacteriology, University of Wisconsin-Madison, Madison, WI 53705

Introduction

Understanding how bacterial pathogens adapt to host environments is critical to developing novel antimicrobials. The cytosolic bacterial pathogen Listeria monocytogenes requires glmR, a gene of previously unknown function, for cytosolic survival, resistance to cell wall stress, and virulence in

vivo. Using metabolomics we observed that ΔglmR mutants are impaired in the production of UDP-GlcNAc, an essential bacterial cell wall precursor. A suppressor selection revealed that blocking flux of UDP-GlcNAc into a non-essential pathway restored resistance to cell wall stress and virulence of ΔglmR mutants. We next demonstrated that purified GlmR can directly catalyze the synthesis of UDP-GlcNAc. Finally, transcomplementation of L. monocytogenes ΔglmR mutants with GlmR ortho–logues and assays with purified orthologues suggest that relevant functions are evolutionarily conserved.

Methods

Metabolites were extracted from wild-type and ΔglmR L. monocytogenes, normalized by OD600, dried and resuspended. Resuspended extracts were separated by UHPLC, ionized and analyzed by coupled ESI negative mode hybrid quadrupole Orbitrap MS. Metabolites were identified using Metabolomics Analysis and Visualization Engine (MAVEN). A transposon mutant library in a ΔglmR background was selected for suppressors with the cell wall degrading enzyme lysozyme. To determine In vivo virulence of suppressor mutants, mice infected IV with 1 LD50 of wild-type were sacrificed at 48 hours post infection and spleens and livers were harvested, homogenized, and plated for CFU. His-tagged GlmR homologues were expressed and purified from E. coli using Ni chromatography. Substrate catalysis in enzymatic assays was determined by coupled UHPLC-MS.

Preliminary Data

GlmR is required for virulence of several related bacterial pathogens. The glmR mutant is also sensitive to some cell wall stresses and we hypothesized that the gene is involved in cell wall metabolism. To test this hypothesis, we utilized metabolomics to identify metabolites differing in abundance in ΔglmR mutants relative to wild type L. monocytogenes. In our analysis the essential cell wall precursor metabolite UDP-GlcNAc was found as the most differentially abundant Kyoto Encyclopedia of Genes and Genomes (KEGG) identifiable metabolite across 4 biological replicates. UDP-GlcNAc is essential for both major components of the gram positive bacterial cell wall (peptidoglycan and Wall Teichoic Acid) and levels of UDP-GlcNAc are reduced by 73% in the ΔglmR mutant. In a parallel approach we performed a suppressor selection using lysozyme and a transposon mutant library. Two suppressors that also rescued in vitro measures of virulence are gtcA and yfhO mutants. Both of these genes are required for a non-essential pathway and inactivating them restores UDP-GlcNAc levels. These suppressor mutations also partially rescue in vivo virulence. We hypothesized that GlmR may be an enzyme in the UDP-GlcNAc synthesis pathway. To test this hypothesis we purified GlmR and tested for catalytic activity with known substrates of the pathway. We determined GlmR can directly catalyze the synthesis of UDP-GlcNAc from UTP and GlcNAc-1P. Homologues from other related pathogens also exhibit uridyl-transferase activity. Not all homologues could complement in vitro phenotypes indicating that regulation mechanisms such as localization or phosphorylation may be important to protein function in the bacteria.

Novel Aspect

Discovery of a novel enzyme producing a metabolite required by all bacteria cell walls using metabolomics.

83. Eric Pereira

Molecular characterization of the in vivo effects of the hemorrhagic metalloproteinase HF3: analysis of the proteome of mice muscle tissue

Dilza Trevisan Silva[1]; Solange Maria de Toledo Serrano[1]
[1]Laboratãrio Especial de Toxinologia Aplicada, Center of Toxins, Immune-response and Cell Signaling, Instituto Butantan, São Paulo—SP—Brasil.

Introduction

Hemorrhagic factor 3 (HF3) is a potent hemorrhagic and myotoxic snake venom metalloproteinase (SVMP) isolated from the venom of Bothrops jararaca. Studies on this toxin have shown that it can degrade proteins of the extracellular matrix, the cytoskeleton, and the plasma. It has also been shown that HF3 promotes pro-inflammatory events, such as phagocytic macrophage activity and leukocyte rolling. The present study aims to expand the molecular characterization of the myotoxicity and the inflammatory and cell death pathways induced by HF3, evaluating the changes on the proteome of mouse gastrocnemius muscle after 2 h and 6 h of HF3 injection.

Methods

For this purpose, mice were injected with 5 µg of HF3 or saline solution in their right thigh muscle, and after 2 h and 6 h the gasctrocnemius was collected (CEUA 8174030915), frozen in liquid nitrogen and stored at -80°C. Two different methods for muscle protein extraction were evaluated by comparing the protein yield and the electrophoretic profile. In order to characterize the proteome of the thigh muscle, samples were pooled according to the type of treatment and subjected to in-solution or in-gel trypsin digestion. The peptides obtained were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Preliminary Data

The use of PTS lysis buffer and Precellys-24 sample homogenizer was the most effective combination in extracting muscle tissue proteins. All MS and MS/MS spectra obtained from muscle samples were submitted to a search in Peaks X software. This analysis resulted in the identification of a total of 435-517 peptide sequences and 72-95 protein groups among the samples analyzed. The low number of protein groups identification is probably related to the high dynamic range of protein concentration in this tissue. In order to decrease the protein complexity, muscle samples were submitted to SDS-PAGE. Each gel lane was divided in 9 slices and in-gel trypsin digestion was performed. Tryptic peptides derived from this procedure were stored at -20 °C and LC-MS/MS is under analysis.

Novel Aspect

This work will shed more light in the cellular death pathways that are triggered in the snakebite envenomation context.

84. Jakob Petereit

Characterization of the mitochondrial CLPXP complex in Arabidopsis thaliana

Shaobai Huang, Harvey Millar
School of Molecular Sciences, ARC Centre of Excellence in Plant Energy Biology, The University of Western Australia

Introduction

Protein degradation is necessary to recycle proteins after the end of their life, trigger receptor cascades or simply remove unwanted proteins from the current protein pool. Mitochondria have no access to the proteasome in the cytosol, so they employ their own degradative network: presequence proteases, proteasome-like protease complexes and oligo-peptidases.

CLPXP is a major protease complex in mitochondria and chloroplasts. Although a lot of research has been done on the complex in mammals, bacteria and chloroplasts, CLPXP in plant mitochondria remains uncharacterized mainly due to the unavailability of knock out mutants for CLPXP. We are now characterising the first KO mutant for the proteolytic subunit of the CLPXP complex in plant mitochondria.

Methods

In order to create a CLPXP KO, we employ CRISPR-Cas9 guided DNA strand break-repair impairments, which result in two independent mutants for the proteolytic subunit of the CLPXP complex in plant mitochondria. The mutants show complete absence of CLPp on the protein level.

The mutants are characterised with a combined omic approaches. Shotgun proteomics, label-free quantitated with Max Quant are combined with RNA-seq transcript abundances. Using omic approaches, it is possible to predict the nature of changes in mitochondrial protein abundances.

Additionally, we use the N-terminal enrichment strategy ChaFRADIC to identify protease target candidates in collaboration with the Leibniz Institute for Analytical Sciences in Dortmund, Germany.

Preliminary Data

Using Untargeted shotgun MS, we quantify 1600 proteins, showing 39 significantly changed proteins ($p < 0.05$, FC > +-30%) in two individual mutants. Targeted MRMs are used to quantify changes of specific proteins more precisely, such as the 24KDa and 51KDa subunit of complex I. Both approaches show the virtual absence of the CLPp subunit of the CLPXP protease complex and reveal that three groups of mitochondrial proteins are increased in abundance in both mutants: (1) mitochondrial proteases, such as CLPX and PREP1; (2) mitochondrial translation machinery proteins such as PPR 336 and RPL21M; and (3) N-module subunits of Complex I such as 24KDa, 51KDa and B8 proteins.

The causes of protein accumulation can be explained by (a) simply a lack of a suitable degradation mechanism; (b) a transcript upregulation feedback to counter the KO of CLPp; or (c) impairments in complex assembly and maintenance.

To disentangle the cause of these protein accumulations, the proteomic data needs to be connected to other experiments. RNA-seq will be employed to determine if changes of protein abundances are cause by an upregulation of genes that encode them at the transcript level. Impairments of complex I assembly, will be determined by import assays of the 51KDa subunit of Complex I, as well as Western Blots of multiple Complex I subunits on native gels. If the increased abundance of other mitochondrial proteases relates to a change of proteolytic patterns or activity, N-terminal enrichment studies (ChaFRADIC) will be applied, to reveal changes in abundance or new or existing endogenous peptides.

Novel Aspect

ChaFRADIC – Charge-Based Fractional Diagonal Chromatography Proteomics; CRISPR-Cas9 KO mutants; Quantitative Shotgun MS, MaxQuant, DEP, RNA-seq.

85. Arati Poudel

Amino acid profiling of soybean roots infected by soybean cyst nematode reveals tight regulation of amino acid metabolism

Arati N. Poudel[1], Pramod K. Kandoth[2], Abou Yobi [3], Ruthie Angelovici[3] and Melissa G. Mitchum[1]
[1]Division of Plant Sciences and Bond Life Sciences Center, University of Missouri, Columbia
[2]National Agri-Food Biotechnology Institute, Department of Biotechnology, India
[3]Division of Biological Sciences and Bond Life Sciences Center, University of Missouri, Columbia

Introduction

Soybean cyst nematode (SCN, Heterodera glycines Ichinohe) is a major cause of soybean yield loss worldwide. Planting of resistant soybean cultivars is the primary strategy for combating SCN-related production loss. In resistant cultivars, infective juveniles are unable to establish and maintain a feeding site within the roots, unlike in susceptible cultivars where feeding site establishment is unimpeded and provides the nutrients needed for the nematodes to complete their life cycle. The rhg1-b gene on chromosome 18 encoding for α-SNAP (GmSNAP18) and the Rhg4 gene on chromosome 8 encoding for a cytoplasmic serine hydroxymethyltransferase (GmSHMT8) are required for soybean cultivar Forrest resistance to SCN. GmSHMT8 catalyzes the reversible interconversion of serine and tetrahydrofolate (THF) to glycine and 5,10-methylene THF for one carbon metabolism. Free amino acid content has been correlated with crop plant disease resistance to other pathogens. Thus, we sought to explore potential differences in primary amino acid metabolism among the SCN-resistant cultivar Forrest, Forrest mutants in GmSHMT8, and the SCN-susceptible cultivar Essex upon infection with SCN.

Methods

We measured the free amino acids content from lyophilized root tissues with appropriate standard using an electrospray ionization (ESI) triple quadrupole mass spectrometer (Xevo TQ-S, Waters) interfaced with the ACUITY Ultra Performance Liquid Chromatography (UPLC) system (ACUITY H-class, Waters).

