POSTER CONTEST & SESSION

NOTE: Each submitter's name is in bold and italicized.

CONTEST FINALISTS

(1) Proteomic Analysis of Functional Inward Rectifier Potassium Channel (Kir) 2.1 Reveals Several Novel Phosphorylation Sites

Kyle A. Brown1,2, Corey Anderson3, Louise Reilly3, Kunal Sondhi3, Ying Ge4,5, Lee L. Eckhardt3
Department of Surgery1; Department of Chemistry2; Cellular and Molecular Arrhythmia Research Program, Division of Cardiovascular Medicine, Department of Medicine3; Department of Cell and Regenerative Biology4, Human Proteomics Program5, University of Wisconsin-Madison, WI, 53706, USA.
kbrown33@wisc.edu

Background: Membrane proteins represent a large family of proteins that perform vital physiological roles and represent key drug targets. Despite their importance, bioanalytical methods aiming to comprehensively characterize the post-translational modification (PTM) of membrane proteins remain challenging compared to other classes of proteins in part because of their inherent low expression and hydrophobicity. The inward rectifier potassium channel (Kir) 2.1 is crucial for the maintenance of the resting membrane potential and phase-3 repolarization of the cardiac action potential. Several sudden arrhythmic death syndromes including Andersen-Tawil and Short QT syndrome are associated with loss or gain of function mutations in Kir2.1 that are often triggered by changes in β-adrenergic tone. Thus, understanding the posttranslational modifications (PTMs) of this channel (particularly β-adrenergic driven phosphorylation) is important for arrhythmia prevention. Here, we employ proteomic analysis, top-down, middle-down, and bottom-up approaches, for comprehensive characterization of the PTMs of recombinant Kir2.1, resulting in the successful mapping of six novel sites of phosphorylation. In parallel, we utilized whole-cell patch-clamp analysis to verify the function of the channel. Together the proteomics and functional studies suggest that the overall level of phosphorylation may influence function rather than the specific site; however, further functional analysis is needed to access this trend. Our study provides a framework for future work to assess the role of PTMs in regulating Kir2.1 functions as well as other ion channels implicated in channelopathies.

(2) TOne-Pot Exosome Proteomics Enabled by a Photocleavable Surfactant

Kevin M. Buck, Kyle A. Brown, David S. Roberts, Timothy J. Aballo, David R. Inman, Suzanne M. Ponik, Ying Ge
kmbuck2@wisc.edu

Exosomes are nano-sized extracellular vesicles (EVs) containing nucleic acids, lipids, and proteins that are implicated in tumorigenesis, metastasis, and cardiac regeneration, serving as potentially useful biomarkers from fluids. Global mass spectrometry (MS)-based proteomics have been used to profile exosome cargo previously for diagnostic purposes. However generating data with significant coverage of exosome proteins often requires lengthy sample preparations and multi-dimensional chromatographic

separations. Hence we have developed a high-throughput exosome proteomics method enabled by our recently developed photocleavable surfactant 4-hexylphenylazosulfonate (Azo) for effective solubilization

and digestion of exosome proteins followed by one-dimensional LC-MS for highly reproducible and quantitative exosome proteomics. This method provides deep coverage of exosome proteins while avoiding lengthy sample preparation, separation, and digestion steps common in traditional workflows.

We identified 3466 protein groups in three replicates of mammary fibroblast-derived exosomes. Label-free quantification (LFQ) intensities were log2 normalized and matrices strictly filtered for valid LFQ values, with 2288 protein groups reliably quantified. These data had normally distributed LFQ intensities and high Pearson correlation coefficients (PCCs) indicating a highly quantitative and reproducible, label-free experiment. Additionally, this method captured approximately 45% of previously annotated exosomal proteins from the database ExoCarta and data were 91% overlapping with EV databases generally, demonstrating the deep coverage of a highly heterogenous proteome. Quantitative exosomal protein markers were detected with high abundance, including syntenin-1 and CD63. Further, STRING interaction network and gene ontology analysis showed densely connected networks of interactors, as well as cellular components confidently assigned to extracellular exosomes. With this method as the basis for future exosomal development, we envision its use in biomarker discovery and validation for precision disease treatment, detection, and monitoring.

(3) Structural Elucidation of Human Cardiac Troponin Complexes by Native Mass Spectrometry

Emily A. Chapman[1], Timothy N. Tiambeng[1], David S. Roberts[1], Jake A. Melby[1], Kyle A. Brown[1,2], Daniel Kim[1], Andrew Alpert[3], Song Jin[1], Ying Ge[1,4]

1. Depart. Of Chemistry, University of Wisconsin (UW)-Madison
2. Depart. Surgery, UW-Madison
3. PolyLC, Columbia, MD
4. Human Proteomics Program, UW-Madison

eachapman2@wisc.edu

The cardiac troponin complex (cTn; ~77 kDa) plays critical roles in cardiac contractility and is composed of three subunits (troponin I, T, C). Calcium ions (Ca^{2+}) bind to cTnC, the Ca^{2+}-binding sensor of cTn, inducing a conformational change of cTn that initiates muscle contraction. While alterations in cTn Ca^{2+} sensitivity and binding are associated with cardiac dysfunction, proteomics methods for characterizing endogenous cTn by native mass spectrometry (MS) remain challenging due to difficulties in the isolation and stability of cTn under non-denaturing conditions. Thus, we have developed a novel “native nanoproteomics” technique incorporating surface-functionalized nanoparticles and native MS-analysis to enrich and structurally characterize endogenous cTn complexes from human cardiac tissues to provide new insights into cTn structure and Ca^{2+} binding interactions.

Our native nanoproteomics technique coupled with native MS revealed distinct perturbations in cTn complex stoichiometry and Ca^{2+} binding interactions. To probe the role of Ca^{2+} in stabilizing the cTn complex, we introduced EGTA as a metallic ion chelator to remove bound Ca^{2+} ions from up to three occupied Ca^{2+} binding sites. cTnC with three Ca^{2+} bound within the cTn heterotrimeric complex were destabilized upon addition of EGTA (25 mM), with only the cTn (I-T-C) proteoforms consisting of doubly Ca^{2+}-bound cTnC retained. Surprisingly, excess EGTA (100 mM) was unable to dissociate or reduce the doubly bound Ca^{2+} proteoform in the cTn complex, implying that Ca^{2+} interactions cause a substantial difference in cTn structure. Additionally, native MS revealed that the binding affinities of Ca^{2+} is lower in free cTnC monomer compared to Ca^{2+} bound in cTn complexes suggesting that the Ca^{2+} dissociation rate from cTnC significantly slows upon addition of cTnI and cTnT.

Overall, these results uncovered the varying stoichiometry of non-covalent Ca^{2+} cofactors which regulate cardiac contractility in endogenous cTn monomers and trimers. This provides important foundation for future elucidation of structure-function relationships within human cardiac cTn complexes. Moreover, the structural elucidation of endogenous cTn complexes will provide the strongest connection between molecular mechanisms and disease for new precision medicine approaches.

(4) Isobaric Labeling of Phospholipids toward Multiplexed LC–MS/MS-Based Multiplexed Quantitative Analysis

Ting-Jia Gu1, Peng-Kai Liu2, Shuling Xu1, Lingjun Li1, 2
1School of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA
2Biophysics Program, University of Wisconsin-Madison, Madison, WI, USA
tgu338@wisc.edu

Phospholipids are essential biomolecules that are involved in many biological processes, including membrane fusion, apoptosis, and the regulation of membrane proteins. Disturbance in the homeostasis of lipids is associated with many diseases, such as cancer. To understand the roles of phospholipids in these disease progressions, it is desirable to measure the changes in phospholipids during the progressions. However, the studies of quantification in high-throughput manners were still limited, and the current methods were not able to target all phospholipid classes simultaneously due to the high structural diversity of phospholipids. Herein, we propose a novel two-step derivatization strategy to enable high-throughput quantification for 6 major phospholipid classes using isobaric labeling. First, we utilized diazo compounds which are able to react with phosphate groups on lipids and coupled with carbonyl groups to be specifically targeted by aminoxyTMT for multiplexed quantification. The conditions of derivatization with diazo compounds and aminoxyTMT were carefully optimized using different solvents, catalysts, and reagents to achieve maximum derivatization efficiency. Currently, the derivatization efficiency and labeling efficiency were able to reach more than 95% for each phospholipid class with minimum side reaction. To evaluate the quantitative performance and applicability on complex systems, 5 aliquots of phospholipids extracted from human cells were labeled with 5-plex aminoxyTMT respectively and pooled prior to MS analysis. Upon LC-MS/MS, the intensities of reporter ions exhibited an expected ratio 1:1:1:1:1 suggesting that our method is compatible with complex samples and showing an accurate quantification. This method features high labeling efficiency, quantitative accuracy, multiplexed quantification, and most importantly, simultaneous analysis of all major phospholipid classes, providing a powerful tool for comprehensively high-throughput phospholipid quantitative analysis. In the future, we plan to apply this method to human cell lipid extract from disease and control groups to discover the lipids that are relevant to specific diseases.

