PROGRAM AND ABSTRACTS FOR THE 2012 JOINT MEETING OF THE SOCIETY FOR GLYCOBIOLOGY & AMERICAN SOCIETY FOR MATRIX BIOLOGY

November 11–14, 2012
San Diego, CA

PROGRAM
Joint Meeting of the Society for Glycobiology & American Society for Matrix Biology

November 11 – 14, 2012
Sheraton Hotel and Marina, San Diego, CA

Organizers: Jeff Davidson, Hudson Freeze, Robert Haltiwanger, David Roberts, Jean Schwarzbauer

Sunday, November 11	8:30 am – 1:30 pm	**SFG Guest Symposia I Advances in Glycoprotein Production, Characterization and Modification** *(Nautilus II)* Registration required: $50 (includes scientific program, workshop and coffee breaks)
	9:00 am – 5:30 pm	**ASMB Guest Symposia I - Wound Healing Society** **Quantifying Heterogeneity and Modeling Complexity in the Injury Response. Implications for Therapeutics Development** *(Nautilus I)* *Registration Required: $50 WHS members/ $100 WHS non-members
	12:00 – 5:30 pm	**SFG Guest Symposia II - Consortium for Functional Glycomics (CFG) Participating Investigator Meeting** **CFG Satellite** *(Grande Ballroom B)* Registration: $50 – CFG membership not required
	1:00 – 4:00 pm	**ASMB Guest Symposia II - TERMIS: Tissue Engineering & Regenerative Medicine International Society** *(Grande Ballroom C)*
	6:00 – 7:00 pm	**Opening Reception** *(Grande Foyer and Grande Ballroom A)*
	7:00 – 8:00 pm	**ASMB and SFG Society Welcome & Keynote Lecture** *(Grande Ballroom B & C)* **Bioorthogonal Chemistry for Glycoprofiling and Beyond** **Carolyn Bertozzi, *University of California, Berkeley***
Monday, November 12	7:30 – 8:30 am	**Continental Breakfast** *(Grande Foyer and Grande Ballroom A)*
	7:30 – 8:30 am	**Career Mentoring Breakfast (*pre-registration required)** *(Marina 5)*
	8:30 – 10:00 am	**Plenary I: Stem and Progenitor Cells, Genetic Therapy and Regenerative Medicine** *(Grande Ballroom B & C)*
	10:00 – 10:30 am	**Coffee Break** *(Grande Foyer and Grande Ballroom A)*
	10:30 – 12:00 pm	**Plenary II: Biomaterials and Matrix Engineering** *(Grande Ballroom B & C)*
	12:00 – 2:00 pm	**Poster Session I Lunch** *(Nautilus III, IV, V)*
	2:00 – 3:30 pm	**Concurrent Sessions 1 – 4** **CS1: Matrix and Carbohydrate Immunology** *(Grande Ballroom C)* **CS2: Matricellular Proteins** *(Nautilus I)*

		CS3: Receptors, Signaling, and Cytoplasmic Glycosylation
		(Grande Ballroom B)
		CS4: Vascular Biology, Elastic Fibers, and Related Diseases
		(Nautilus II)
	3:30 – 4:00 pm	**Coffee Break**
		(Grande Foyer and Grande Ballroom A)
	4:00 – 5:30 pm	**Concurrent Sessions 5 – 8**
		CS5: Carbohydrate and Matrix Metabolism in Disease States
		(Grande Ballroom C)
		CS6: Basement Membranes
		(Nautilus I)
		CS7: Genetics and Gene Expression
		(Grande Ballroom B)
		CS8: Mechanobiology and Biomedical Engineering
		(Nautilus II)
	6:00 – 7:30 pm	**SFG Award Lectures**
		(Grande Ballroom C)
	6:00 – 8:00 pm	**ASMB Award Lectures**
		(Grande Ballroom B)
Tuesday, November 13	7:30 – 8:30 am	**Continental Breakfast**
		(Grande Foyer and Grande Ballroom A)
	7:30 – 8:30 am	**Career Mentoring Breakfast (*pre-registration required)**
		(Marina 5)
	8:30 – 10:00 am	**Plenary III: Biosynthesis, Secretion and Assembly**
		(Grande Ballroom B & C)
	10:00 – 10:30 am	**Coffee Break**
		(Grande Foyer and Grande Ballroom A)
	10:30 am – 12:00 pm	**Plenary IV: Immunology and Inflammation**
		(Grande Ballroom B & C)
	12:00 – 1:00 pm	**Poster Session II Lunch**
		(Nautilus III, IV, V)
	12:00 – 2:00 pm	**National Academy of Sciences Luncheon – Future of Glycoscience**
		(Spinnaker)
	2:00 – 2:30 pm	**ASMB Business Meeting**
		(Grande Ballroom C)
	2:00 – 2:30 pm	**SFG Business Meeting**
		(Grande Ballroom B)
	2:30 – 4:00 pm	**Concurrent Sessions 9 – 12**
		CS9: Stem/Progenitor Cells and Their Environment
		(*Grande Ballroom C*)
		CS10: Biosynthesis, Protein Folding, Secretion, and Matrix Assembly
		(*Nautilus I*)
		CS11: Cancer Microenvironment
		(Grande Ballroom B)
		CS12: Glycomics/Chemical Biology I: Function
		(*Nautilus II*)
	4:00 – 4:30 pm	**Coffee Break**
		(Grande Foyer and Grande Ballroom A)
	4:30 – 6:00 pm	**Concurrent Sessions 13 – 16**
		CS13: Development
		(*Grande Ballroom C*)
		CS14: Fibrosis, Proteolysis, and Tissue Repair
		(*Nautilus I*)