Preliminary Data

The results compared the quantities of free amino acids in infected and noninfected root segments of each soybean genotype at four days post-inoculation with SCN. Infection-related and genotype-specific increases and decreases in free amino acid levels indicate a tight regulation of amino acid metabolism in SCN-infected soybean roots.

Novel Aspect

The results provide new insight into the biochemical and physiological relationship of SCN to its host.

86. Ellen Quillen

High-fructose diet alters proteins involved in glucose and cholesterol metabolism in the liver

Biswapriya B. Misra (Wake Forest School of Medicine)
Jeannie Chan (Wake Forest School of Medicine)
Sobha Puppala (Wake Forest School of Medicine)
Laura A. Cox (Wake Forest School of Medicine)
Michael Olivier (Wake Forest School of Medicine)

Introduction

Fructose intake from high-fructose corn syrup in prepared foods has increased markedly over the past thirty years and now comprises a substantial proportion of the caloric intake of many Americans. Among the metabolic complications associated with extremely high levels of fructose consumption is non-alcoholic fatty liver disease which occurs when fructose triggers lipogenesis in liver cells. To evaluate the immediate changes induced in the liver by a high-fat diet, we performed a proteomic evaluation of the liver biopsies collected after a six-week high-fructose diet challenge in a non-human primate model. Despite the relatively short-term exposure to a high-fructose diet, metabolic indicators such as enzymatic liver markers, insulin, cholesterol and triglycerides increased.

Methods

Female vervet monkeys (Cercopithecus aethiops) were fed chow (n=5) or a high-fructose diet (n=5; 24% of calories) for 6 weeks. Animals were matched for age and weight prior to the diet challenge. We performed label-free LC-MS/MS (liquid chromatography-tandem mass spectrometry) on liver biopsies collected at the end of the challenge. Samples were analyzed on a Thermo Orbitrap Elite with repeat injections and raw data processing was performed using MaxQuant. Proteins were identified based on peptide spectral matching to custom search databases generated from RNA sequencing data in the same biopsies using the customProDB R package and implemented in GALAXY-P.

Preliminary Data

A total of 2,243 proteins were identified across all liver samples using the customProDB method. This method improves matching relative to public databases by capturing novel mutations and reducing the search space for spectral matches to those predicted from transcripts. 1,298 passed

cutoffs for missingness and variance and were retained for downstream analysis. Networks of proteins covarying across samples were identified using the WGCNA R package. 16 modules ranging in size from 30 to 217 proteins were identified. These groups of proteins may interact with one another in vivo based on their similar variation across samples independent of dietary challenge. One of these modules was also associated with the difference in diet. Proteins in this module show increased abundance in animals on the high-fructose diet as well as consistent correlations across transcript networks also upregulated in response to diet. Ontologies for the most strongly upregulated proteins within this module include glucose metabolic processes (H6PD, FAHD1), cholesterol biosynthesis (IDI1), and intracellular protein transport (AP2B1, SEC24C, and COPG1). These pathways highlight the means through which the liver processes increased fructose in the diet. We are currently analyzing the proteomic response in conjunction with the transcriptomic data to better model how the transcripts and proteins may be interacting within the cell.

Novel Aspect

Proteomic datasets from non-human primate tissues are essential for translational models but entirely absent from the literature.

87. Dushani Ranasinghe

Exploring EMAPII/STAT3 signaling axis in lung development and lung diseases

Dushani Ranasinghe[1,A,B], Daniel Lee[2,A,C], Margaret Schwarz[1,2,A,B,C]
[1]Department of Chemistry and Biochemistry, University of Notre Dame
[2]Department of Pediatrics,
[A]Harper Cancer Research Institute, [B]University of Notre Dame, [C]Indiana University.

Introduction

Inflammation is a high-risk factor in lung development and hyperoxia-induced lung diseases of prematurity. Accumulating studies have implied the physiological role of Endothelial Monocyte Activating Polypeptide (EMAP) II in various biological processes such as inflammation and angiogenesis in developing lung and lung diseases. Our preliminary studies identified that EMAPII promotes the phosphorylation of signal transducer and activator of transcription 3 protein (STAT3). However, the underlying molecular mechanism by which EMAPII exerts adverse inflammatory effects is yet unknown. As sphingosine-1-phosphate (S1P) also mediates inflammation, angiogenesis and phosphorylates STAT3, we hypothesized that EMAPII induced STAT3 activation is linked to S1P signaling axis.

Methods

1) Mouse macrophages (RAW 264.7) were treated with of 2 µg/ml EMAPII for 24 hours after blocking S1P generation (sphingosine kinase 1 (SPHK1)) and signaling through its known receptors (S1PR1-3). Phospho-STAT3(Y705) levels were measured in whole cell extracts by immunoblotting. 2) A genetic reporter assay (luciferase assay) was performed using pGL4.47[luc2P/SIE/Hygro] to assess the activation and nuclear translocation of STAT3 following the treatment of EMAPII after blocking S1PRs in HEK293T. 3) Expression levels of SPHK1 after EMAPII treatment was studied using immunoblotting of whole cell lysate of EMAPII treated macrophages and immunohistochemistry (IHC) of FFPE- tissue

slides of EMAPII treated Bronchopulmonary Dysplasia (BPD) mouse lungs. 4) EMAPII induced activation of Extracellular signal-regulated protein kinases 1 and 2 (ERK 1/2) was measured by immunoblotting. 5) Expression of Early Growth Response Gene 1 (EGR-1) which is a mammalian transcription factor was measured in whole cell lysate, nuclear and cytoplasmic fractions using immunoblotting.

Preliminary Data

Both immunoblotting and luciferase assays reported that EMAPII treatment increases pSTAT3 relative to the control and the blocking of S1PRs and SPHK1 reduce pSTAT3 level generated by EMAPII signaling while demonstrating the connection between EMAPII induced STAT3 activation and S1P signaling axis. While validating the significant role of SPHK1 in EMAPII signaling, immunoblotting studies of EMAPII-treated macrophages and IHC studies of EMAPII-treated BPD mouse lungs showed EMAPII induced elevation of SPHK1 protein expression. In this backdrop, a potential activator of SPHK1, ERK1/2 showed stimulated phosphorylation/activation by EMAPII just as in few minutes (about 5 minutes) after the treatment and the highest elevation is showed after 1 hour in macrophages. Interestingly, an increased expression of a reported downstream target of ERK1/2, EGR-1 which is a mammalian transcription factor is observed after 2 hours of EMAPII treatment in macrophages in both whole cell lysates and nuclear fraction while showing the absence of EGR-1 in cytoplasmic fraction indicating the EMAPII-induced nuclear targeting of EGR-1.

Novel Aspect

Discovering a small molecular inhibitor for EGR1 is an area to be explored.

88. Val Ressler

Novel mass spec compatible surfactant for in-solution protein mass spec sample preparation

Valerie Ressler, Promega Corporation, Madison WI

Introduction

Mass spec (MS) compatible surfactants are commonly used in proteomics to improve protein extraction and solubilization, enhance proteolysis and address other needs of protein sample preparation. However, performance of these surfactants often falls short of expectations. SDS is generally accepted as the most popular surfactant, due to its powerful protein solubilizing and denaturing ability. MS compatible surfactants currently fail to match SDS's performance. Peptide loss during surfactant degradation used to remove surfactants from a reaction is another problem. To address these and other shortcomings of MS compatible surfactants, we have conducted a study in which the structure of mass spec compatible surfactants was systematically optimized. Using this approach, we have significantly improved on the critical properties of these surfactants.

Methods

In this study, we screened a large library of mass spec compatible surfactants synthesized in-house. We identified a surfactant showing advanced protein solubilizing and denaturing properties. This surfactant performed equally well in terms of resistance to adverse conditions used in protein sample preparation including boiling and overnight incubation at 50-60oC. The selected surfactant

was fully compatible with trypsin even at relatively high concentrations. Moreover, it supported tryptic proteolytic activity at high reaction temperatures (50oC) for extended time periods. These beneficial properties provided conditions for the most efficient protein digestion. Furthermore, the surfactant was optimized to prevent peptide loss, commonly observed during degradation of MS compatible surfactants.

Preliminary Data

Improvement of the surfactant properties had a critical impact on the efficiency of proteomic analysis. The selected surfactant provided a significant increase in the peptide and protein IDs as well as protein sequence coverage in a proteomic study as compared to commercially available MS compatible surfactants. The results of the surfactant testing with additional applications including deglycosylation, peptide quantitation with TMT labeling and others will be presented.

Novel Aspect

Structural optimization of MS compatible surfactants to improve critical surfactant properties and minimize side effects.

89. Angela Ricono

Clarifying the role of glucosinolates during abiotic stress in the Brassicaceae

Dr. Kathleen Greenham; University of Minnesota

Introduction

Understanding how plants respond to stress is paramount to our ability to maintain robust crops. Varieties in the genus Brassica are known for their extensive variation in morphology, climatic niches, and stress responses. How might less tolerant varieties cope with extreme temperatures as predicted by climate change? Modifying the production of secondary metabolites (i.e. glucosinolates) appears to be an effective defense strategy against biotic stress, yet these compounds are costly to synthesize and likely to decrease productivity and fitness. One-way plants balance this cost-to-fitness ratio is through circadian regulation of physiology and metabolism; however, how these temporal metabolic processes change under abiotic stress is relatively unclear.

Methods

To mimic spring planting conditions of B. rapa, 24 replicates of five B. rapa morphotypes (ssp. trilocularis,ssp. pekinensis, ssp. rapa, ssp. chinensis, ssp. nipposinica) will be grown with 14h, 24°C days and 14°C nights. Three weeks post germination, half of the morphotypes will be moved into cold-stress conditions (14h, 10°C days and 4°C nights) to mimic a cold snap during early development. Leaf tissue will be collected every 4h for 48h to measure glucosinolates. Additional isotope labeling and pulse-chase experiments will be done to assess turnover rates. Extraction and characterization of metabolites will be performed using a combination of HPLC and MS protocols.[2] These high resolution metabolomic data will then be used to generate metabolic flux models.

Preliminary Data

Glucosinolates (GLS's) are secondary metabolites (SM's) that are widely known for their beneficial role in plant defense and pathogen resistance, and to a lesser degree, plant growth regulators. Largely grouped into three major classes (aliphatic, aromatic and indolic), these SM's are primarily

found in the Brassicaceae family which includes the model plant Arabidopsis thaliana and proposed B. rapa varieties. Within A. thaliana's glucosinolate biosynthetic pathway, twelve genes related to aliphatic GLS's have been linked to desiccation stress response, indicating that these SM's have an important role in drought tolerance. These regulatory genes have been associated with drought and cold responses making them good targets to modify cold tolerance.

Prior to genetic modification, we must first understand the energy cost to make these compounds. Metabolic flux models in A. thaliana have shown that GLS production can increase photosynthetic requirements by at least 15%. This considerable shift in resource allocation raises an important question: when and how does this allocation become beneficial? In Brassica, the concentration of aliphatic GLS's varies temporally between morphotypes within a 24h cycle; although other GLS's do not necessarily share this pattern. This differential pattern between morphotypes leads to the hypothesis that these varieties may respond differently to environmental stress. Without improved flux models that incorporate time of day and stress responses we are limited in our ability to predict gene targets effectively in such a variable system.