(5) Progranulin as a biomarker for disease progression in the Niemann-Pick type C1 I1061T mouse model

Wenping Li, Thu T. A. Nguyen, Stephanie M. Cologna
jklwp007@uic.edu

Introduction: Niemann-Pick Type C (NPC) is a fatal, and progressive cerebellar neurodegenerative disease. Mutations of either NPC1 or NPC2 lead to the accumulation of unesterified cholesterol and sphingolipids in the late endo/lysosomal system. To date, most comparative studies have utilized the Npc1-null mouse model, which recapitulates the human disease, however, does not accurately mimic the most common genetic mutation in patients. To address this, a recently developed mouse model containing a homozygous point mutation, I1061T, a most common and detected on 18% of NPC patient alleles, was developed. Here, we investigated the differential proteomic profile of this newly developed mouse model to understand retained differences from prior studies using the null model and to identify new protein markers of NPC disease.

Methods: Cerebellar tissue lysates (n=5 for each genotype) from 9-week-old control and NPC1I1061T mice were digested using the S-Trap method followed by TMT10plex labeling. The multiplexed sample was further fractionated into 20 fractions using high pH reversed- phase liquid chromatography. Data were acquired using nanoLC coupled to a Q-Exactive mass spectrometer and analyzed using SEQUEST via Proteome Discoverer software. Differential proteins were subject to pathway analysis using Ingenuity Pathway Analysis (IPA). Western blot and immunohistochemistry (IHC) were performed on control and NPC1I1061T mouse cerebella to validate proteomic findings and to investigate the potential biomarker for disease progression. The spinning-disc confocal microscopy (Nikon) is used to image the IHC brain slices. All western blot and IHC images were analyzed using ImageJ.

Preliminary Data: Cerebella from 9-week-old mice were used to study the altered proteome. In total, we observed 141 significantly upregulated proteins, including progranulin (PGRN), and 123 downregulated proteins in NPC1I1061T compared to control. The top three altered pathways in IPA included autophagy, phagosome maturation, and the complement system, which are in accordance with previous studies. In the current study, PGRN was chosen to be further studied due to its multiple roles in the brain, such as regulating lysosomal biogenesis, (anti-)inflammation properties, wound repair, and aging. The defect of PGRN was reported to be related to many neurodegenerative diseases, including neuronal ceroid lipofuscinosis, and frontotemporal lobar degeneration. In this study, the increased expression of PGRN in the whole cerebellum lysate was validated by western blot on 9-week-old mice. Furthermore, we observed the PGRN may be an NPC1 disease progression marker since PGRN was detected on 7-, 9-, and 15-week-old NPC1I1061T mice but not on 4-week-old mice, and the expression level increases with age.

To test whether PGRN level increases in the brain at the cellular level, IHC was performed on 4-, 9-, and 15-week-old mouse brain slices which represents a range of disease progression. The number of IBA1-positive microglia, representing activated microglia, was increased in 9- and 15-week-old NPC1I1061T mice. At the same time, the PGRN was upregulated in these activated microglia in NPC1I1061T mice, which is consistent with a previous report studied in Npc1-null mice. These results further indicates that PGRN can be a disease progression marker for NPC1 particularly from microglia. Interestingly, PGRN is downregulated in the Purkinje neurons only at 15-week-old NPC1I1061T mice, indicating that the upregulated PGRN we observed in MS and western blot was contributed by mainly microglia. Finally, to translate this observation in patients, CSF or blood samples will be used to measure PGRN expression level.

Novel aspect: This study reports progranulin as a disease progression marker in a neurodegenerative disease.

(6) Infrared photoactivation boosts sensitivity of quantitative single-cell proteomics

Trenton M. Peters-Clarke1,2, Kenneth W. Lee2, Keaton L. Mertz1, Yiran Liang3, Michael S. Westphall2,4, Ryan T. Kelly3, and Joshua J. Coon1,2,4,5*
1Department of Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA
2Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI, 53706, USA
3Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT, 84602, USA
4National Center for Quantitative Biology of Complex Systems, Madison, WI, 53706, USA
5Morgridge Institute for Research, Madison, WI, 53515
petersclarke@wisc.edu

Isobaric tagging facilitates multiplexed experiments that can determine sequences and relative amounts of peptides in biological samples using tandem mass spectrometry. Limited reporter ion generation limits quantitative accuracy and precision. Further, unintended co-isolation of contaminating peaks in MS2 experiments distorts reporter intensities. MS3 experiments address contamination by generating reporter ions via collisional activation (HCD) of peptide product ions rather than the precursor ion. Because HCD performance is related to m/z, activation of synchronously isolated products generates suboptimal reporter intensities. We supposed that a combination of IRMPD and ion parking would maximize reporter generation from tagged peptides by eliminating the m/z dependence inherent in HCD. We applied this method to single-cell mixtures to highlight quantitative sensitivity improvements for limited sample amounts.

One and ten cell TMT-18plex samples were prepared. For one cell samples, a carrier channel of 50 cells was used. We illustrate the inefficiency of HCD due when activating individual peptide fragments. When synchronously activating multiple precursors, as is the case with MS3-based quantitation, IRMPD with ion parking of reporter ions gives compounded benefits. For a yeast triple-knockout standard, we show that using IRMPD to generate reporter ions from ten synchronously isolated product ions results in a 2.4-fold increase in reporter intensities, significantly enhancing the sensitivity and dynamic range of quantitation via isobaric tagging. However, when lower peptide concentrations are analyzed, up to 5-fold gains are seen with IRMPD.

To further illustrate benefits of improved reporter ion yield with IRMPD, HeLa and A549 single-cell tryptic peptides were quantified at the MS3-level with HCD and IRMPD. HCD MS3 allowed for the detection of all 18 TMT channels for only 10% of its 16,233 precursors, whereas IRMPD SPS MS3 allowed for detection of all 18 TMT channels for 57% of its 16,233 precursors. Altogether, we identify similar number of peptides and proteins between methods, but the increase in sensitivity gains of our IRMPD method allows us to quantify many more peptides and proteins. We demonstrate that IRMPD boosts the number of single-cell quantified peptides by about 2-fold, without perturbing expected quantitative ratios.

(7) MALDI-MSI mapping of lipids in Niemann-Pick disease, type C: Exploring potential treatments

***Dominick Pierre-Jacques*, Nigina, Chandimal, Cristin Davidson, William J Pavan, Cologna**
dperr4@uic.edu

Introduction - Niemann Pick disease, type C (NPC) is a fatal, autosomal recessive, lysosomal storage disorder that is characterized by progressive cerebellar neurodegeneration. NPC is caused by mutations in either the NPC1 or NPC2 genes which result in their respective protein dysfunction and inability to traffic cholesterol out of the lysosome. Following cholesterol storage, a cascade of events occur which ultimately results in cell death. Currently there is no FDA-approved therapy for NPC disease. However, 2-hydroxypropyl-beta-cyclodextrin (HPBCD) and gene therapy strategies are currently being investigated. In the current work, we utilize MALDI mass spectrometry imaging to understand the lipid distribution profile of mice with NPC1 disease, as well as those treated with these experimental therapies.

Methods – The very well-characterized NPC1-null mouse model was used in these studies. HPBCD treatment was administered by intraperitoneal injection (4000 mg/kg HPBCD or PBS) every other day following weaning and until euthanasia (7 weeks). AAV9 gene therapy was administered via retro-orbital injection (1.21e11 vector genomes/mouse) at weaning and mice were euthanized at 10 weeks old. Brain tissue was harvested and flash frozen. The brains were sectioned in the sagittal direction to a 10µm thickness, and then thaw mounted onto a stainless steel MALDI plate. Matrix was applied using a home-built sublimation system onto the samples in a uniform coating. Lipids were imaged in both positive and negative mode from 500-1600m/z using a Sciex 4800 MALDI-TOF/TOF. All images were processed using MSiReader.

Preliminary data – Prior studies in multiple animal models have shown that lipids other than cholesterol are altered in NPC1 brain tissue. For example, bioactive sphingolipids such as ceramides and gangliosides have been reported as altered and have also shown unique spatial distributions in the cerebellum of mutant animals. In the current study, we further investigated these lipids owing to their involvement in signaling pathways and regulation of biological functions. For instance, ceramides are known to induce apoptotic cell death and are increased in NPC1 brain areas such as the cerebellum where a well-documented patterned loss of Purkinje cells occurs. Whether the neuronal cell death seen in NPC1 is caused by an accumulation of ceramides or an accumulation of ceramides is present because of the ongoing neuronal cell death is currently unknown. In MSI analysis of animals treated with HPβCD, we observe ceramide accumulation decreases significantly in the mutant brain while the drug induces no change in wild type mice. This effect is most striking in the cerebellum. Similar results are seen with gangliosides, GM2 and GM3, in the NPC1 brain. That is, untreated mutant animals have elevated ganglioside levels whereas HPBCD treatment appears to normalize ganglioside levels and distribution. Interestingly, ganglioside accumulation is also reduced in treated mutant animals across the brain with the exception of the caudate putamen region, an area associated with motor impairment disorders such as Parkinson's disease.

Currently, our work is focused on defining lipid distribution changes in mice that have received gene therapy treatment. This will give us the opportunity to see how different treatments affect lipid accumulation in NPC1 with particular focus on the central nervous system.

Novel aspect – This work highlights changes in lipid distribution within the brain of Npc1 mutant mice following therapeutic intervention.