		CS15: Glycomics/Chemical Biology II: Analytical Methods (*Grande Ballroom B*) **CS16: Host-Pathogen Interactions** (*Nautilus II*)
	7:00 – 10:00 pm	**Banquet (ticketed event)** *(Grande Ballroom C)*
Wednesday, November 14	7:30 – 8:30 am	**Continental Breakfast** *(Grande Foyer and Grande Ballroom A)*
	7:30 – 8:30 am	**Special Guest Lecture: Jennifer Zeitzer, Director of Legislative Relations, FASEB Office of Public Affairs** **Biomedical Research: A Perspective from Washington, DC** (*Grande Ballroom B & C)*
	8:30 – 10:00 am	**Plenary V: Rare Glycosylation and Matrix Diseases** *(Grande Ballroom B & C)*
	10:00 – 10:30 am	**Coffee Break** *(Grande Foyer and Grande Ballroom A)*
	10:30 – 12:00 pm	**Plenary VI: Development and Morphogenesis** *(Grande Ballroom B & C)*

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Joint Meeting of the Society for Glycobiology & American Society for Matrix Biology

November 11–14, 2012
Sheraton Hotel and Marina
San Diego, CA
Organizers: Jeff Davidson, Hudson Freeze, Robert Haltiwanger, David Roberts, Jean Schwarzbauer

SUNDAY, NOVEMBER 11

8:00 am – 7:30 pm
Registration *(Grande Foyer)*

8:00 am – 6:00 pm
Speaker Ready Room and Cyber Café *(Marina 6)*

8:30 am – 1:30 pm
SFG Guest Symposia I Advances in Glycoprotein Production, Characterization and Modification *(Nautilus II)*
Co-Chairs: Qun Zhou (Genzyme), John Briggs (Genentech), and Sam Tep (Biogenidec)
Registration required: $50 (includes scientific program, workshop and coffee breaks)

8:00 am – 12:00 pm
ASMB Council Meeting (by invitation only) *(Marina 5)*

9:00 am – 5:30 pm
ASMB Guest Symposia I - Wound Healing Society
Quantifying Heterogeneity and Modeling Complexity in the Injury Response.
Implications for Therapeutics Development
Andrew Baird, *UC San Diego* (*Nautilus I*)
*Registration Required: $50 WHS members/ $100 WHS non-members

12:00 – 5:30 pm
SFG Guest Symposia II - Consortium for Functional Glycomics (CFG) Participating Investigator Meeting
James Prestegard, *University of Georgia* and *(Grande Ballroom B)*
Registration for CFG Satellite will open March 15 ($50 – CFG membership not required)

Time	
12:00	CFG Participating Investigator Meeting
12:30	Get acquainted lunch – organized by CFG subgroup leaders
2:00	Symposium: Polysaccharide Analysis, Synthesis and Pathophysiology

12:00 – 7:00 pm
Exhibits *(Grande Ballroom A)*

1:00 – 4:00 pm
ASMB Guest Symposia II - TERMIS: Tissue Engineering & Regenerative Medicine International Society
Organized by Anthony Ratcliffe, Synthasome, Inc and Karen Christman, UCSD *(Grande Ballroom C)*
Matrix for Vascular and Lung Regeneration
Laura E. Niklason, *Yale University*
Extracellular Matrix Scaffolds for Airway Repair
Thomas W. Gilbert, *University of Pittsburgh*

Injectable Extracellular Matrix Based Hydrogels for Treating Cardiovascular Disease
Karen L. Christman, *University of California San Diego*
The Extracellular Matrix in Biomaterials Design and Tissue Engineering
Jennifer H. Elisseeff, *Johns Hopkins University*

3:00 – 5:00 pm
SFG Board Meeting (by invitation only) *(Marina 5)*

6:00 – 7:00 pm
Opening Reception *(Grande Foyer and Grande Ballroom A)*

7:00 – 8:00 pm
ASMB and SFG Society Welcome *(Grande Ballroom B & C)*
Chairs: Hudson Freeze and Jean Schwarzbauer
Keynote Lecture Chairs: Hudson Freeze and Jeff Davidson
Bioorthogonal Chemistry for Glycoprofiling and Beyond
Carolyn Bertozzi, *University of California, Berkeley*