Novel Aspect

To date, no study has used fine-scale temporal data to link high resolution glucosinolate response and turnover under cold stress.

90. Rosanna Rossi

Acute Ischemic Stroke Clot Project: Focus on a Proteomic Approach for Novel Biomarker Discovery

Rosanna Rossi [1,2] , Oana Madalina Mereuta[1,2] , Séan Fitzgerald [1,2] , Andrew Douglas [1,2] , Abhay Pandit[2], Karen Doyle [1,2]

[1] School of Medicine, Department of Physiology, National University of Ireland, Galway (IE)

[2] CÚRAM, Centre for Research in Medical Devices, Biomedical Sciences, National University of Ireland Galway (IE).

Introduction

Acute ischemic stroke (AIS) remains a major cause of death and disability worldwide (15 million people each year). Until recently, the treatment approach for AIS was limited to intravenous thrombolysis with recombinant tissue-type plasminogen activator (IV rtPA). Since 2015, the use of medical devices (aspiration catheters and stentrievers) to mechanically remove the clot (thrombectomy) has become the optimum treatment strategy for many patients. Furthermore, thrombectomy has made blood clots available for analysis and characterization, leading to an emerging area of research. We hypothesize that the responsiveness to rtPA and mechanical thrombectomy treatment depends on the composition of the specific AIS clot.

Methods

Clot composition will be investigated by using several kinds of complementary techniques including Mass Spectrometry. Mass Spectrometric part will involve in first instance the analysis by LC-HRMS of human clot analogues with varying levels of Red Blood Cells and fibrin aiming to provide for a basal indication of core proteins within the clots, in order to define tighter search criteria. A bottom-up

proteomics approach will be optimized, starting from fresh/frozen human clot analogues. Sample preparation procedure for proteomics analysis will be optimized also for formalin-fixed paraffin-embedded tissues (FFPE) and the two methods will be compared. Both the analysis will be performed by using the Q-Orbitrap mass analyzer.

Preliminary Data

The use of "classical" techniques as Histological staining and Immunohistochemistry analysis showed a considerable variability among clot composition. To date, the optimization of proteomics sample preparation for clot analysis is still in progress. However, the use of Mass Spectrometry as a complementary technique will give us the possibility to more deeply investigate also the molecular proteome of the thrombus, to obtain a complete overview of clot composition.

Novel Aspect

To discover potential biomarkers for AIS pathology and stroke etiology and to identify novel diagnostic tools and therapeutic targets.

91.Bianca Ruiz

Functional selections to investigate the effects of amino acid substitutions across the proteome

Department of Genome Sciences, University of Washington
Ricard Rodriguez-Mias, Judit Villén
Stephanie Zimmerman, Stanley Fields
Department of Biochemistry, University of Western Ontario
Matthew Berg, Christopher Brandl

Introduction

Rapid advancement in sequencing technology is identifying growing numbers of missense mutations, but many have poorly understood biological consequences. To extend functional studies of missense mutations to the proteome scale, the Fields and Villén labs are developing a technology called Mistranslation Mutagenesis. Mistranslation Mutagenesis consists of three general steps: induce proteome-wide mistranslation to create a large number of protein variants, apply a functional selection to the proteome, and read out the selection by mass spectrometry. Data from such an experiment are used to measure enrichment or depletion of mistranslated peptides relative to wild-type peptides.

Methods

One of several approaches to produce amino acid substitutions is through genetic perturbations. By mutating the anticodon of a tRNA, we have enabled amino acid substitution at incorrect codons during translation. In our case, the wild-type anticodon of a serine tRNA, UGA (recognizes serine codon UCA), was mutated to UGG (recognizes proline codon CCA). This mutation enables substitution of serine at up to six percent of proline sites across the proteome in S. cerevisiae.

Preliminary Data

The first functional selection we applied to this mistranslated proteome was a phospho-enrichment. Since serine is a commonly phosphorylated residue and many kinases are directed by proline-

containing phosphomotifs, we hypothesized that mistranslating proline as serine across the proteome would induce aberrant phosphorylation by introducing new phosphosites. Preliminary data from these experiments suggest that phosphorylation of [Pro-Thr/Ser] phosphomotifs is higher when the proline within the motif is substituted to serine than the rest of the phosphoproteome.

Novel Aspect

Mistranslation Mutagenesis provides a novel approach to investigating the functional consequences amino acid substitutions at the proteome scale.

92. Scott Rusin

Quantitative Mass Spectrometry-based Proteomics to decode rules of Targeted Protein Degradation

Kirti Sharma, Kymera Therapeutics
Karen Yuan, Kymera Therapeutics
Nello Mainolfi, Kymera Therapeutics

Introduction

Small molecule inhibitors of proteins have revolutionized the field of medicine. However, nearly 90% of the human proteome remains untargeted by these class of molecules, due to the lack of enzymatic activity or substrate binding regions. Recently, the popularization of proteasome-targeting heterobifunctional molecules has allowed the recruitment of previously undrugged proteins to the proteasome for degradation, accessing a new portion of the proteome to therapeutics. At Kymera Therapeutics we seek to better understand the rules of protein degradation in part through the use of mass spectrometry-based proteomics. Our initial work has focused on IRAK4, a critical component of the Myddosome, important in NFκB and MAPK signaling.

Methods

We utilize mass spectrometry-based shotgun proteomics and isobaric tagging to understand the effects of targeted protein degraders on protein levels in cultured cancer cells and human PBMCs. Cells are treated with protein-degrading compound or inactive analog, collected, lysed and digested using the Thermo EasyPep MS sample kit, and desalted. Peptides are then labeled using Tandem Mass Tag (TMTTM) reagent, mixed, and desalted. Labeled peptides are fractionated using off-line basic pH reversed phase HPLC fractionation on an Agilent 1290 system, concatenated into 24 fractions and analyzed on a Q-Exactive HF-X in high-resolution MS2 mode. Data is processed in MaxQuant (v1.6.2.10) and further analyzed in Perseus (v1.6.2.3).

Preliminary Data

Using proteomics, treatment with the compound KYM-001 showed specific (sole protein observed with greater than two-fold degradation) and reproducible degradation of the target protein IRAK4 in the diffuse large B-cell lymphoma cell line OCI-LY10 after 8 hours of treatment. Our focus has been to increase depth and accuracy to ascertain changes in relatively low abundance proteins, and we have optimized parameters in both the off-line and on-line fractionation to attempt to achieve routine deep proteome coverage. Interestingly, though peptides are TMT labeled, we observe a majority of peptide eluting early from the on-line column (Thermo EasySpray 75uM ID, 500mm

length, 2uM particle size). Therefore, we optimized the steepness of uHPLC gradient leading to equal distribution of MS/MS events thereby enabling a 16% increase in total MS/MS scans. We have also optimized the off-line fractionation to increase peptide uniqueness per fraction and increase the contribution of each fraction to the total proteome depth. Through lengthening of the gradient and spacing of the primary elution window we increased our overall efficiency (percent unique peptides per fraction) from 53% (with 77% found in only 2 fractions) to 73% (with 91% found in only 2 fractions), allowing more unique peptide identification per fraction. This led to about 10% increase in protein identifications, increasing from 8,725 to 9,546 unique protein groups. MS2-based TMT quantification has suffered from ratio compression; however, our data agrees with published literature that increased fractionation efficiency or total number of fractions have led to increased precision in quantification. The correction is evident by an increase in quantitative accuracy of our target protein, IRAK4, upon degrader treatment when corroborated with other orthogonal analysis methods. This coupled to tools that would enable reporter ions quantification based on 3D features (m/z, intensity and time) will lead to robust quantification in the MS2 space.

Novel Aspect

Characterization of targeted protein degraders utilizing mass spectrometry-based proteomics through understanding of specificity and mechanism of action.

93. Aya Saleh

Analysis of protein dynamics in developing mouse using non-canonical amino acids labeling

Kathryn R. Jacobson, Tamara L. Kinzer-Ursem and Sarah Calve
Weldon School of Biomedical Engineering, Purdue University

Introduction

Mapping the spatiotemporal protein dynamics in complex organisms as a function of development is indispensable for proper understanding of mechanisms of tissue repair as a consequence of disease or injury. This goal is, however, hampered by lack of tools. To address this gap, we developed a technique that enables labeling of newly synthesized proteins based on systemic injection of the non-canonical amino acid azidohomoalanine (Aha). The azide functionality of Aha allows selective enrichment of labeled proteins via click chemistry using alkyne-bearing affinity tags. Using this technique, we were able to examine the dynamics of protein synthesis and turnover in various cellular compartments in developing murine tissues.

Methods

C57Bl/6 murine dams were time mated and injected with Aha or PBS (control) at different embryonic time points. Embryos at embryonic time point E15.5 were homogenized and Aha-labeled proteins were conjugated to biotin-alkyne via copper-catalyzed click chemistry. Biotinylated proteins were affinity purified using NeutrAvidin beads and enriched proteins were analyzed using LC-MS/MS. For turnover studies, embryos were harvested 0-48 h following Aha injection at E12.5, fractionated into different cellular compartments based on their solubility profiles, clicked to biotin-alkyne, and then analyzed via western blotting using Streptavidin fluorophore to quantify the change in fluorescence intensity over time.

Preliminary Data

Using the injection-based non-canonical amino acid labeling technique, we were able to selectively isolate the newly synthesized proteins with minimal background of old preexisting proteins in developing embryos. In addition, we were able to probe the dynamics of protein synthesis and turnover in different cellular fractions. Our data indicates that the rate of protein turnover differs significantly between various fractions. As such, successful completion of this work will ultimately have a significant impact on our understanding of the regulation of protein dynamics as a function of development.

Novel Aspect

This technique enables quantifying the turnover rates of different tissue fractions during development to understand functional mechanisms of tissue regeneration.

94. Tommy Saunders

Improving Accuracy and Repeatability with Single Injection MS/Polyarc Split for Extractables and Leachables

Introduction

The analysis of extractables and leachables (E&L) and residual solvents found in pharmaceuticals is often performed using gas chromatography with mass spectrometry (GC-MS). Unfortunately, these measurements are often complicated by the varied and unpredictable responses of different molecules in the GC-MS leading to various MDLs and tenuous concentration estimates without exhaustive calibrations. Here, we show how companies are using the Polyarc/FID combined with GC-MS to improve the accuracy and speed of E&L, and solvent analyses, and meet the requirements of the US Food and Drug Administration (FDA), European Medicines Agency (EMA), ISO, and the Product Quality Research Institute (PQRI).