(8) Informed Match Between Runs Improves Quantitative Confidence in Single Cell Proteomics

Alexander Solivais, Hannah Boekweg, Michael R. Shortreed, Samuel H. Payne, Lloyd M. Smith
solivais@wisc.edu

Single cell proteomics has potential to identify thousands of proteins per cell, advancing precision medicine by revealing information on cellular variation. Single cell interrogation of the proteome can reveal information about disease progression, local signaling environments, and the presence of post-translational modifications. However, analysis of the proteome at the single cell level pushes the limits of mass spectrometry. Limited amounts of protein and their corresponding peptides produce signal at the limit of sensitivity, resulting in missed identifications. To address this problem, we have modified the MetaMorpheus platform to employ a modified form of match between runs (MBR) that is able to recover peptide spectra that are missed during a traditional database search. Our informed approach to MBR uses spectral angle comparison to recover MS2 spectra that could not be confidently identified in the original search. In traditional MBR, intact mass measurements with no corresponding MS2 events are matched to "donors": runs where a peak with a similar mass and retention time was observed and fragmented, resulting in a confident MS2 identification. The traditional approach patches holes in the data, enabling quantification of peptides that would otherwise be missed but at the cost of increased uncertainty. In single cell proteomics experiments, almost every observable peak has an associated MS2 spectrum, albeit often one of low quality. Our modified algorithm rescues these lower quality spectra, yielding additional information that is leveraged to lower the false discovery rate and increase quantitative certainty. This informed match between runs approach will maximize the quantitative impact of single cell proteomics data and be made available to all researchers in the single cell proteomics community as a new feature in our open-source software.

(9) Multiomic Analyses of Pancreatic Islets via Laser Capture Microdissection-Mass Spectrometry and timsTOF fleX MALDI-2 Imaging

Dylan Nicholas Tabang1, Daniel M. Tremmel2, Zihui Li1, Sara Dutton Sackett2, Matthew T. Flowers3, Jon S. Odorico2, Dawn Belt Davis3,4, Lingjun Li1,5*
1Department of Chemistry, University of Wisconsin-Madison; 2Department of Surgery, Division of Transplantation, School of Medicine and Public Health, University of Wisconsin-Madison; 3Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Wisconsin-Madison; 4William S. Middleton Memorial Veterans Hospital, Madison, Wisconsin; 5School of Pharmacy, University of Wisconsin-Madison.
tabang@wisc.edu

Pancreatic islets are small cell clusters that produce hormones, like insulin. Islets comprise less than 3% of total pancreatic mass. In diabetes, islet dysfunction leads to characteristic high blood glucose with long-term complications. Enzymatic isolation and culturing of islets removes them from the surrounding extracellular matrix (ECM), potentially leading to major artifactual changes in their biomolecular profiles. To preserve native biomolecular profiles for multiomic analyses, laser capture microdissection (LCM) was used to collect islets from intact tissue. In combination with high-resolution mass spectrometry imaging (MSI), a biomolecular profile of pancreatic islets with increased analyte coverage and increased in vivo fidelity was obtained.

Proteomic and peptidomic analyses were performed after extraction in either detergent (for subsequent enzymatic digestion) or organic solvent (for intact hormones). For matrix-assisted laser desorption/ionization (MALDI) imaging, tissue sections were imaged using a timsTOF fleX MALDI-2 or rapifleX mass spectrometer.

Due to the small amount of protein from LCM samples, enzymatic digestion with trypsin was performed using suspension trapping. Using tissue from a single non-diabetic donor, 880 proteins were identified (67 from the ECM). Acidified methanol was used to extract intact peptide hormones from islets. From the same donor, 106 hormones were identified from many peptide hormone families. After tryptic digestion, 15 hormones were identified, including 6 glucagon isoforms and insulin B chain.

Subsequent future MALDI-MS imaging analyses from individual diabetic donors will seek to use the optimized multiomic methods to identify biological heterogeneity in the localization and originating cell

types of species identified using LC-MS/MS, as well as other analyte types like lipids and glycans, with signal enhancement afforded by ion mobility separation and MALDI post-ionization to determine biomolecular changes in diabetes. This work, for the first time, combines LC-MS/MS with MALDI-MSI for comprehensive analysis of pancreatic islets in their native environment.

(10) sn-position Resolved Quantification of Aminophospholipids by Isotopic N, N-dimethyl leucine Isobaric Labeling via High-resolution Ion Mobility Mass Spectrometry

Shuling Xu1, Zhijun Zhu 2, Ting-jia Gu1, and Lingjun Li1,2
1School of Pharmacy, 2Department of Chemistry, University of Wisconsin-Madison, WI, 53705
Sxu374@wisc.edu

The aminophospholipids (APLs), composed of phosphatidylethanolamine (PE) and phosphatidylserine (PS), are the main constituents of mammalian cell membranes and lipoproteins, displaying both structural and signaling functions. Dysfunctional metabolic processes have been linked to human diseases and can result in altered APL structural features, such as permutation of fatty acid connectivity (sn-positions). The unambiguous assignment and accurate quantification of APL sn-isomers by routine tandem mass spectrometry (MS/MS) continue to present a major challenge. In this study, we developed a novel method for sensitive discernment and accurate absolute quantification of APL sn-isomers using isotopic N, N-dimethyl leucine (iDiLeu) labeling coupled with high-resolution ion mobility mass spectrometry (IM-MS). Utilizing Agilent 6560 IM-QTOF platform with high resolution demultiplexing (HRdm) data processing, the IM resolution > 200 could be achieved. The sn-isomeric pairs of PE16:0_18:1(9Z) and PS16:0_18:1(9Z) with small structural differences (ΔCCS less than 1%) could be all very well separated in IM dimension. Furthermore, derivatizing the primary amine of APLs with iDiLeu could further increase the sensitivity without diminishing the IM discrimination of sn-position. Through 5-plex iDiLeu labeling strategy, by which APLs in samples were labeled to the first channel followed by a four-point standard curve, APL sn-isomers priorly resolved by high-resolution IM were absolutely quantified. Through the investigation of mouse cortex tissues by this strategy, we discovered that both PE18:1(9Z)/16:0 and PE16:0/18:1(9Z) increased over aging for wild-type (WT) mice, but the contrary trend was observed for APP/PS1 Alzheimer's Disease (AD) model mice, indicating their strong correlation to AD progression. Incorporating both iDiLeu labeling and high-resolution IM, this strategy could unravel more detailed alteration of APL sn-isomers, which have long been considered as the "dark matters" from traditional lipidomics workflow, leading to more precise interpolation of molecular mechanisms of various diseases.

GENERAL POSTER SESSION

(11) Proteomics Perspective of Postnatal Swine Heart Development

Timothy J. Aballo[1], David S. Roberts[2], Elizabeth F. Bayne[2], Wuqiang Zhu[3], Gregory Walcott[3], Ahmed I. Mahmoud[1], Jianyi Zhang[3], Ying Ge[1],[2]
Department of Cell and Regenerative Biology[1], Department of Chemistry[2], University of Wisconsin-Madison, Madison, WI
Department of Biomedical Engineering[3], University of Alabama at Birmingham, Birmingham, AL
aballo@wisc.edu

Purpose: The neonatal swine heart possesses an ability to regenerate injured myocardium through the proliferation of pre-existing cardiomyocytes (CMs), but this regenerative capacity is lost shortly after birth. The mechanisms governing the proliferative capacity of CMs during this early postnatal stage are unknown; therefore, there is great need to define the proteomic landscape during postnatal development to identify regulators of this regenerative process.

Methods: Left ventricular (LV) tissue was isolated from swine at postnatal days (P) 1, 7, 28 and 56 (n=3). For top-down analysis, myofilament proteins were enriched using a differential pH extraction and analyzed by reversed phase liquid chromatography (RPLC) interfaced with a Q-TOF mass spectrometer. For bottom-up analysis, LV tissue was homogenized in Azo, and proteins were digested with trypsin prior to RPLC interfaced with the timsTOF Pro.

Results: Top-down proteomics of the sarcomere revealed a concerted transition of fetal sarcomeric proteoforms into their more mature counterparts. More specifically, we observed a marked decrease in

ssTnI expression, cTnT2 expression, and α-Tpm phosphorylation throughout postnatal cardiac development.

Global bottom-up proteomics quantified over 4,000 protein groups in the swine heart. We identified >900 differentially expressed proteins (adjusted $p<0.05$) and mapped these proteins to different biological processes, revealing a steep decrease in cardiomyocyte developmental processes and cell cycle activity and a sharp increase in metabolic proteins throughout development.

Conclusion: Using these methods, we comprehensively defined how the proteomic landscape of swine hearts changes throughout postnatal development. We identified significant alterations in sarcomere proteoform expression patterns, which may influence sarcomere disassembly, a process necessary for CM proliferation. Additionally, we mapped global changes in protein abundance to determine there was a significant increase in metabolic activity and a substantial decrease in processes related to developmental and cell cycle related processes. Overall, we will use these results to guide future investigations into swine heart regeneration.