Methods

A set of test solutions comprised of molecules commonly found in E&L studies was prepared for evaluating the method, as well as five level calibration curves of several surrogate molecules. Data for this application was gathered on an Agilent 7890 GC with a two way He-purged post column splitter. The two outputs were to an Agilent 5973 MSD and an Activated Research Company Polyarc/FID detector system. The Polyarc converts all organic compounds to methane, resulting a uniform response per mole of carbon in the FID. This allows for calibration-free quantification paired with accurate identification. Calibrated mass spectra were obtained in Raw mode and analyzed with Cerno MassWorks to improve spectral accuracy.

Preliminary Data

E&L samples from a pharmaceutical laboratory were analyzed on this split system at Activated Research Company's laboratory. Ten compounds were detected and quantified, using n-hexadecane-d34 as an internal standard with its known concentration. The compounds detected include benzyl chloride, NMP, cyclomethicone, BHT, n-hexadecane-d34 (IS), 1-HPK, DBP, stearic acid, ATBC, and octadecanamide. In a single injection, every compound was accurately identified and quantified to within 10% of gravimetric values. The two exceptions were cyclomethicone and stearic

acid, which had errors of -49% and -61%, respectively. These errors could be due to sample degradation, inaccurate sample preparation, or inefficient transfer of the sample through the GC system to the Polyarc (specifically with respect to the GC inlet). This quantification was done using the assumption that the FID gives an equivalent response per mole of carbon, which the Polyarc allows for. Using this assumption, these compounds were also quantified with the MSD, and errors ranged from -15% to -85%, showing a clear improvement of using the Polyarc in a single injection compared to the MSD. Because the conventional method of quantification employs the use of calibration curves, three 5-point calibration curves of similar molecules were used to quantify, but this resulted in worse errors with the MSD. Linearity studies were also performed, and the Polyarc/FID showed 1.9% RSDs, where the MSD showed 8.7% RSDs. In conclusion, use of the Polyarc/FID is more effective when paired with MSD for identification than using a MSD to quantify alone because of accuracy, linearity, and the uniform response.

MS identification was also improved through spectral calibration with PFTBA and subsequent software analysis with Cerno Massworks, which showed promising results This entire approach of identification, calibration and quantification has been automated to greatly increase the speed and accuracy of E&L analysis.

Novel Aspect

The accuracy and reproducibility of E&L analysis was improved using a MSD and Polyarc/FID for single injection identification and quantification.

95. Naviya Schuster-Little

Development of a Mass Spectrometry Assay to Analyze the Ovarian Cancer Biomarker CA125

Sergio Madera (University of Notre Dame), Roberta Fritz-Klaus (University of Wisconsin), Mark Etzel (University of Wisconsin), Manish Patankar (University of Wisconsin), Rebecca Whelan (University of Notre Dame)

Introduction

We want to use mass spectrometry to investigate the primary amino acid sequence of the ovarian cancer biomarker protein CA125. CA125 is a large, 3-5 MDa protein that is used as a biomarker for ovarian cancer. It is composed of a core domain that is repeated more than 60 times. The CA125 protein is heavily glycosylated at its N-terminus and within the repeat domains. Currently, CA125 is used in a clinical setting, however, the test produces many false positives and false negatives. We aim to develop an analytical assay with greater sensitivity than antibodies. We raise the analytical question, does the primary sequence of the protein vary from patient to patient, and if so, does that impact detection?

Methods

We are currently optimizing a bottom-up proteomics workflow that enables us to take purified CA125, digest it trypsin, analyze it using mass spectrometry, and identify peptides that correspond to CA125. We denature the protein with SDS, reduce disulfide bonds with TCEP, and alkylate free thiols with IAA. CA125 is heavily glycosylated which motivates us to include DCA as a passivating

agent to prevent the protein from sticking to the tube. Following alkylation, we load the solution onto a suspension-trap and wash away residual reagents. The protein is digested overnight using trypsin. The next morning peptides are eluted off the s-trap, concentrated, and then desalted using C18 ZipTips. Samples are run with nLC-MS and CZE-MS and data analyzed using Proteome Discoverer.

Preliminary Data

Our lab has been successful with digesting and detecting peptides from a smaller, recombinant version of CA125 (rCA125). Using the methods outlined above, we were able to successfully generate peptides from rCA125 and detect them using nanoLC-MS. Amanda Hummon's lab recently compared in-solution, FASP, and s-trap protein digestions and found the s-trap to be the best method. Because of this, we will use a suspension trap (s-trap) during our digestion. During the initial digestion experiments we included BSA as a way to prevent CA125 from sticking to tips and tubes. While this method worked and provided us a positive control to ensure our digestion was working, we did not want to overload the mass spectrometer detector with BSA peptides. To overcome this problem, we looked into work from Joe Loo's group, which uses a small molecule, deoxycholic acid (DCA), as a passivating agent during in-solution and FASP digestions.

Before performing a digestion using DCA, we needed to ensure that DCA did not interfere with the s-trap method. We did a series of experiments, using BSA as a model protein, to make sure that our peptide yields did not decrease when we included DCA. We found that including 0.2% DCA in the digestion buffer did not impact digestion.

We then included 0.2% DCA in a protein digest using rCA125 and were able to successfully identify 34 peptides, which resulted in 43% coverage of the protein. Our future steps will be to apply this method to the native CA125 protein and then the ascites fluid samples from our collaborator. We will also explore the effects of deglycosylating CA125 and the peptides we identify before and after deglycosylation.

Novel Aspect

The novel aspect is comparing the ascites fluid peptide profiles of different individuals.

96. Liudmila Shcherbakova

Characterisation of function and regulation of chromatin remodelling complex subunit ARID1A and its role in oncogenesis

Liudmila Shcherbakova [1], Mercedes Pardo [1], Lu Yu [1] and Jyoti Choudhary [1].
[1] The Institute of Cancer Research (London, UK)

Introduction

A number of genes with high frequency mutations in cancer patients are reported to encode ATP-chromating remodelers of the SWI/SNF family, specifically BRG/Brahma-associated factors (BAF). One of the subunits of BAF complex is an AT-rich interactive domain containing protein 1a (ARID1a) is found to be the most mutated subunit of SWI/SNF in cancer. Literature shows that ARID1a is involved in many protein-protein interactions within, as well as outside of the BAF complex, for example proteins of Polycomb repressive complex 2. Nevertheless, the role of ARID1a is poorly

understood. Using proteomic mass spectrometry, my research is directed toward understanding the functions of ARID1a, as well as identifying its interactors, their functional relationship with ARID1a and therefore their role in disease progression.

Methods

To study the interactions, interactors will be co-immunoprecipitate with tagged and untagged ARID1a in number colorectal cell lines, as well as human embryonic stem cells and followed by mass spectrometry analysis. Cross-linking mass spectrometry will be used to study structural information of ARID1a containing complexes. Blue native PAGE will also be utilised to study the complexes that ARID1a forms a part of and the effect of the ARID1a knock-out on all complexes within the cells. Size fractionation will be also be utilised to resolve sub-complexes within the cells.

Preliminary Data

Previous work from our group has shown that the knock-out of ARID1a in colorectal cancer cell lines does not only result in reduction of ARID1a abundance but also other BAF complex subunits (Roumeliotis et al., 2017). This suggests that BAF complex stability might be regulated post-translationally. Moreover, immunoprecipitation on the tagged-ARID1a in mouse embryonic stem cells has shown a number of potential novel interactors that are to be explored in the context of the human cells.

Novel Aspect

Understanding functions of ARID1a, identifying its interactors, their functional relationship with ARID1a and therefore their role in disease progression.

97. Aaron Simmons

Developing methods for the scalable monitoring and maturation of induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) through 'omics' and systems biology

Dr. Sean Palecek (Department of Chemical and Biological Engineering, UW-Madison)

Introduction

Induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) present rich opportunities for developmental biology, drug screening, and cellular therapy applications. Translation; however, is hindered by high variability, poor characterization, poor maturation, and a lack of real-time, scalable, and robust quality monitoring metrics. To address these, we propose a multi-omic, systems biology scheme to comprehensively characterize iPSC-CM development in vitro. The resulting data sets will be integrated and analyzed for 1) the identification of temporal biomarkers capable of predicting cell status, trajectory, and end-point function and 2) the generation of novel methods to further mature these cells via temporal modulation of key identified pathways to mimic in vivo developmental trajectories.

Methods

iPSCs will be differentiated into iPSC-CMs per established protocols in the WTC11 cell line. At intermittent times throughout the culture period, parallel samples will be sacrificed for various

analyses (molecular, structural, and functional). Molecular analyses will include transcriptomics, proteomics (LC-MS/MS), metabolomics (LC-MS/MS and NMR), and exometabolomics (NMR). Structural analyses will include flow cytometry and immunocytochemistry characterization of cell morphology, targeted protein abundance/localization, sarcomere alignment, and mitochondrial localization/content/morphology. Functional analyses will include electrophysiology assessment via multi-electrode array (MEA), and energy metabolic pathway utilization/flexibility characterization via Seahorse extracellular flux assay. Individual data sets will be integrated and analyzed via various workflows, including flux balance analysis (FBA) and various pathway, multivariate, and machine learning analyses.

Preliminary Data

Dramatic changes are well known to occur in the cascade from embryonic stem cell to fully mature adult cardiomyocyte in vivo, as is readily evident in the stark contrast between the initial and final cell types. Among these changes are included large alterations in structure, function, and metabolic demands. Many of these temporal trends have been observed in vitro in stem cell-based models by our lab and many others, though none have been successful as yet in completely recapitulating full adult cardiomyocyte phenotypes. Initial studies have identified small subsets of protein and/or transcript markers which are conserved between in vitro and in vivo, though such metrics alone are not amenable to real-time, non-destructive monitoring. Some work has identified altered metabolic pathway utilization may present a promising readout of cell progression. Our lab has shown previously that certain metabolite readouts (phosphocholine, glycerophosphocholine, glycine) can discriminate between less and more mature iPSC-CMs, and that said metabolic trends are conserved in in vivo mouse studies. Further studies have demonstrated temporal transitions in mitochondrial dynamics throughout the iPSC-CM progression, mirrored by differences in metabolic pathway utilization trends and accompanying transcriptional and protein abundances. Current efforts to optimize sample preparation and co-extraction methods are underway prior to full-scale implementation.

Novel Aspect

Temporal ‘omics’ profiling for systems biology and metabolic flux balance approaches to tracking and improving iPSC-CM production in vitro.

98. Ben Stocks

A proteomic insight into liver dysfunction and metabolism during obesity

Ben Stocks[1], Alba Gonzalez-Franquesa[1], Juleen R. Zierath[1,2], Atul S. Deshmukh[1,3]

[1] Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen Denmark.

[2] Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden.

[3]Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen Denmark.

Introduction

Obesity and insulin resistance are characterised by altered metabolism and function in various tissues. In the liver, ectopic fat deposits contribute to impaired glucose metabolism, insulin signaling

and mitochondrial function. Ultimately, obesity contributes to liver-related metabolic diseases including insulin resistance and non-alcoholic fatty liver disease. The ob/ob mouse, which produces a truncated inactive leptin protein, is widely used as a model of obesity-induced metabolic disease. Whilst the physiological phenotype of ob/ob mice has been well characterised, the underlying alterations in protein content and function remain relatively understudied. Using a deep-proteomic approach, we have identified divergent proteins and pathways that may underpin the mechanistic basis of obesity-induced liver dysfunction.

Methods

Liver samples were harvested from four-month old ob/ob mice and lean littermates (n = 4 per group, C57BL/6J background) following a four-hour fast. Mice had free access to water and standard rodent chow and were maintained in a temperature- and light-controlled (12-h light/dark cycle) environment. Liver samples were lysed in a 4% SDS buffer and processed according to the MED-FASP protocol using Lys-C and trypsin. Peptides were separated on an Easy nano-flow HPLC system coupled to a LTQ Orbitrap mass spectrometer (HFX) via a nanoelectrospray source (Thermo Fisher Scientific). MS and MS/MS spectra were acquired in a data-dependent manner and analysed using MaxQuant software. Downstream data analysis was performed in Perseus software using label-free quantification (LFQ) intensities.

Preliminary Data

Proteomics analysis of liver from lean and ob/ob mice led to the quantification of 5551 proteins. 330 proteins were differentially regulated with obesity, of which 172 and 158 proteins were up- or downregulated, respectively, in ob/ob mice. Gene ontology annotations related to fatty acid metabolism were upregulated in the liver of ob/ob mice as well as the KEGG pathways PPAR signalling and peroxisome, indicating an adaptation to excess fatty acid availability. Furthermore, protein families involved in bile acid metabolism were also over-represented in ob/ob livers. Conversely, several gene ontology cellular component terms related to the endoplasmic reticulum were enriched in the downregulated proteins. Furthermore, protein categories involved in the metabolism of arachidonic acid (a membrane phospholipid), including oxidoreductase activity and the cytochrome p450 family, were reduced. Reduced arachidonic acid concentrations have been identified in the liver of obese Zucker rats and linked to insulin resistance in human skeletal muscle, whilst arachidonic acid supplementation improves glucose metabolism in ob/ob mice. In addition, lipocalins, specifically major urinary protein (MUP) isoforms, were significantly downregulated in ob/ob liver. MUPs are known to regulate gluconeogenesis in the liver and upon secretion from the liver can increase skeletal muscle mitochondrial function and, consequently, whole-body energy expenditure, glucose tolerance and insulin sensitivity. Furthermore, we have previously identified a reduction in MUP8 protein content within skeletal muscle of ob/ob mice, despite a lack of MUP transcription within skeletal muscle, providing an indication of MUP-mediated cross-talk between liver and skeletal muscle during obesity in mice. Overall, we provide a deep-proteomic analysis of liver from ob/ob and wild-type mice, identifying known and emerging regulators of insulin sensitivity within the liver and other tissues. Further analysis of this dataset will provide additional mechanistic insight into the development of liver dysfunction during obesity.

Novel Aspect

The quantification of over 5000 proteins within liver of ob/ob mice will further our understanding of liver dysfunction in obesity.

99. Arjun Sukumaran

Examining the role of zinc on the proteome, growth and morphology of Klebsiella pneumonia

Arjun Sukumaran and Jennifer Geddes-McAlister
Molecular and Cellular Biology Department, University of Guelph, Guelph, Ontario, Canada

Introduction

Microbial organisms encounter a variety of environmental conditions including changes to pH, temperature, and nutrient levels, which influence their cellular protein regulation and secretion patterns. Recently, a study investigating the transport and regulation of iron, copper, zinc, and manganese in Enterobacteria defined correlations between bacterial strains and the relative importance of different metal transport systems in survival and virulence. Here, we focus on Klebsiella pneumoniae, a commensal and pathogenic bacterium associated with respiratory and urinary tract infections, which produces a polysaccharide capsule and shows nutrient-dependent patterns of regulation. Using K. pneumoniae as a model system, we aim to determine how a nutrient-limited environment (e.g. zinc) modulates the cellular proteome and secretome of the bacteria.

Methods

K. pneumoniae strain 52145 (WT) was grown under zinc-limited and zinc-replete (+10 µM zinc) conditions to mid-log phase. Cell pellets and supernatant samples were collected, processed, and digested with LysC/trypsin. Samples were measured over a 60 min gradient on a Hybrid Orbitrap-Quadrupole Mass Spectrometer. Raw data files were processed using MaxQuant and data analysis, statistical processing, and visualization was performed in Perseus. Proteins with abundance profiles significantly influenced by zinc were subjected to in silico characterization. Two candidates were selected for follow-up experimentation, including mutant construction by Lambda Red recombineering and transcript quantification of zinc-regulated genes by quantitative real-time PCR. Phenotypic characterization of the WT and mutant strains is currently underway.

Preliminary Data

Our results provide an in-depth analysis of the effect of zinc availability on the proteome and secretome of K. pneumoniae in vitro. Here, we identified 2,380 proteins from the total cellular proteome (i.e. cell pellet) and 246 secreted proteins, representing the deepest proteome of K. pneumoniae to date. A principal component analysis (PCA) of our data showed the largest separating component between the samples to be associated with the zinc treatment. Statistical analysis using a Student's t-test (p-value < 0.05, FDR = 0.05) identified 19 proteins from the total cellular proteome and 21 secreted proteins that were noted as significantly different. Within the significantly different proteins, a histidine utilization repressor (hutC) was identified. This protein and its targets have been linked to zinc homeostasis and therefore, represent a candidate for validating our study. Given the function of hutC to repress genes on the histidine utilization (hut) operon, we

will confirm its increase in protein abundance by quantifying the suppression in gene expression of the hutC targets using qPCR. Simultaneously, an uncharacterized protein, chaB was identified, and upon in silico characterization, was proposed to be a cation transport regulator. To evaluate the potential role this protein has in zinc transport, STRING analysis was performed to identify putative interacting partners, from which, a capsule biosynthesis protein was indicated. Upon knocking out chaB, the proteome and secretome of the mutant will be evaluated to assess the role of this potential transporter on cellular homeostasis. Concurrently, the morphology of the mutant will be compared to the WT strain by using phase-contrast microscopy to detect differences in capsule production and to quantify changes in cell size influenced by zinc.

Novel Aspect

Proteomic profiling of K. pneumoniae defines the influence of zinc availability on cellular regulation, protein secretion, and virulence factor production.

100. Mehmet Tatli

Overexpression and Metabolic Regulation of Z. mobilis MEP Pathway Enzymes

Alexander Hebert[1,3], Julio Rivera Vazquez[1,2], Joshua J. Coon[1,4,5,6], and Daniel Amador-Noguez[1,2]
[1]DOE Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI
[2]Department of Bacteriology, University of Wisconsin-Madison, Madison, WI
[3]Genome Center of Wisconsin, Madison, WI
[4]Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI
[5]Department of Chemistry, University of Wisconsin-Madison, Madison, WI; [6]Morgridge Institute for Research, Madison, WI

Introduction

Zymomonas mobilis, a facultative anaerobe, can convert 96% of the glucose consumed to ethanol at high yields. This highly catabolic metabolism can also be redirected towards generation of isoprenoid-derived biofuels via the 2-C-Methyl-D-erythritol 4-phosphate (MEP) pathway.

Methods

The genes of interest from the MEP pathway were cloned into a plasmid with an inducable T7A promoter and spectinomycin resistance. Those were transferred into Zymomonas mobilis ZM4 strain and over-expressed by inducing cells at O.D.600=0.5 with 0.45 mM IPTG. The metabolites from the over-expression strains were extracted and analyzed by using the VanquishTM UHPLC system (Thermo Scientific). For data analysis, LC-MS raw files were converted to mzXML format and visualized using the software, MAVEN.

Preliminary Data

Here, we have individually over-expressed the MEP pathway enzymes (DXS, DXR, IspDF, and IspE) of Z. mobilis in the ZM4 strain to better understand the metabolic regulations in the first part of the MEP pathway, and investigate the effect of the enzymes on directing carbon flux into the MEP pathway and down to the cyclic intermediate, MEcDP. Initial results showed that DXS2 over-expression increases flux through MEP pathway, leading to a 70-fold increase in intracellular MEcDP

levels, and also increases levels of the two end products of this pathway, IDP/DMADP. These results have indicated that DXS2 is a rate limiting enzyme in the MEP pathway of Z. mobilis. Moreover, coupling DXS2 with isoprene synthase, IspS, allows for production of isoprene in ZM4. This over-expression strategy also revealed interesting metabolic changes in the MEP pathway, which might bring new insights into understanding metabolic regulation of the MEP pathway.

Novel Aspect

This work aims to bring novel insights into the metabolic regulation of the MEP pathway in Zymomonas mobilis.

101.John Tilton

A novel selective reaction monitoring mass spectrometry (SRM-MS) assay to monitor HIV protease activation in intact viruses

Daniela Schlatzer (Case Western Reserve University)
Aiman Haqqani (Case Western Reserve University)

Introduction and Methods

HIV-1 encodes an aspartyl protease (PR) that cleaves the viral polyproteins into subunits in a highly regulated process essential for viral infectivity. PR is traditionally measured by western blotting; however, this technique provides limited quantitative measurements of processing and requires large amounts of protein, making it challenging to monitor PR cleavage reactions shortly after viruses are released from cells. We have developed a mass spectrometry assay that monitors unprocessed and processed peptides corresponding to multiple PR cleavage sites simultaneously and are assessing methods to monitor PR with nanogram or lower input quantities to quantify processing in small amounts of virus shortly after release from cells. This assay will provide critical information about HIV PR activation in intact viruses.

Preliminary Data

HIV-1 PR cuts viral polyproteins at 11 canonical cleavage sites that are not processed by LysC and trypsin. In a previous study, we identified peptides spanning cleavage sites that are not cut by PR (unprocessed peptides) and peptides that are cut (processed peptides). These data raised the possibility that we could develop a sensitive assay to monitor HIV PR activity across many or all of the 11 canonical PR cleavage sites simultaneously, generating a quantitative and comprehensive picture of HIV PR processing under physiological conditions. We performed DDA MS on purified, concentrated HIV-1 particles. Using LC-MS/MS with LysC digestion, we were able to detect processed and unprocessed peptides corresponding to 8 of the 11 cleavage sites, a dramatic improvement over western blot methods. For the remaining 3 sites, we detected a single peptide (either processor or unprocessed) but not both; with further method optimization we expect to increase the number of sites that can be simultaneously detected. Next, we sought to minimize the amount of protein input required in order to understand the earliest steps in PR processing of the viral polyproteins. We tested several lysis and purification strategies – including filter-aided sample preparation (FASP), urea lysis, and acetone precipitation – at 1:10, 1:100, and 1:1000 dilutions of viral input and observed that certain peptides were preferentially retained with different methods. The most robust method was acetone precipitation and we were able to detect peptides corresponding to 8, 7, and 6 of the

11 canonical sites in the increasingly dilute samples. Finally, to validate the assay, we generated virus in the presence or absence of the HIV PR inhibitor saquinavir and found a complete loss of processing in the presence of drug, as expected.