(12) Multiomic Analysis of Human Pluripotent Stem Cell-Derived Cardiomyocytes for Discovery of Maturation Markers

Elizabeth F. Bayne1, Aaron D. Simmons2, Yanlong Zhu3,4, Melissa R. Pergande3,4, Austin Feeney2,5, Zhuoxin Shi3, Timothy J. Kamp3,5, Sean P. Palecek2, Ying Ge1,3,4

1Department of Chemistry, 2Department of Chemical and Biological Engineering, 3Department of Cell and Regenerative Biology, 4Human Proteomics Program, 5School of Medicine and Public Health, University of Wisconsin – Madison

ebayne@wisc.edu

Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) show immense promise in cardiac regeneration and precision medicine. However, hPSC-CMs lack phenotypic and metabolic maturity compared to adult CMs in vivo, hindering their use in biotherapeutic applications. To identify metabolic transitions in long-term culture, we profiled the hPSC-CM metabolome and proteome across developmental stages spanning 1-6 months in vitro. Intracellular metabolites and proteins were sequentially extracted and analyzed by unbiased high-resolution mass spectrometry (MS). The multiomic analysis was benchmarked against known markers of maturation evaluated by top-down proteomics. Using global trends identified in each dataset, we identified biomarker candidates to monitor and facilitate hPSC-CM maturation.

Changes in isoform expression of key contractile proteins occurred between 1-3 months. The conversion of atrial to ventricular isoforms of essential and regulatory light chains began at 1month and plateaued at 3month with relative expression of RLCv remaining at ~57% from 3-6 months. Cardiac troponin I, a known in vivo marker indicating adult phenotype, remained undetected up to 6 months.

We quantified 950 metabolite features using untargeted metabolomics, 274 of which were annotated using accurate mass (<2.0 ppm). 78 metabolites were confirmed by tandem MS. To analyze residual proteins, we used label-free bottom-up proteomics, quantitating 3,581 proteins across groups. Differential enrichment analysis identified global trends of metabolites and proteins, including 314 metabolite features and 1,171 proteins significantly changed across time (one-way ANOVA, $p<0.05$). Metabolites and proteins increasing in abundance in from 3-6m revealed a marked shift in energy metabolism, including upregulation of amino acid synthesis and fatty acid metabolism. Significantly altered metabolite and protein constituents of these pathways were identified as biologically important biomarker candidates which can be used to monitor hPSC-CM maturation.

(13) Increasing Signal-to-Noise for Poorly Resolved Low Abundance Peaks Through a Novel Spectrum Averaging Algorithm

Nicholas E. Bollis, Austin V. Carr, Lloyd M. Smith

nbollis@wisc.edu

Mass spectrometry is a powerful tool for proteomic analysis. In the analysis of complex samples, it is often difficult to identify low abundance and/or poorly resolved species. One approach that can help to improve data quality and thus increase identifications is spectral averaging. We present here a novel binning averaging algorithm adapting outlier rejection methods from astronomical image enhancement

techniques and weighting binned values against statistical distributions. These methods included sigma clipping and winsorized sigma clipping, iterative rejection algorithms in which a central value and dispersion (sigma) are determined, and values are rejected based upon their sigma distance from the central value1. Two dimensional nLC/MS-MS was performed on Pierce intact protein standard mix in a Thermo Scientific Q Exactive Plus Quadrupole-Orbitrap, acquiring successive individual microscans at orbitrap resolutions of 15k, 30k, 45k, 60k. Averaging methods were compared using spectra of Bovine Carbonic Anhydrase II (29 kDa). Winsorized sigma clipping yielded a significant increase in signal-to-noise for low abundance species when averaging at least twenty spectra. When fewer spectra were averaged, sigma clipping showed the greatest increase in signal to noise for the same species at low resolution. Averaging five scans at 15k and 30k resolution yielded up to six additional charge state envelope peaks above twenty percent relative abundance when compared to the default instrument averaging method. These findings suggest adjusting the averaging method used and collecting lower resolution spectra with fewer microscans will decrease the duty cycle of the instrument while producing an overall increase in signal to noise, particularly for low abundance and poorly resolved peaks. Future application in a clinical laboratory setting will allow for an increase in both the sensitivity and throughput in precision medicine multi-omic analyses.

(1) Starck, J.-L.; Murtagh, Fionn. Astronomical Image and Data Analysis; Astrononmy and Astrophysics; 2002.significantly up-regulated by Dox treatment, while not changing much in the other two groups.

(14) Variation in mouse islet Ca2+ responses reveals novel regulators of human islet function.

Lauren Clark1*, Christopher H. Emfinger1*, Kathryn Schueler1, Shane Simonett1, Donnie Stapleton1, Kelly Mitok1, Matt Merrins2, Mark Keller1, Alan Attie1
1Department of Biochemistry, University of Wisconsin-Madison
2Department of Medicine, Division of Endocrinology, University of Wisconsin-Madison
*Both authors contributed equally
leclark4@wisc.edu

Impaired insulin secretion is a major contributor to type 2 diabetes (T2D). The intracellular flux of calcium (Ca2+) into β-cells triggers insulin release. Since inter-individual variation in insulin secretion is primarily due to genetics, we surveyed -cell Ca2+ oscillations in islets from eight genetically diverse mouse strains. With fluorescence microscopy, we measured relative Ca2+ levels in response to three conditions: 1) 8mM glucose; 2) 8mM glucose with amino acids; and 3) 8mM glucose, amino acids, plus 10nM GIP. These conditions interrogate β-cell function, α-cell to β-cell signaling, and incretin responses. To determine whether these differences in islet Ca2+ led to altered insulin secretion, we measured insulin secreted from perifused islets of male 129 and WSB mice using the same secretagogues. These mice have similar average Ca2+ but different levels of insulin secretion. We then nominated candidate genes driving the inter-strain Ca2+ differences by calculating the correlation between Ca2+ waveform parameters and islet protein abundance. Some proteins highly correlated to certain Ca2+ wave metrics have human orthologues encoded by genes located near GWAS SNPs associated with T2D. Several of these, while poorly explored regarding islet function, nonetheless have drug targets or are secreted. Therefore, understanding the role these proteins play may identify novel biomarkers or therapeutic targets for diabetes. Utilizing genomics, proteomics, and human GWAS data enables us to prioritize genes that affect islet function and display genetic variation contributing to risk of diabetes in humans.

(15) Development of a novel hybrid search strategy by combining spectral library and database searches for bottom-up and top-down proteomic analysis

***Yuling Dai*, Lab: Lloyd Smith, University of Wisconsin Madison**
dai59@wisc.edu

Protein database searching and spectral library searching are widely used peptide identification methods. However, each search strategy has its shortcomings. The sequence database used in database searching lacks peak intensity information, which limits the sensitivity of scoring function. Spectral libraries often contain fewer peptides than repository databases, which limits the number of peptides capable of identification by spectral match to a subset. To address these limitations, we developed a hybrid search strategy, combining protein database search and spectral library search. This hybrid search strategy, an algorithm for spectral library generation, and a visualization tool for spectra comparison were integrated

into the MetaMorpheus search engine, improving peptide identification rate and sensitivity, and making it easy to visualize and evaluate PSM results.

Methods: The hybrid search strategy involves two steps. First, the raw spectra are searched against a protein sequence database to obtain the database search scores. Second, the results are further evaluated with an imported spectral library, and the library search scores (spectral angles) are obtained. The target-decoy search strategy for estimating incorrect peptides and proteins is used in our workflow. In this process, spectral angles are calculated between each observed spectrum and their assigned library spectrum. These related search scores are then employed in a binary decision tree calculation to compute a posterior error probability, which provides a measure of uncertainty for each peptide-spectrum match.

Preliminary Data: To evaluate the spectrum similarity function based on spectral angle calculation, we utilized a public dataset generated from the human epithelial cell line, Hela, and searched it against a spectral library predicted by pdeep, a published deep neural network algorithm for predicting spectral libraries. The algorithm efficiently differentiated target libraries from decoy libraries as the spectral angle values increased. More than 95% of the target peptide spectrum matches (PSMs) can be separated from decoy PSMs at a spectral angle value of 0.6, suggesting that the spectrum similarity function has high sensitivity in detecting target PSMs against the decoy PSMs.

To further evaluate the hybrid strategy, we further compared the results from the new hybrid search and a conventional database search. A total of 176,454 target PSMs were found at PEP q-values <0.01 by hybrid search, while 172,161 target PSMs were found at PEP q-values <0.01 by database search. The PSMs generated by spectral library search increased by 2.5% compared to the results from database search only.

We then applied the hybrid search strategy to top-down analysis. The results showed that more than 95% of the target Proteoform spectrum matches (PrSMs) were separated from decoy PrSMs at a spectral angle value of 0.6, suggesting that the whole workflow worked well for top-down analysis.

PrSMs obtained using either the conventional database search or the hybrid search were then compared. When searching the same dataset, 11,937 PSMs were identified by the hybrid search and 11,833 PSMs were identified by the database search only. There were 0.87% more PrSMs identified by the hybrid search than PrSMs identified by the database search.

Novel Aspects: Developed a novel hybrid search strategy by combining spectral library and database search, improving the identification rate and the sensitivity

(16) Decoding the Epigenetic Language of Gut Microbiome

Jessica Han
jhhan3@wisc.edu

Trimethylamine-N-Oxide (TMAO) is a metabolite generated by gut microbial consumption of choline that is enriched in red meat, nuts, and fish. While choline is essential for our body to function, its derivative – TMAO – produced from the dysbiosis has been associated with the exacerbation of many inflammatory and metabolic diseases. More recently, TMAO has been linked to various neuronal diseases from mild cognitive impairment, Alzheimer's Disease, to autism spectrum, suggesting that microbial TMAO may be involved in regulating Gut-Brain-Axis in the host. While the underlying mechanisms remain elusive, our preliminary data suggest that the microbially derived TMAO crosses the blood-brain-barrier and induces the dysregulation of multiple neuroinflammatory and mitochondrial proteins in the mouse hippocampus. Further, we demonstrated that TMAO modifies the chromatin states in the host cells by placing post-translational modifications on histones, which can affect the folding and accessibility of DNA. In all, our data suggest that the microbially derived TMAO modulates protein homeostasis by remodeling the chromatin states in the host cells. To interrogate how TMAO remodels the chromatin biology of the host on a molecular level, we use germ-free mice and cell culture models treated with pathophysiological concentrations of TMAO and leverage multi-omics approaches and biochemical assays to examine key epigenetic changes.