Novel Aspect

We are developing a SRM-MS assay to monitor HIV protease activity simultaneously across multiple cleavage sites in intact viruses.

102. Bhairavi Tolani

Use of Mass Spectrometry for Cancer Therapeutics

Introduction

The emergence of cutting-edge mass spectrometric tools has helped elucidate the Mechanism of Action (MoA) of small molecules. One experiment can concurrently identify which proteins are up- and down-regulated at the whole proteome level. Our lab identifies small molecules as potential drug candidates for lung cancer. However, while these molecules exhibit highly potent anti-tumor effects, their MoA remains unknown. Identification of relevant cellular targets and their associated MoA has been a critical barrier to exploiting small molecules with therapeutic potential from being developed into approved drugs for patient benefit.

Methods

To identify the unknown (MoA) of our family of compounds, I performed an initial proteomic screen of molecule-treated tumor cells using quantitative, stable isotope labeling with amino acids (SILAC)-based mass spectrometry. We anticipate that this experiment will provide mechanistic and functional insight into the MoA of anti-tumor activity for this family of compounds.

Preliminary Data

From this screen, I identified two top hits upon molecule treatment, ATP6V1E1 and LAMTOR1, proteins involved in autophagic lysosomal degradation and endosomal sorting in cancer cells. Both ATP6E1V1 and/ LAMTOR1 proteins were strongly downregulated in response to treatment, indicating potential roles in the MoA of said molecules. In several cancers, resistance to key oncogenic drivers is mediated in part by upregulation of autophagic-lysosomal activity. Thus, development of lysosome-based inhibitors that can turn off this resistance could serve as therapies for multiple cancer types.

Novel Aspect

I propose a potentially useful and broadly applicable approach to MoA identification of small-molecules.

103. Jose Victorino

Ssu72-L84F Disrupts RNAPII Termination and Outlines Modules of CPF

Whitney R. Smith-Kinnaman, Neil McCracken, Samuel Ogunsanya[1], Rachel A. Chan, Melanie Fox, Hongyu Gao, Yunlong Liu, Amber L. Mosley

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana

Introduction

Polyadenylation dependent transcription termination is dependent on the Cleavage and Polyadenylation Factor complex (CPF) which is essential for the termination and processing of mature RNA. The disruption of the polyadenylation dependent pathways can impact expression of nearby genes, both protein coding and non-coding. Ssu72 is a phosphatase that is an essential member of the CPF complex and that regulates phosphorylation levels of the largest subunit of RNAPII referred to as the C-terminal domain (CTD), and acts as a docking site for transcription regulatory proteins. The role of Ssu72 in the regulation of termination is unknown. We propose that Ssu72 regulates recruitment of proteins to the transcription machinery as well as to the CPF complex.

Methods

Our studies are focused on a Ssu72-L84F mutant yeast strain which shows evidence of termination abnormalities. In order to study RNAPII localization, we performed chromatin immunoprecipitation followed by exonuclease treatment (ChIP-exo), a single nucleotide resolution ChIP assay, with a Ssu72-L84F yeast strain. We performed total RNA sequencing to analyze the mutant's effect on the transcriptome. Analysis of defective termination was done by measuring length of RNA transcripts via Northern blots. Protein-protein interactions where assessed by purifying CPF complex members and performing multidimensional peptide separation in combination with tandem mass spectrometry.

Preliminary Data

Our results show many genes including mRNA encoding genes had differential RNAPII occupancy as a result of the Ssu72-L84F mutation. Global studies highlight the read-through effects of ncRNA as well as mRNA genes. Read-through with the Ssu72-L84F mutant was confirmed with northern blot analysis and RNA sequencing analysis. We show that the levels RNAPII CTD phosphorylation are decreased in Ssu72-L84F mutants by western blot, implying that Ssu72-L84F confers a gain of function. Our proteomic studies have identified differential protein-protein interaction networks in the CPF complex in the Ssu72-L84F mutant.

Novel Aspect

Increased Ssu72 activity alters noncoding and protein coding RNA production and transcription termination of RNAPII.

104. Jinlong Wang

PABPs regulate plant immunity through association with R-motifs

Guoyong Xu[1], Duke University.
George H. Greene[1], Duke University.
Heejin Yoo, Duke University.

Introduction and Methods

Translation initiation in most eukaryotes is initiated by recruiting 40S small ribosome subunit and its associated initiation factors (eIFs) to the 5-prime 7-methylguanosine (m7G) cap structure, followed by 40S ribosome scanning and 80S ribosome assembling. Through interacting with a trimeric eIF4F cap-binding complex, Poly(A) binding proteins (PABPs) on poly(A) tail promote circularization of an mRNA, thereby facilitate protein synthesis.

Preliminary Data

We found that translation is tightly regulated during pattern-triggered immunity. Further investigation revealed that R-motifs (an element contains almost exclusively purines) locating in the 5' leader sequences of transcripts with increased translational efficiency play an essential role in regulating translation during pattern-triggered immunity through association with PABPs. After mutating PABs, Arabidopsis plants were more resistant to bacterial infection in mock treatment, indicating that PABs are negative regulators in plant basal resistance. Interestingly, after recognizing pathogen-associated molecular elf18, PABPs switch their functions from repression to activation. Moreover, both are dependent on R-motifs. Now, we are focusing on elucidating the regulatory mechanisms of R-motifs and understanding the roles of PABPs in different translation mechanisms.

Novel Aspect

Translation regulation in plant immunity and the switch of PABP function.

105. Yu Wang

Analytical Strategy for Bacterial Metabolites Identification by MS Technologies

Introduction

The small metabolites generated from bacteria normally include a broad category of chemical ingredients, from volatiles to non-volatiles and from polar to non-polar compounds. With rapid MS technology development, theoretically, most bacterial metabolites can be identified. In personal care industry, besides common non-volatile metabolites, the study of malodor and beneficial aroma ingredients from bacteria is a very interesting aspect. From our routine job of metabolites identification with LC-MS technology, we realized volatiles can't be identified because they could be evaporated at ESI or APCI interface. Meanwhile, the identification of metabolites from multiple bacteria species become more challenging. This is why we developed this analytical strategy to combine solid-phase microextraction (SPME)-GC in headspace (HS) mode, MSTFA chemical derivatization, and high-resolution LC-MS to lead to a reasonable conclusion.

Methods

GC analysis was carried out with an Agilent 6890N GC system fitted with a SLBTM-5ms capillary column (30 m x 0.25 mm I.D. x 0.25 µm film thickness) from Supelco, which is linked between a Gerstel autosampler MPS2 with SPME device and an Agilent 5975 single quadruple MS detector. LC-MS analyses were performed with Thermo Q-Exactive and Sciex Triple Quad 4500 system. HILIC and C18 columns were the main separation tools for metabolites characterization. The derivatization reagent, N-methyl-N-TMS-trifluoroacetamide (MSTFA), was purchased from Resteck. The bacteria

samples were run with SPME first for volatile metabolites and then dried with Savant SPD2010 SpeedVac concentrator. The dried sample then reacted with MSTFA for GC-MS and NIST library analysis, from which the results were applied for LC-MS characterization.

Preliminary Data

In the beginning, both bacterial metabolites (bacteria name is confidential here) and broth media samples were analyzed by SPME-GC-MS in HS mode. Volatile compounds such as 2-amino-1-propanol, acetic acid, isovaleryl, methyl glyoxal, isobutyric acid, butanoic acid and nonanone were identified from metabolites samples by comparison with broth media sample. Both samples (1 mL each) were then dried using Thermo SpeedVac concentrator at 45 °C for 1 hour. MSTFA reagent was added into the dried sample vials (1 mL each) and mixed well while shaking. The sealed vials were put into an 80 °C oven to react for another 1 hour. The corresponding trimethylsilyl derivatives of most polar metabolites would be formed. The reacted solutions were filtrated with 0.2 μm PTFE filter for liquid GC-MS analysis. The metabolites structures can be proposed according to NIST library. For LC-MS identification, same broth and metabolite solutions were diluted 10 times in methanol without MSTFA derivatization. The clear solutions were filtrated with 0.2 μm PTFE filter for a SeQuant ZIC cHILIC column separation and MS detection. The mobile phase was composed of A and B. A is 100% water and B is 90% ACN plus 10% water, both containing 10 mM NH4F and 0.2% FA. An elution gradient from 5% to 35% A within 30 minutes was applied to separate metabolites in both positive and negative modes on MS detectors and most metabolites such as lactic acid, citric acid, rabitol, glycolic acid and more than ten essential amino acids from GC-MS prediction were confirmed either in negative or positive detections. The most important metabolites were further confirmed in LC-MS by the chemical standards.

Novel Aspect

Combination of SPME, MSTFA derivatization, GC-MS, NIST library and LC-MS provided a systematic tool to fully identify metabolites.

106. Pamela Westmark

Identification of Proteomic Biomarkers in Response to Soy-Based Diets

Pamela R. Westmark Department of Neurology, University of Wisconsin-Madison
Jenna A. Loewus Department of Neurology, University of Wisconsin-Madison
Qinying Yu School of Pharmacy, University of Wisconsin-Madison
Min Ma School of Pharmacy, University of Wisconsin-Madison
David W. Nelson Department of Nutritional Sciences, University of Wisconsin-Madison
Chi-Liang “Eric” Yen Department of Nutritional Sciences, University of Wisconsin-Madison
Lingjun Li School of Pharmacy, University of Wisconsin-Madison
Cara J. Westmark Department of Neurology, University of Wisconsin-Madison

Introduction

Soy-based diets greatly affects seizure propensity, weight gain and behavior in Fmr1KO mice, a rodent model for fragile X syndrome (FXS). FXS is a genetic disorder that causes developmental problems such as learning disabilities, cognitive impairment and autistic behaviors. We hypothesize that soy-based diets are contributing to the childhood obesity epidemic and exacerbating

neurological phenotypes in genetically vulnerable populations such as children with FXS. There is a dearth of knowledge regarding the molecular signature of soy consumption or the resulting neurological and metabolic phenotypes. Herein, we present our most recent findings and describe future experiments to identify and validate metabolic phenotypes and proteomic profiles that change in response to dietary consumption of single-source soy-based diets.

Methods

Anthropometric measurements (weight, length, percent body fat and bone density) are being used to ascertain differences in growth as a function of diet (exactly matched purified ingredient diets differing in the protein source, casein versus soy protein) in male and female WT and Fmr1KO mice. Energy expenditure is being measured by indirect calorimetry. Blood biochemistry will quantitate cholesterol, triglyceride, glucose and insulin levels. Behavior is being assessed by actigraphy, rotarod and passive avoidance tasks. Proteomic profiles in brain are being generated by MALDI-MSI.

Preliminary Data

Juvenile mice fed a soy-based chow throughout gestation and postnatal development exhibit increased body weight compared to mice fed a casein-based purified ingredient diet. The effects are more pronounced in Fmr1KO mice. Adolescent WT and Fmr1KO mice weaned onto soy-based infant formula diet exhibit increased weight gain compared to mice weaned onto a casein-based infant formula diet. Adult Fmr1KO mice transferred to soy infant formula diet also exhibit excessive weight gain. Thus, at the systems level, our findings indicate that the consumption of single-source soy-based diets increases weight gain in mice irrespective of age with the most pronounced effects in juvenile Fmr1KO mice. A recently funded R01 proposes to: (1) determine the effects of soy protein consumption on metabolic phenotypes (monitoring anthropometrics, energy expenditure, biochemistry, activity, hematology and necropsy phenotypes) in wild type and Fmr1KO mice; (2) identify and quantitate proteomic profiles in response to soy protein consumption by proteomic mass spectrometry analysis and in situ labeling of MALDI tissue sampling; and (3) validate soy responsive molecular biomarkers by western blot and ELISA. Preliminary findings indicate increased body weight and lean mass in response to the soy-based purified ingredient diet in young adult mice.

Novel Aspect

Because many infants consume soy formula and many vivariums use soy-based chows, it is critical to identify associated molecular biomarkers.

107. Khadija Wilson

Understanding epigenome and proteome remodeling caused by novel germline histone H3.3 mutations during neurodevelopment

Khadija D Wilson[1]; Geoffrey P. Dann[1]; Elizabeth J. Bhoj[2]; Hakon H Hakonarson[2]; Benjamin A. Garcia[1]
[1]University of Pennsylvania, Philadelphia, PA;
[2]Children's Hospital of Philadelphia, Philadelphia, PA

Introduction

Histone H3.3 (H3.3), a histone variant, is often found at actively transcribed loci. H3.3 plays a role in cellular inheritance as ablation of H3.3 expression leads to loss of active gene states and dysfunction of heterochromatin telomeric structures. H3F3A and H3F3B, the two genes known to encode H3.3, are expressed in all human cells with higher expression in the gonads and brain. Recently, our collaborators discovered novel germline mutations in both H3F3 genes in a small cohort of patients who suffer from a common pattern of neurodevelopmental disorders. We aim to investigate the role of the mutant H3.3 (mH3.3) in the regulatory capacity of H3.3 containing nucleosomes.

Methods

Stable murine astrocyte cell lines were developed expressing either wild type or mH3.3 (G90R, T45I) containing 3xFLAG constructs using lentiviral transfection. Global histone post translational modifications (PTMs) were then quantified by analysis of histones from these astrocytic cell lines by liquid chromatography mass spectrometry (nano-LC MS/MS). To determine local PTM changes caused by these mutations, FLAG immunoprecipitation was performed followed by quantification of the isolated mH3.3 using nano-LC MS/MS. Investigation of proteomic changes due to mH3.3 was analyzed using bottom-up proteomic analysis.

Preliminary Data

Results show modest changes to the global histone PTMs as compared to wild type, however, significant decrease in H3.3 K27me3 was seen in both mutant lines. These results suggest that unlike somatic mutations of the H3F3 genes seen in glioblastomas and chrondoblastomas these mutants do not have a dominant negative effect and more likely exhibit local changes on mH3.3 containing nucleosomes. Proteomic analysis identified a downregulation of chromatin remodeler proteins like Eed, Kdm6a, and SSu72 in the G90R mutant as well as an upregulation of H2B variants. Interestingly, a few proteins appeared to be downregulated in both mutants suggesting similar regulation of these mH3.3 containing nucleosomes in conjunction to an upregulation on translocase mitochondrial proteins. Nevertheless, further studies involve identifying the deposition regions in the genome of these mutants which would shed a light towards the changes of the regulatory capacity of these H3.3 containing nucleosomes.

Novel Aspect

Understanding the basic biology of these mutations will shed light into the molecular mechanisms of H3.3 in neurodevelopment.

108. Nate Wlodarchak

Using plasma induced modification of biomolecules to assess changes in exposed protein surface with inhibitor bound kinases

Nathan Wlodarchak, UW-Madison
Ben Minkoff, UW-Madison
Michael Sussman, UW-Madison
Rob Striker, UW-Madison & William F. Middleton Veterans Memorial Hospital.

Introduction

Inhibiting specific protein kinase activity can treat many diseases including several cancers and opportunistic infections. Kinases undergo dynamic local conformational changes both in isolation and with other protein or ligand binding partners. These conformational changes are critical to create an ideal fit and improve specificity. When available, a kinase-inhibitor structure guides inhibitor modifications, but elucidating multiple inhibitor-bound structures is often prohibitive. "Protein footprinting" using mass spectrometry can rapidly and economically provide information on structural changes associated with several inhibitors, improving structure-guided drug development. The goal of this research is to use a novel structural biology technique, Plasma Induced Modification of Biomolecules (PLIMB), to measure changes in conformation on the surface of kinase drug targets when bound to structurally distinct inhibitors.

Methods

The mycobacterial kinase PknB was used to assess surface changes in the presence and absence of a known inhibitor. The protein, plus or minus inhibitor, was exposed to cold atmospheric plasma to generate hydroxyl radicals from bulk water and footprint the protein. The protein was digested with trypsin and analyzed using MS/MS in an Orbitrap. Raw data was searched for oxidation falling on any residue and quantified using precursor mass extracted ion chromatograms (EICs). Percentage oxidation was thus calculated as the sum of the areas under the EIC of oxidized peptides over all identified variants of the respective peptides. Differences from the apo and inhibitor bound samples were statistically analyzed in excel using two-tailed and unpaired t-tests with equal variance.

Preliminary Data

We performed initial experiments with PLIMB on PknB with and without a known inhibitor, GSK690693, for which we previously solved a co-crystal structure. I confirmed activity in the non-reductive buffer and we did initial PLIMB tests to check for protein stability. The protein was stable with plasma treatment and we were able to obtain > 95% sequence coverage on MS. After confirming stability, we tested PknB in the presence or absence of GSK690693 to assess areas of movement. The results revealed increased oxidation of areas known to be solvent exposed, such as the activation loop; however, these areas are quickly saturated and changes between inhibitor bound and apo forms may still occur on shorter timescales that we cannot assess. We found an increase in solvent exposed residues on the N-terminal region of the C-helix when bound to GSK690693 corresponding to amino acids 42-60. Although there is no apo PknB structure available, these results are consistent with changes seen in inhibitor bound PknB structures, and the apo form of Cdk2. This preliminary data shows that this technique can assess small changes in solvent exposure of PknB regions upon ligand binding, broadening the use of the technique to more novel targets.

Novel Aspect

This is the first application of protein footprinting and PLIMB to bacterial kinases.

109. Yuan Xu

Establishing and applying mass spectrometric tools to measure levels and 13C-labeling kinetics of metabolites in Camelina sativa leaves and seeds

Bibin Paulose; Hesham Abdullah; Danny Schnell; Yair Shachar-Hill
Michigan State University, East Lansing, MI

Introduction

The oilseed crop plant Camelina sativa shows considerable promise as a dedicated industrial oilseed crop with advantages of low agronomic inputs and natural resistance to biotic and abiotic stresses. The major limitation for Camelina as a commercially viable industrial oilseed crop is its modest seed yield. We aim to substantially increase the Camelina's yields by understanding and improving photosynthetic CO2 capture and conversion to triacylglycerols (TAGs) in wild type and transgenic plants. We established and applied a mass spectrometric method to track carbon assimilation in Camelina sativa wild type and a transgenic line with higher productivity using time course labelling with 13CO2.

Methods

Camelina sativa plants were grown under 500 µmol m-2 s-1, temperatures of 22/20°C, and 50% relative humidity in 16/8-daylight for 4 weeks. Individual Camelina leaves were labeled in a LI-6800 Portable Photosynthesis System with gas mixture prepared by mass controllers containing 13CO2 (Sigma) at a 13CO2/N2/O2 ratio of 0.033/78/21.967. Samples were collected 0, 30, 90, and 180 second of labelling followed by rapid quenching with liquid nitrogen. Seeds were harvested 17 days after pollination (DAP). Metabolite extraction, LC-MS/MS, and GC-MS methods were modified from Arrivault et al., 2009, and used with Camelina sativa leaves and developing seeds to quantify levels and labeling in sugar phosphates, amino acids and organic acids.

Preliminary Data

We established 13CO2 time course labeling and mass spectrometric tools to measure labeling in, and levels of, metabolic intermediates in mature (source) leaves in wild type and transgenic Camelina sativa to understand carbon capture through the Calvin – Benson cycle (CBC) pathway and carbon distribution in related pools. The levels and labeling kinetics of glyceraldehyde-3-phosphate (GAP), dihydroxyacetone-phosphate (DHAP), glycerate-3-phosphate (3PGA), erythrose-4-phosphate (E4P), ribose-5-phosphate (R5P), xylulose-5-phosphate (X5P), ribulose-5-phosphate (Ru5P), glucose-6-phosphate (G6P), frutose-6-phosphate (F6P), 6-phosphogluconate (6PG), sedoheptulose-7-phosphate (S7P), ribulose-1,5-bisphosphate (RuBP), fructose-1,6-bisphosphate (FBP), glycolate, glycine, serine, and other amino- and organic- acids of central metabolism were measured. Levels of most, but not all CBC and photorespiration pathway intermediates in leaves were similar to those reported in Arabidopsis; developing seeds showed elevated levels of 6-phosphogluconate.

Novel Aspect

Levels and 13C labeling kinetics of central metabolites in wild type and transgenic Camelina sativa leaves and seeds were measured.

110. Katharina Yandrofski

Size and Mass Heterogeneity of the NISTmAb: From Reference Values to Reference Cell Line

Lila Kashi; NIST, Rockville, MD
Zvi Kelman; NIST, Rockville, MD
John Schiel; NIST, Rockville, MD

Introduction

The NISTmAb IgG1k is a NIST Reference Material (RM 8671) that embodies the quality and characteristics of a biopharmaceutical product, is widely available to the biopharmaceutical community, and is an open innovation tool for development and dissemination of results. It is intended to provide a well characterized, longitudinally available test material that is expected to greatly facilitate development of originator and follow-on biologics for the foreseeable future. Size exclusion chromatography and intact/middle-down MS were optimized for characterization of the NISTmAb. These methods are now being implemented for characterization and similarity assessment of prototype non-originator NISTmAb materials.

Methods

The NIST monoclonal antibody Reference Material (RM 8671) is an IgG1k intended to serve as an open innovation test case for development of innovative product characterization strategies. Extension of these principles to development of non-originator materials was pursued through the heterologous NS0 expression of material comprising the same constituent amino acid sequence. All materials were characterized using size exclusion chromatography, intact mass spectrometry, and related MS-based techniques.

Preliminary Data

The NISTmAb has been shown to contain modifications common to this pharmaceutical class including N-terminal pyroglutamination, C-terminal lysine clipping, and glycosylation of the heavy chains. LC-MS-based characterization of the first non-originator batches revealed a relatively high degree of sameness, however deviations from the molecular profile were identified. An increase in high molecular weight species was observed via size exclusion chromatography as well as an increase in heterogeneity observed during intact mass spectrometry analysis.