(17) Time-course single-cell RNA-Seq analysis reveals ligand-receptor protein signaling interactions in burn injuries.

Parth Khatri, Anqi Gao, Julie Rindy, Anna Huttenlocher, Huy Q. Dinh
McArdle Laboratory for Cancer Research, Department of Oncology Department of Biostatistics and Medical Informatics
Department of Medical Microbiology and Immunology Department of Pediatrics
University of Wisconsin School of Medicine and Public Health, Madison, WI
pkhatri@wisc.edu

Cell-cell interactions, defined by protein-protein signaling between a ligand and its corresponding receptor, plays important roles in disease progression. Single-cell RNA transcriptomics (scRNA-seq) has provided a novel way to identify potential interactions between cell types via statistical analysis of ligand-receptor co-expression. However, the lack of mechanistic and experimental models makes further functional studies of ligand-receptor interactions in specific biological conditions a challenge. Here, we are developing an analysis framework to evaluate the changes of ligand-receptor interactions between immune cells for time course scRNA-seq data. This pipeline enables the investigation of differentially interacting cell types between conditions at a timepoint, while also capturing the dynamics of changing interactions time in a particular condition. To this end, we employed multiple scRNA-seq integration methods accounting for technical and biological variation and performed cell-cell interaction inference using CellChat, which models mass action kinetics of differentially expressed signaling genes to identify statistically significant interactions by permutation tests. We applied our pipeline to an in-house generated time-course scRNA-seq data of innate immune cells using burn injury zebrafish larvae models. Our analysis identified known interactions between neutrophils and macrophages, such as IL6-IL6R, and potentially novel interactions including THY1 signaling between different neutrophil subsets. The list of potential candidates will be candidates for further investigation using proteomics approaches such as quantitative mass spectrometry to validate and guide further in vivo study of the burn wound healing process. In addition, our approach will be used for a comparative bioinformatic study between zebrafish and mouse burn models using publicly available data and compare with our generated human burn data. In summary, our analysis shows that scRNA-seq and statistical analysis can inform further proteomics experiments to understand how cell-cell interaction impacts wound healing across species and time.

(18) Evaluation and Application of Dialkylated Amino Acids as Isobaric Tags with Different Amine-Reactive Groups for Quantitative Proteomics

Peng-Kai Liu[1], Ting-Jia Gu[2], Lingjun Li[1], [2]
1Biophysics Program, University of Wisconsin-Madison, Madison, WI, USA
2School of Pharmacy, University of Wisconsin-Madison, Madison, WI, USA
pliu235@wisc.edu

Quantitative mass spectrometry is widely applied in proteomic and metabolomic studies to investigate complex biological systems. Quantitative approach measures the global dynamics of proteins and metabolites in a cell, tissue, or organism. Various quantitative proteomics strategies are available to determine relative changes in protein metrics between samples, using isotopic labels or in a label-free manner. Isobaric labeling-based method allows quantitative proteomics and metabolites in high-throughput manners, revealing protein roles in many physiological processes and disease progression. Multiplexing capabilities with isobaric mass tags have expanded its applicability to a wide range of sample types simultaneously.

Currently, our group has developed several cost-effective isobaric tags, DiLeu and DiAla, for quantitative proteomics. However, the labeling efficiency and identification rates for DiLeu and DiAla tags with different reactive groups haven't been examined thoroughly. Furthermore, to further expand the applicability of mass tags to other fields, the removal of all reagents other than activated mass tags is necessary. Herein, we evaluated the performance of two amine-reactive groups, DMTMM and NHS, from the aspects of the stability of activated tags, the labeling efficiency, protein identification, and quantification.

Our result suggested that the newly developed tag (DeAla) is a potential labeling reagent for quantitative proteomics. DeAla-NHS ester is highly reactive but relatively stable compared to the DeAla-DMTMM

ester. DeAla labeled peptides exhibit more abundant backbone fragmentation and enhanced sensitivity, rendering more identifications. DeAla-NHS tag relatively generates a greater number of PSMs, peptides, and proteins, increasing overall proteome coverage. DeAla-NHS features high stability, labeling efficiency, and peptide backbone ion formation, enabling simultaneous identification and quantification of a large number of proteins in complex biological systems.

(19) Exploring Crown Ether as the Shift Reagent for the Differentiation of Amino Acid Enantiomers and Peptide Epimers via Ion Mobility Spectrometry

Gaoyuan Lu1, Zhijun Zhu2, Shuling Xu1, Lingjun Li1,2
1School of Pharmacy, University of Wisconsin-Madison, Madison, Wisconsin 53705, United States
2Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin 53705, United States

Introduction: The differentiation and quantification of chiral biomolecules such as amino acid enantiomers and peptide epimers are necessary to elucidate their physiological roles, especially in food, the environment, and human health. Meanwhile, ion mobility spectrometry (IMS) is becoming an effective tool to study stereochemistry. However, it is extremely challenging to separate enantiomers by IMS. Peptide epimers such as D-amino acid-containing peptides (DAACPs) and their L-analogs also share small collision cross-section (CCS) differences (~1%) in most cases. These factors hinder the application of IMS in chiral analysis. Here we introduced a chiral crown ether, (−)-(18-Crown-6)-2,3,11,12-tetracarboxylic acid (18C6TA), as the shift reagent in IMS to differentiate chiral biomolecules. Amino acids can form a non-covalent ion complex diastereomer with crown ether to enable enantiomeric separation via IMS. The D/L structural differences (DLSDs) in peptide epimers can also be enhanced with the complexation.
Methods: Chiral molecules (amino acid enantiomers, peptide epimers standard) and crown ether were mixed together in solution and sprayed into the mass spectrometer via direct infusion. Waters Synapt G2 and Agilent 6560 IM Q-TOF were used for drift time measurement.
Preliminary Data: [Amino Acid+18C6TA+H]+ and [Peptide+18C6TA+H]+ ion complexes can be obtained in ESI experiments. Within different IMS platforms, the enantiomeric differentiation of the D/L-phenylalanine enantiomer can be well achieved. Neuropeptides including achatin-1 (GDFAD), dermorphin 1–4 (YDRFG), and a series of other tetrapeptides with a D-residue at the second position from the N-terminus were examined as peptide epimer models. The DLSDs of the above DAACPs and their L-analogs can be enhanced by incorporating CCS coordinates of [Peptide+18C6TA+H]+ in addition to [Peptide+H]+. The experimental CCS values of ion complexes can be further matched with theoretical CCS calculation.
Novel Aspect: The chiral crown ether as the shift reagent in IMS for rapid differentiation of chiral biomolecules has been demonstrated.

(20) DiLeu isobaric labeling coupled with limited proteolysis mass spectrometry for high-throughput structural protein quantitation in Alzheimer's disease

Haiyan Lu1, Bin Wang1, Yuan Liu1, Danqing Wang2, Lauren Fields2, Hua Zhang1, Miyang Li2, Xudong Shi3, and Lingjun Li1,2
1School of Pharmacy, University of Wisconsin-Madison, Madison, WI, 53705, USA
2Department of Chemistry, University of Wisconsin-Madison, Madison, WI, 53705, USA
3Division of Otolaryngology, Department of Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, Madison, WI, 53792, USA
*Corresponding author: lingjun.li@wisc.edu
hlu244@wisc.edu

High-throughput quantitative analysis of protein conformational change has a profound impact on our understanding of the pathological mechanisms of Alzheimer's disease (AD). To establish a useful workflow enabling quantitative analysis of changes in protein conformation within multiple samples simultaneously, here we report the combination of N,N-Dimethyl Leucine (DiLeu) isobaric tag labeling with limited-proteolysis mass spectrometry (DiLeu-LiP-MS) for high-throughput structural protein quantitation in AD serum samples. The feasibility of DiLeu-LiP-MS was initially validated through the holomyoglobin and apomyoglobin model system. Upon demonstration of proof-of-concept results, the DiLeu-LiP-MS method was employed for AD human serum sample analysis. The method results in the identification of 28 proteins exhibiting structural changes, mapping to 140 unique conformotypic peptides with significant changes between the AD group and the control group. Among the 28 proteins, 10 proteins, including

A2MG, APOB, TRHB, CO3, ITIH2, ANT3, TRFE, CO8B, C4BPA, and TTHY, exhibited potential correlation with AD. Moreover, we found that complement proteins (e.g., CO3, CO8B and C4BPA) related to AD showed elevated levels in the AD group compared to that in the control group. Although larger and more diverse cohorts are required in future studies to explore the underlying molecular mechanism of valuable complement protein as well as structural changes of proteins in AD. Current results provide evidence that the established DiLeu-LiP-MS method provides a powerful tool for high-throughput structural protein quantitation, which also showed great potential for in-depth quantitative analysis of protein conformational changes in other biological systems.

(21) Cell-Surface Glycomics Study at Single-cell Level Enabled by Mass-defect Multiplex Isobaric Labeling

Min Ma1, Miyang Li2, Lingjun Li1,2*
1School of Pharmacy, 2Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, 53705, United States
mma58@wisc.edu

While the analysis of single-cell transcriptomes is well established and the single-cell proteomics is gaining popularity recently, the glycomics study at single-cell level lags far behind. This is mainly due to the inherent analytical difficulties of glycan species and the lack of appropriate tools reaching enough sensitivity. Here, we developed a set of 6-plex mass defect isobaric tags (mdSUGAR) to facilitate glycomics analysis of cell-surface glycans at single-cell level. By employing triplicate labeling strategy and utilizing high resolution mass spectrometer, the low abundance glycans can be recognized by the miliDelton mass shift signature imparted by the chemical labeling on MS1 level, while enhanced intensity of precursor ions promote MS/MS fragmentation and therefore provide rich information of glycan structures.

Cell-surface N-glycans were released directly from the harvested cells using PNGase F, and mdSUGAR tag were used to derivatize the reducing end of glycans through reductive amination. Each sample was aliquoted into three parts evenly and labeled with adjacent three channels of mdSUGAR. The labeling reaction was performed in aqueous buffer and borane-pyridine were selected as the reducing agent. Samples were pooled together and desalted using HLB column before running LC-MS/MS analysis on a self-fabricated HILIC column. High-resolution (1,000k @ 200 m/z) survey scan was performed first to collect the full MS spectrum where an inclusion list of the mass and retention time of those pre-identified precursors can be generated to guide the sequential targeted MS/MS analysis.

(22) Efficient isolation of RNA-protein complexes from formaldehyde-crosslinked cells for the characterization of the RNA-binding proteome

***Samuel Markovich*, Rachel Knoener, Brian L. Frey, and Lloyd M. Smith**
smarkovich@wisc.edu

Abstract: Characterization of the RNA-binding proteome is important to understanding interactions between RNAs and proteins. A methodology capable of isolating RNA-protein complexes, Orthogonal Organic Phase Separation (OOPS), was developed for UV-crosslinked cells. However, UV-crosslinking is inefficient and does not reveal entire protein complexes, leading to increased sample requirements and missed protein identifications. We addressed these issues by reoptimizing OOPS parameters for formaldehyde-crosslinked cells. The resultant workflow, which we refer to as OOPS-FX, consistently recovers up to 90% of total RNA while retaining 30% of the protein. Preliminary results suggest that we will be able to estimate cellular crosslinking efficiencies, which are currently unquantifiable. Due to the increased efficiency of OOPS-FX, ten-fold fewer cells are needed to study the RNA-binding proteome compared to the original OOPS method, enabling the study of low quantity, precious samples.

This method will be employed with our previously developed technique, Hybridization Purification of RNA-protein complexes followed by Mass Spectrometry (HyPR-MS). HyPR-MS is an RNA-centric proteomics approach, allowing the experimentalist to determine the protein interactors of specific RNAs present in cell lysate. The HyPR-MS protocol currently requires a large amount of starting material due to inconsistent and nonspecific captures of targeted RNAs, yielding low protein quantities for analysis. We hypothesize that the simpler sample OOPS-FX generates will lead to better capture efficiencies and specificities for targets of HyPR-MS and other similar approaches, while lowering the cost

of each experiment due to the reduced sample volume requirement. Ultimately, the adapted HyPR-MS protocol employing OOPS-FX will be applied to identify diagnostic markers in prostate cancer tumors.

(23) Combined protein and transcriptional single-cell analysis reveals extensive neutrophil heterogeneity in multiple myeloma

Rishil Mehta, Joshua Brand, Aisha Mergaert, Athena E. Golfinos, Huy Q. Dinh
McArdle Laboratory for Cancer Research, Department of Oncology
Department of Biostatistics and Medical Informatics
University of Wisconsin of Medicine and Public Health, Madison, WI
rmehta2@wisc.edu

Single-cell transcriptome analysis (scRNA-seq), a state-of-the-art genomic profiling technology, has revolutionized the characterization of cellular heterogeneity in the tumormicroenvironment (TME). However, scRNA-Seq profiling of human neutrophils, the most abundant immune cell type in the TME, has been challenging due to their low RNA expression contents and the need for fresh clinical samples due to their short lifespans. Previous scRNAseq profiling of neutrophils in multiple myeloma (MM) patients underestimated their abundance and heterogeneity in bone marrow despite recent in-depth characterization in healthy individuals via protein antibody profiling approaches by us and others. The lack of a transcriptional neutrophil atlas limits understanding their implications in disease progression and drug resistance. Here, we use CITE-seq, a new multimodal single-cell technology, to profile both RNA and surface protein expression to better characterize cellular heterogeneity in both the blood and bone marrow of MM patients. The power of having both protein and RNA expression in individual cells helped us to define extensive neutrophil heterogeneity that could not be identified with scRNA-seq. Specifically, we identified 7 subsets of neutrophils, including CD66b-CD64+ early neutrophil precursors, a subset of mature neutrophils with platelet-related protein expression (CD9+CD62p+CD41+CD49b+), and interferon-related mature neutrophils (IFIT1/2/3hi) that were not known in MM. Additionally, using neutrophil signatures from the CITE-seq data, we identified missing neutrophils in a publicly available MM scRNA-Seq dataset, with potential links to disease progression and treatment response. Our results demonstrate that proteomic data can significantly improve the analysis of scRNA-Seq data to better characterize the TME at the single-cell level. We anticipate that further investigation of the role of these neutrophil subsets in the TME using both protein and RNA expression analysis will improve our understanding of cancer progression and potentially identify new immunotherapeutic targets.

(24) DiLeu-Enabled Quantitative Analysis of Sphingolipids for Biomarker Discovery in Alzheimer's Disease

Jericha Milla, Dustin Frost*b*, Jordan Appel*c*, Ting-Jia Gu*b*, Thomas Raife*b*, Lingjun Li*a,b*
aDepartment of Chemistry, University of Wisconsin-Madison, Madison, WI, United States
bSchool of Pharmacy, University of Wisconsin-Madison, Madison, WI, United States
cCollege of Agricultural and Life Sciences, University of Wisconsin-Madison, Madison, WI, United States
dDepartment of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, Madison, WI, United States
mill@wisc.edu

Alzheimer's Disease (AD) is the most common cause of dementia; despite extensive research, there are no effective treatments. Diagnosis is based on observations of decline in behavioral/cognitive function. However, by symptom onset, cellular decline has long been occurring.

The role of lipids in AD has been recognized since the APOE gene was implicated in AD. Recently, we identified several increased sphingolipid species in AD. Because sphingolipids can be modulated both through lifestyle changes and pharmacologically, the AD sphingolipid profile could be both a therapeutic target and a means to track treatment efficacy.

Untargeted mass spectrometry (MS)-based metabolomics is a valuable tool for biomarker discovery, but targeted quantitation can be time-consuming. Isobaric labeling increases throughput by enabling multiplexing, making the AD sphingolipid profile a viable clinical biomarker. DiLeu is an amine-targeting isobaric label made in-house and used widely in the Li laboratory; it has not been used for lipids and requires method optimization.

Labeling efficiency (LE) increased with increasing DiLeu from a 5:1 to 200:1 ratio but peaked at an 80:1 ratio with 95% LE. Reaction time did not have a strong effect LE, as 95% LE was met after 30 minutes and did not improve with additional time. Interestingly, LE decreased at 120 minutes, suggesting extended reaction time may be detrimental.

For DiLeu labeling, sphingolipids must be hydrolyzed to create a free amine. Following proof-of-principle labeling experiments with pre-hydrolyzed lipids, enzymatic hydrolysis was performed on intact sphingomyelin. Because sphingolipid-ceramide N-deacylase catalyzes both hydrolysis and condensation, Ca2+ ions were used to encourage the forward reaction by precipitating fatty acids. More optimization is ongoing. Current research is focused on a new reaction wherein intact sphingolipids are derivatized to have a carbonyl group that can then be labeled by a carbonyl-targeting isobaric label. Experiments with phospholipids have been promising, with >95% LE.

(25) Novel NeuCode Tagging Reagents for Proteoform Identification

John G Pavek1; Dustin Frost1; Brian L Frey1; Lingjun Li1; Lloyd Smith1
1University of Wisconsin-Madison, Madison, WI
jpavek@wisc.edu

Proteoforms arising from the same gene can have unique functions and subcellular localizations. It is accordingly important to be able to accurately and efficiently identify proteoforms in biologically interesting systems. There are currently two methods for proteoform identification: top-down and intact-mass analysis. Top-down analyses are time consuming and require highly specialized instrumentation. Intact mass analysis is less time-consuming, but identifications are often ambiguous. Proteoform identification using intact mass in conjunction with lysine counting via NeuCode SILAC, a method that leverages minute differences in isotope mass shifts incorporated through cell culture, is a viable alternative. However, SILAC is a method that is only applicable to cell culture samples, thus is quite limited. To address this limitation, a NeuCode pair of chemical labels targeting cysteine has been designed and synthesized to enable proteoform identification via intact mass and cysteine count on any protein sample, including tumor and tissue samples. Conditions to achieve sufficient labeling efficiency as well as specificity for cysteine have been optimized. The efficacy of the synthesized NeuCode pair of tags for cysteine counting and proteoform identification have been assessed on a sample of Promega Human protein extract spiked with a known protein, lysozyme. The accuracy of cysteine counting using the synthesized NeuCode pair of labels has been confirmed by analysis of the spiked lysozyme. Additionally, approximately 25 native human proteoforms have been identified at a 30% false-discovery rate (FDR), suggesting the potential of this method for efficient proteoform identification in clinically-relevant samples. Improvements in data processing analysis, as well as the implementation of an enrichment strategy for cysteine-containing proteoforms are likely to drastically improve these results.