Novel Aspect

First characterization of pre-competitive non-originator monoclonal antibody test case.

111. Montwaun Young

Multimodal Chemical Imaging Approach Based on Optical and Mass Spectrometries to Simultaneously Generate High-Resolution Element- and Molecule- Specific Chemical Images

Jessica R. Hellinger, Sunil P. Badal, Garett M. MacLean, Jacob T. Shelley
Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute

Introduction

The functionality and roles of materials, both naturally occurring and manufactured, depend on the composition and spatial distribution of chemical species within a sample. With recent advances in analytical techniques, the ability to generate high-resolution chemical images, which provides a two-dimensional chemical map of a sample's composition has gained popularity. However, to provide unambiguous analyte identification, multiple analytical techniques are required to analyze a sample, which provides complementary physical and chemical information, termed 'multimodal imaging'. Although tandem imaging techniques offer an abundance of information, they suffer from poor chemical specificity, extensive sample preparation, compromised selectivity, and the need for a high-vacuum environment depending on the spectroscopic technique is employed. Here we demonstrate an approach towards simultaneous elemental and molecular images.

Methods

The multimodal approach is achieved through laser sampling of solid samples followed by simultaneous optical and mass spectrometric analysis. During laser ablation events, a laser-induced plasma (LIP) is formed on the surface of the sample and the light emitted from the plasma can be measured via fiber optic cable coupled with a 6-channel spectrometer. This chemical information reveals the elemental composition of the ablated sample. At the same time, tiny particles are ejected from the sample surface and transferred by a stream of helium gas to the afterglow of the flowing atmospheric-pressure afterglow (FAPA) ionization source and mass analyzed with a Q-Exactive Orbitrap mass analyzer. After the data has been processed, elemental and molecular chemical images can be constructed.

Preliminary Data

Mass spectrometry imaging provides highly specific molecular information with accurate and high-resolution mass analysis. The data is processed and presented as 2-dimensional map with a false color scale representing specific ions. Resolution is affected by aerosol transport efficiency and laser spot size. The spatial resolution for LIBS is dictated by the laser spot size (≤15μm). We show proof-of-concept for the generation of simultaneous elemental and molecular images at high-resolution (≤40μm). Additionally, by laser drilling in a fixed lateral position, a depth profile of the sample can be obtained revealing the composition of the sample as a function of depth. This multimodal chemical imaging approach is capable of providing simultaneous element- and molecule--specific chemical images from the exact same location at high resolution. Analytical figures-of-merit and approaches for simultaneous elemental and molecular information from depth resolution experiments will be presented.

Novel Aspect

The demonstration of simultaneous elemental and molecular chemical information with images at high-resolution and depth information.

112. Stephen Zambrzycki

High-Throughput MALDI-TOF Stem Cell Quality Assurance

Gilad Doron (Department of Biomedical Engineering, Georgia Institute of Technology)
Dr. Johnna Temenoff (Department of Biomedical Engineering, Georgia Institute of Technology)

Dr. Facundo M. Fernández (School of Chemistry and Biochemistry, Georgia Institute of Technology)

Introduction

The emerging field of stem cell (SC) therapeutics has shown significant promise in regenerative medicine. Many SC treatments are approved for investigational use, and a Crohn's disease SC treatment has been fully approved in Europe. As with small molecule medicines (e.g. anti-infectives), SCs can also suffer from quality issues. Degradation due to improper transport and storage conditions, lack of sterility, and poor manufacturing practices affect SC quality to various degrees, endangering patients and reducing the public's confidence in these emerging therapies. Falsification, although not yet reported for SCs, could also become a problem in the future. Our study evaluates the possibility of using high-throughput MALDI to rapidly assess the quality of SCs in pre- or post-market surveillance scenarios.

Methods

The MALDI mass spectrometer used in this study was a Bruker rapifleX with a spatial resolution of 50x50 µm2, mass resolution of 20,000 FWHM at m/z 800, and a repetition rate of 50 pixels/s. Samples were deposited onto a polished steel MALDI target plate via the dried droplet technique. Two matrices were evaluated for their effectiveness at yielding information-rich SC metabolomic profiles: α-cyano-4-hydroxycinnamic acid (HCCA) and 2,5-dihydroxybenzoic acid (DHB). The two primary analytes used were 1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine (18:0 lyso PC), and a sample of mesenchymal SCs suspended and frozen in phosphate buffer solution.

Preliminary Data

First, 18:0 lyso PC was used to optimize the various parameters of the rapifleX mass spectrometer, which included the diameter of the sample area tested, laser shot frequency, laser power, number of raster shots at a single point, and the total shot count collected and averaged. The percent relative standard deviation for 18:0 lyso PC ion abundances for each measurement was used as the metric for reproducibility and to select optimal instrument parameters. Once optimized, the instrument and sample/matrix preparation protocol were streamlined to enable quantitation and as a means to monitor cell count. A calibration curve for 18:0 lyso PC was tested from 0 µM to 25 µM in 5 µM increments. Using linear regression, the HCCA matrix yielded an R^2 of 0.9913, and the DHB matrix resulted in a R^2 0.9321 for the [M+H]+ species at m/z 524.37. Following these experiments each matrix was tested with SCs. At a concentration of 2.5*10^6 cells/mL, DHB showed significantly more features than HCCA. The primary features were located between 700 m/z and 900 m/z in positive mode. Using one of the features (782.57 m/z), we tested two calibration ranges. One concentration range was from 0 to 5*10^6 cells/mL and another from 0 to 1.5*10^6 cells/mL. We were able to obtain R^2 values of 0.9117 and 0.8785 for a linear regression based on the analyte peak area, respectively; however, the confidence intervals for each data point were still relatively large. To minimize the confidence intervals, further refinement of our sample preparation protocol prior to testing and the inclusion of an internal standard will be investigated. After further refinement, other varieties of SCs, and controlled contamination experiments will be pursued to determine the extent by which MALDI can accurately predict SC quality.

Novel Aspect

Application of MALDI MS metabolomics to biotype and ensure the quality of the stem cells.

113. Zhenbin Zhang

Filter aided, single tip based (FAST) method for high throughput, ultrasensitive proteomics analysis

Norman J Dovichi, University of Notre Dame

Introduction

One of the major goals of proteomic research is to be able to monitor all proteins in a particular biological system. However, the behavior of cells in a population cannot always be reliably approximated by averaging the population results. The performance of traditional workflows in proteomic experiments drops steeply when sub microgram of starting materials are available due to inefficiencies related to sample processing and instrument sensitivity. These drawbacks have largely precluded the use of proteomics in applications where high reproducibility, sensitivity, and throughput are necessary, such as in clinical studies or population screening. To address these challenges, here we introduce a novel protocol using filter aided, single tip (FAST) method.

Methods

The FAST microreactor is fabricated in a 20 μL pipette tip. Cell lysate was loaded onto the microreactor with centrifugation at 600-10 000x g. Then 5 μL of 20 mM NH4HCO3 was loaded onto the microreactor for washing away the detergents and salts. Finally, the trypsin was loaded onto the microreactor at 200x for 30 seconds. The microractor was put in a sealed 1.5 mL centrifuge tube and placed in 37 °C water bath for overnight. After digestion, the digest on the filter was directly eluted into the insert vial for LC-ESI-MS/MS analysis

Preliminary Data

30377 (n=2, RSD=3.9%) unique peptides and 3487 (n=2, RSD=1.4%) protein groups were identified from 1 μg of K562 cell lysate by FAST method. In contrast, only 2142(n=2, RSD=56%) unique peptides and 821 (n=2, RSD=37%) protein groups were identified from 1 μg of starting material by filter aided, sample preparation (FASP) method and the reproducibility was not satisfied. The FAST method was also applied to single cell analysis, 20943 unique peptides and 2597 protein groups were identified from single blastomere dissected from 50-cell stage xenopus laevis embryo.

Novel Aspect

The FAST method retained the merits of FASP method but with greatly improved sample recovery and reproducibility when processing sub microgram of starting material.

114. Katharina Zittlau

A proteomic approach to study the mitochondrial outer membrane proteome

Prof. Dr. Boris Maček (Interfaculty Institute for Cell Biology)

Introduction

The MOM is the place of action for many cellular processes such as mitophagy and apoptosis. Impairments of these processes have been linked to severe disorders such as Parkinson's or

Alzheimer's disease. Aberrations in the mitochondrial serine/threonine-protein kinase PINK1 or the E3-ubiquitin-protein ligase PARKIN are linked to these diseases. Impairments of the apoptotic pathway in which MOM permeabilization plays a central role and which is regulated by the Bcl-2 family are connected to Alzheimer's disease. Post-translational modifications such as phosphorylation and ubiquitination might play an important regulatory role during mitophagy and apoptosis. Despite its important role in Parkinson's and Alzheimer's disease the investigation of the MOM phospho- and ubiquitinome is still not complete.

Methods

The relatively low number of MOM proteins and their hydrophobic nature make investigation of the MOM proteome particularly challenging. In this project we will establish methods to investigate the MOM-proteome composition and its alterations during apoptosis and mitophagy. To this end, we will apply optimized purification protocols of mitochondria in combination with the localization of organelle proteins by several methods, including isotope tagging (LOPIT) to define the MOM proteome. By applying different peptide labeling strategies such as dimethyl labeling or tandem mass tag (TMT) and optimized ubiquitinylated-protein or phosphopeptide enrichment strategies we will analyze drug-dependent changes in the phosphoproteome and the Ubiquitinome over time. Furthermore, we will take advantage of the new Q Exactive™ HF-X Hybrid Quadrupole-Orbitrap™ Mass Spectrometer.

Preliminary Data

So far I was establishing a workflow for precise quantification of the MOM proteome and changes in the phosphoproteome and ubiquitinome. In order to reduce quantification bias due to proteins of organelles, which are not related to mitophagy or apoptosis, we compared different purification methods of mitochondria. Special emphasis was given to the following aspects: (1) Since some of the important players in mitophagy or apoptosis are only recruited to the MOM after stress induction, we aim for a purification method which does not generate highly purified mitochondria which lost contact to their environment; (2) With the prospect of subsequent enrichment for post-translationally modified peptides, which need high amounts of starting material we target a method giving high protein amounts; (3) In order to keep the time per isolation as short as possible to make the handling of many samples at once visible we finally decided to use the subcellular protein fractionation kit for cultured cells provided by Thermo Scientific™ which meets our first and second requirements, too.

We are currently optimizing different chemical labeling strategies in combination with highly specific phosphopeptide enrichment. During our first testing we identified more than 3,800 protein groups and more than 6,000 phosphorylation sites of samples containing mostly mitochondria and mitochondria associated proteins.

Novel Aspect

Our work will contribute to a better understanding about the MOM proteome and its special role in apoptosis and mitophagy.

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