(26) Comprehensive Analysis of Phospholamban Proteoforms Enabled by Photocleavable Azo Surfactant and Top-down Proteomics

Holden Rogers, Eli Larson, Kalina Reese, Jake Melby, Austin Carr, Kyle Brown and Ying Ge
htrogers@wisc.edu

Phospholamban (PLN) is a regulatory membrane protein in the cardiac sarcoplasmic reticulum; its interaction with Ca2+-ATPase SERCA2a is essential in controlling muscle contraction through calcium ion concentration. The function of PLN is determined by post-translational modifications (PTMs), most notably palmitoylation and phosphorylation. Both palmitoylation and phosphorylation sites have been implicated in cardiomyopathy; however, proteomic-based efforts by mass spectrometry (MS) have not been able to distinguish site occupancy of phosphorylated proteoforms. Reproducible extractions, chromatographic separation performance, fragment coverage of PLN, and localization of phosphorylation sites remain challenging in PLN characterization. Herein, we have developed a reproducible top-down LC-MS/MS method enabled by Azo for extraction and chromatographic separation of PLN from cardiac tissue to comprehensively analyze proteoforms and localize modifications sites.

We implemented top-down proteomics in conjunction with our novel photocleavable anionic surfactant Azo and a robust LC-MS method to analyze PLN in swine and human cardiac tissue. Membrane proteins

were extracted using a two-stage extraction. Cytosolic proteins were depleted using an ammonium bicarbonate extraction. Membrane proteins were extracted by adding Azo to pelleted tissue followed by homogenization and centrifugation. Azo was subsequently cleaved by UV-irradiation and extracts were concentrated and desalted using molecular weight cutoff filters. Proteins were separated with a Waters nanoAcquity LC using online reverse-phase liquid chromatography on a C2 column coupled to a Bruker maXis II quadrupole time-of-flight MS for detection and fragmentation. Data was analyzed using Bruker Compass DataAnalysis v. 4.3 and MASH Native v. 1.0.

Our platform enabled consistent and reliable extraction of hydrophobic proteins from cardiac tissue. The analysis of Azo extracts revealed varying PLN proteoforms, and included single/dual phosphorylation, palmitoylation, and their combination. Tandem MS data demonstrated reproducible fragmentation coverage of the hydrophobic region of PLN. Fragmentation of the cytosolic region where phosphorylation sites reside could be enhanced with electron capture dissociation and electron-transfer dissociation. Prospectively, this method will be crucial in its application to cardiac diseases such as ischemic cardiomyopathy, as well as chamber comparisons that examine the regional heterogeneity in human hearts.

(27) A Shift in the Human Plasma Lipidome with Metformin Monotherapy Independent of Glycemic Control in a Type 2 Diabetes Clinical Cohort.

Benjamin Wancewicz*a*, Yanlong Zhua,b, Rachel J. Fenskec,d,e, Alicia M. Weeksc,d, Kent Wengera,b, Samantha Pabichc, Michael Danielsc,d, Margaret Puntc, Randall Nallc,d, Darby Peterc,d, Allan Brasierc,e, Elizabeth D. Coxf, Dawn B. Davisc,d, Ying Gea,b,g*, and Michelle Kimplea,c,d*

a Department of Cell and Regenerative Biology, b Human Proteomics Program, c Department of Medicine, Division of Endocrinology, Diabetes, and Metabolism, d Research Service, William S. Middleton Memorial Veterans Hospital, e Institute for Clinical and Translational Research, f Department of Pediatrics, g Department of Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, USA

wancewicz@wisc.edu

Type 2 diabetes, a metabolic disorder, is a rising pandemic with current prevalence is estimated to be 9.3% of adults and is expected to rise to 10.9% (700 million individuals) by 2045. Of particular concern, individuals with diabetes are at a greater risk for macro- and microvascular complications. Metformin and diet and lifestyle intervention are the first line of treatment, with varying success in preventing these adverse outcomes.

In this study, we analyzed human plasma from an observational study looking at these first line interventions using our metabolomics platform. Our diabetic groups displayed similar level of glycemic control and BMI, which allowed for comparison in difference of the treatment group. We applied a high-throughput, ultrahigh resolution Flow Injection Electrospray (FIE) Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometry (MS) workflow to biobanked plasma samples from non-diabetic (ND) subjects, T2D subjects treated with diet and lifestyle modifications, and T2D subjects on metformin monotherapy, quantifying the relevant abundance of metabolic features among groups using an untargeted approach.

In total, we detected 2000 metabolic features and annotated over 400 metabolic features with the majority of these annotations being lipids. Of particular interest, we observed a shift in the levels of fatty acids and multiple phospholipids, such as phosphatidylcholines and phosphatidylethanolamines, between the diabetic groups. As a general trend, these lipids were increased in the diet and lifestyle group, while the metformin-treated group had similar levels as the nondiabetic group. This shift in lipid metabolism with similar glycemic control is of interest because metformin has shown a cardioprotective effect and might provide insight into the mechanism of its benefits. By utilizing the FIE-FTICR MS metabolomics platform, differences in lipid metabolism can be identified between the two diabetic groups.

(28) 12-plex DiLeu isobaric labeling enabled high-throughput investigation of citrullination alterations in DNA damage response

Bin Wang1; Zihui Li2; Qinying Yu; Yatao Shi1; Lingjun Li1,2
1School of Pharmacy, 2Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53705, USA
bwang333@wisc.edu

Introduction and preliminary data: Protein citrullination is a key post-translational modification (PTM) which leads to the loss of positive charge and consequent protein structural and functional changes. Though it has been indicated to play critical roles in various physiological and pathological processes, effective tools are limited due to the small mass shift of this PTM and its low-abundance nature. Recently, we developed a biotin-thiol tag which enabled large-scale profiling of protein citrullination. However, a high-throughput quantitative approach is needed to further improve the understanding of this PTM. Herein, we report an efficient pipeline using our custom-developed N,N-dimethyl leucine isobaric tags to achieve a multiplexed quantitative analysis of protein citrullination. We then apply this strategy to investigating citrullination alterations in response to DNA damage stress. Three different methods were used to introduce DNA damage to human MCF7 cells which included UV radiation, oxidative stress using H2O2 and anthracycline drug. In total, we identified and quantified 78 citrullination sites from 63 citrullinated proteins. Hierarchical clustering of all quantified citrullination sites was plotted to explore their profiles in different groups and demonstrated the capability of our methods for reliable quantitative analysis of protein citrullination. ANOVA analysis reveals 31 sites from 26 citrullinated proteins to be significantly changed. Both UV and ADR-treated groups exhibit dramatic alterations compared to the control group while obvious disparities are also observed between them, indicating different mechanisms between these two types of DNA damage. Our results indicate an intimate involvement of this PTM in the regulation of translation and DNA repair processes and provide new insights into DNA damage-associated disease pathogenesis.

(29) ATP-Coated Dual-functionalized Titanium (IV) IMAC Material for Simultaneous Enrichment and Separation of Glycopeptides and Phosphopeptides

Danqing Wang1, Junfeng Huang2, Haoran Zhang1, Min Ma2, Meng Xu1, Yusi Cui1, Xudong Shi3, Lingjun Li1,2
1Department of Chemistry, University of Wisconsin-Madison, Madison, WI 53706, USA
2School of Pharmacy, University of Wisconsin-Madison, Madison, WI 53705, USA
3Department of Surgery, University of Wisconsin–Madison, Madison, WI 53705, USA
dwang356@wisc.edu

Protein glycosylation and phosphorylation are two of the most common post-translational modifications (PTMs), which play an important role in many biological processes. However, low abundance and poor ionization efficiency of phosphopeptides and glycopeptides make direct MS analysis challenging. Previously, we explored the electrostatic and hydrophilic properties of commercial centrifuge-assisted-extraction Titanium (IV) IMAC (CAE-Ti-IMAC) material and its application in simultaneously enriching and separating common N-glycopeptides, phosphopeptides, and M6P glycopeptides with a dual-mode enrichment. In this study, we developed a hydrophilicity-enhanced bifunctional Ti-IMAC material with grafted adenosine triphosphate (denoted as: epoxy-ATP-Ti4+) to achieve better enrichment performance in dual-mode enrichment. The epoxy-ATP-Ti4+ IMAC material was prepared from epoxy-functionalized silica particles via a convenient two-step reaction. The ATP molecule provided strong and active phosphate sites for binding phosphopeptides in conventional IMAC mode and also contributed significantly to the hydrophilicity. This permitted the enrichment of glycopeptides via HILIC. The two modes could be implemented simultaneously, permitting glycopeptides and phosphopeptides to be collected separately. Besides standard protein samples, the material was further applied to HeLa cell digests and mouse lung tissue samples. In total, 2928 glycopeptides and 3051 phosphopeptides were identified from mouse lung tissue sample, which indicates that this material is readily applicable to large-scale PTM analysis of complex biological samples. Overall, our method allows simple and effective enrichment and separation of glycopeptides and phosphopeptides, which could be helpful for studying the potential crosstalk between these two PTMs.

(30) Mass Spectrometry Analysis of O-Glycosylation in Human Pancreatic Tissue

***Feixuan Wu*[1]**, Dylan Nicholas Tabang[2], Daniel M. Tremmel[3], Megan Ford[4], Sara Dutton Sackett[3], Jon S. Odorico[3], Lingjun Li[1,2]*
[1]School of Pharmacy, University of Wisconsin-Madison; [2]Department of Chemistry, University of Wisconsin-Madison; [3]Department of Surgery, Division of Transplantation, School of Medicine and Public Health, University of Wisconsin-Madison; [4]Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI, USA.
feixuan.wu@wisc.edu

Diabetes is a disease that impacts at least 425 million people worldwide. The characterization of its post-translational modifications (PTMs) is very essential because those modifications can alter molecular structure and bioactivity. Unlike N-glycosylation, O-linked glycosylation has neither a single consensus sequence motif nor a single core glycan template. Therefore, it is difficult to identify O-glycopeptides due to a lack of enabling techniques. Here, O-glycosylated peptides were enriched via electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) and analyzed with mass spectrometry (MS).

Tissues are first cryo-pulverized into a fine powder before protein extraction using sodium dodecyl sulfate. Then proteins are subjected to enzymatic digestion with LysC/trypsin. The peptide samples are aliquoted prior to enrichment using ERLIC. The enriched glycopeptides were separated using nano-scale reversed phase liquid chromatography-mass spectrometry (nRPLC-MS) using an Orbitrap Fusion Lumos mass spectrometer. Raw data files were analyzed using Byonic and Proteome Discoverer 2.5.

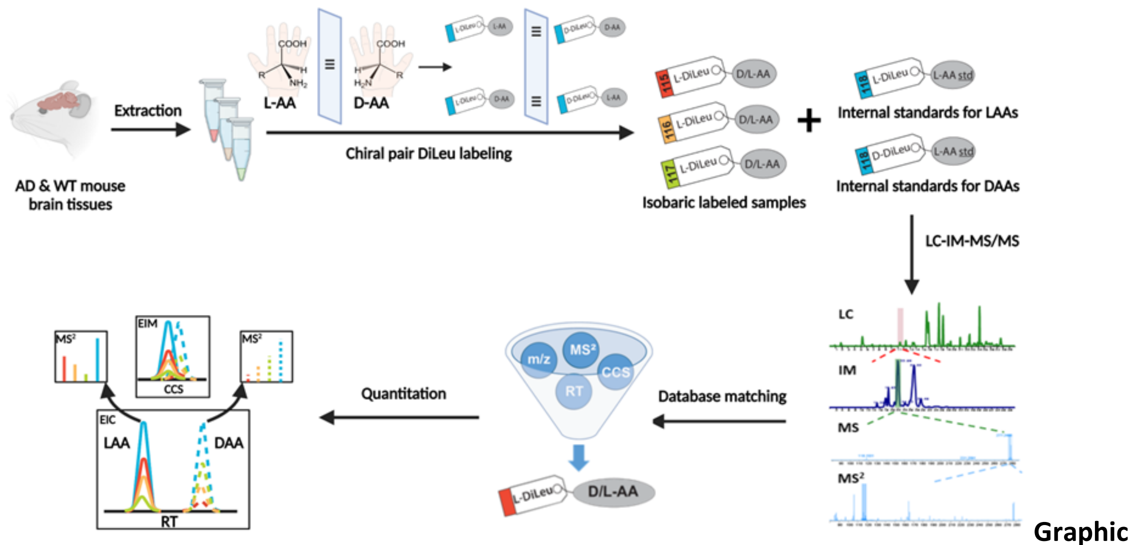
The total number of peptides without any filter were 2931, 2722, 2138, 1306 and 911 for each fraction, while after the filter, the number of identifications was approximately 24, 27, 16, 5, and 4, respectively. This suggests potential matches for O-glycosylation for the peptides, though manual inspection is needed. Ongoing efforts have been directed to the development of improved chromatography conditions, coupled with site-specific tandem MS analysis of O-glycopeptides, and alternative software tools for enhanced analysis of O-glycosylation in pancreatic tissue.

(31) Chiral Pair Isobaric DiLeu Labeling Strategy Enabled Separation and Absolute Quantitation of Enantiomeric Amino Acids

Zhijun Zhu1, Shuling Xu2, Zicong Wang2, Daniel G. Delafield1, Min Ma2, Ting-Jia Gu2, Gaoyuan Lu2, and Lingjun Li1,2
1School of Pharmacy, 2Department of Chemistry, University of Wisconsin-Madison, WI, 53705
zzhu285@wisc.edu

Although L-amino acids (LAAs) are predominant in mammals, D-amino acids (DAAs) play significant neurological functions. Abnormal levels of LAAs and DAAs are related to neurodegenerative diseases like Alzheimer's Disease (AD). Because DAAs and their enantiomeric LAAs demonstrate the same physicochemical properties in achiral environments, it remains challenging to differentiate DAAs from their L-counterparts by either gas-phase/liquid-phase separation or mass spectrometry (MS), let alone enantiomer-resolved quantitation. Here, we developed a high-throughput 4-plex isobaric N, N-dimethyl-L-leucine (L-DiLeu) and N, N-dimethyl-D-leucine (D-DiLeu) paired labeling strategy to qualitatively and quantitatively analyze LAAs and DAAs on LC-ion mobility (IM)-QTOF platform. In this strategy, the DiLeu tag, on the one hand, serves as a novel chiral reagent to generate diastereomers of D/L-AAs to facilitate both LC separation and IM identification. On the other hand, isobaric DiLeu tags also enable multiplexed quantification. Baseline RPLC separation of 19 D/L-AA counterparts (except achiral glycine) after DiLeu labeling was successfully achieved on a C18 column. Moreover, the distinguishable IM drift time differences between labeled D/L-AAs provided additional structural information, effectively reducing the interferences from co-fragmented isomers/isobars. A 4D database (m/z, CCS, RT, MS/MS) was built using chiral pair DiLeu labeled AA standards for confident assignment of labeled D/L-AAs. Utilizing D-DiLeu labeled LAAs as surrogates to L-DiLeu labeled DAAs, LAA standards labeled by the last isobaric channel of both L-DiLeu and D-DiLeu could largely boost the signal of labeled D/L-AAs in MS1 and serve as internal standards for accurate and confident absolute quantitation without extra DAA standards. A higher abundance of L-Ser, L-Lys, D-Ser, and D-Asp were found in the cortex of male APP/PS1 mice than in male wild-type mice, indicating that these AAs may be potential biomarkers of AD. This strategy can be further

applied to other chiral amine-containing metabolites for metabolomic biomarker discoveries, providing insights into precision medicine investigation.



Graphic Abstract

(32) Multi-omics method development with a single set of C. elegans samples and its application to study mitochondrial stress response

Yunyun Zhu1, Annie Jen1, Katherine A. Overmyer1, Arwen W. Gao2, Amélia Lalou2, Johan Auwerx2, and Joshua J. Coon1

1 University of Wisconsin, Madison, WI 53506, USA;

2 Laboratory of Integrative Systems Physiology, Interfaculty Institute of Bioengineering, École Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland.

yzhu338@wisc.edu

Mass spectrometry based multi-omics is powerful for answering biological questions, yet there are several major challenges including sample preparation, data collection, and data analysis. Sample preparation for difficult sample types such as C. elegans is especially challenging, as the worms have thick and highly structured cuticles that can inhibit the release of biomolecules. To develop multi-omics methods with a single set of C. elegans samples, we tested methods for efficiently extracting proteins, lipids, and polar metabolites with less variance for large scale studies. Tissue disruption and biomolecule extraction methods were evaluated in terms of number of identifications, lipid/metabolite class coverage, and feature variance. Specifically, we compared tissue disruption method flash-freezing in liquid nitrogen, bead-beating, bath-sonication, and combinations of these three methods. We found that either flash freezing, bath-sonication or bead-beating alone was able to effectively break worm tissues, and combinations of these had no additional benefits. We then compared four different extraction solvent mixtures: Matyash (MTBE:MEOH:H2O = 4:1.2:1), modified Matyash with M1 (MTBE:MEOH = 3:1): M2 (H2O:MEOH = 3:1) = 1.54:1, Butanol:ACN:H2O = 6:2:2 (BAW), and MEOH:ACN:H2O = 4:4:2 (MAW). The Matyash and modified Matyash methods had good coverage of lipid/metabolite coverage and lower inter-replicate variance. Yet the modified Matyash method was simpler and was thus selected as the preferred method for multi-omics studies with C. elegans. We applied this optimized method to study the biomolecule changes driven by chemical or genetically-induced mitochondrial stress response in C. elegans. We found that chemical and genetically-induced mitochondrial stress response resulted in distinct changes in the proteome, lipidome, and metabolome. While some molecules changes in all instances of mitochondrial stress response, other compounds like L-valine and L-Valine significantly increased in mrps-5 RNAi group, but decreased in both Doxycycline treated and cco-1 RNAi group. Another example of such discrepancy is Q19429_CAEEL DB domain-containing protein, which was significantly up-regulated by Dox treatment, while not changing much in the other two groups.