MONDAY, NOVEMBER 12

7:00 am – 5:00 pm
Registration *(Grande Foyer)*

7:30 – 8:30 am
Continental Breakfast *(Grande Foyer and Grande Ballroom A)*

7:30 – 8:30 am
Career Mentoring Breakfast (*pre-registration required) *(Marina 5)*

7:30 am – 5:30 pm
Exhibits *(Grande Ballroom A)*

8:30 – 10:00 am
Plenary I: Stem and Progenitor Cells, Genetic Therapy and Regenerative Medicine *(Grande Ballroom B & C)*
Chair: Linda Sandell, *Washington University Medical School*

Abstract #	*Time*	
	8:30	**Glycobiology of Human Pluripotent Stem Cells;** Steve Dalton, *University of Georgia*
#1	9:00	**Siglec-Sialoglycan Binding Regulates Cell-Cell Interactions;** Ronald Schnaar, *Johns Hopkins School of Medicine*
	9:30	**Stem Cell Therapies in Epidermolysis Bullosa;** Angela Christiano, *Columbia University Medical Center*

10:00 – 10:30 am
Coffee Break *(Grande Foyer and Grande Ballroom A)*

10:30 am – 12:00 pm
Plenary II: Biomaterials and Matrix Engineering *(Grande Ballroom B & C)*
Chair: Adam Engler, *University of California, San Diego*

	10:30	**Hydrogels as Synthetic Extracellular Matrices;** Kristi Anseth, *University of Colorado at Boulder*
	11:00	**Mechanical Regulation of Cell Adhesion and Function;** Christopher Chen, *University of Pennsylvania*

11:30 **Biofunctional Hydrogels: ECM-mimetic Materials for Tissue Repair;** Andres Garcia, *Georgia Institute of Technology*

12:00 – 2:00 pm
Poster Session I Lunch *(Nautilus III, IV, V)*

12:00 – 1:30 pm
Glycobiology Editorial Board Meeting Lunch
(by invitation only) *(Spinnaker I/II)*

2:00 – 3:30 pm
Concurrent Sessions 1 – 4

CS1: Matrix and Carbohydrate Immunology *(Grande Ballroom C)*
Chair: Jim Paulson

Abstract #	*Time*	
#2	2:00	**Siglecs in B cell Tolerance;** Jim Paulson, *The Scripps Research Institute*
		Selected Talks
#3	2:30	*SFG Travel Award Winner* **Glycosylation of IgG by B cells is Regulated by Immune Stimuli;** Alison Mahan, *Ragon Institute of MGH, MIT and Harvard*
#4	2:50	**Identification of endogenous lung proteins bearing glycan counter-receptors for mouse Siglec-F;** Toshihiko Katoh, *Complex Carbohydrate Research Center, University of Georgia*
#5	3:10	**Listeria monocytogenes subverts 2-O-sulfated HS domains in syndecan-1 to promote its infection** Rafael Aquino, *Children's Hospital, Harvard Medical School*

CS2: Matricellular Proteins *(Nautilus I)*
Chair: David Roberts

Abstract #	*Time*	
#6	2:00	**Thrombospondin-1 signaling through CD47 regulates cell and tissue radiosensitivity via autophagy** David Roberts, *NIH, NCI, CCR*
		Selected Talks
#7	2:30	**The search for proteins bound to C-mannosylated TSR-derived peptides involved in the regulation of TGF-β signaling in cultured fibroblasts;** Yoshito Ihara, *Wakayama Medical University*
#8	2:50	**Collagen signaling promotes hypoxia induced tumor progression in murine models of pancreatic cancer;** Lee Rivera, *UT Southwestern Medical Center*
#9	3:10	**Matrix-associated glycoprotein 2 (MAGP2) binds TGFβ superfamily ligands and MAGP2 loss-of-function has pleiotropic effects in vivo;** Michelle D. Combs, *Washington University School of Medicine*

CS3: Receptors, Signaling, and Cytoplasmic Glycosylation
(Grande Ballroom B)
Co-Chairs: Ambra Pozzi and Gerald Hart

Abstract #	*Time*	
	2:00	**Role of Collagen Binding Receptor in Renal Fibrosis;** Ambra Pozzi, *Vanderbilt University*
		Selected Talks
#10	2:30	***SFG Travel Award Winner*** **O-GlcNAc regulated arginine methylation: a new paradigm in survival signaling;** Albert Lee, *The Johns Hopkins University*
#11	2:50	**Combating Adaptation to Stretch Conditioning Through Prolonged Activation of Extracellular Signal-Regulated Kinase;** Justin Weinbaum, *University of Minnesota*
#12	3:10	**Recognition of hyaluronan by Siglec-9 blunts neutrophil activation: Molecular mimicry by group A Streptococcus;** Ismael Secundino-Vélazquez, *Glycobiology Research and Training Center, University of California, San Diego*

CS4: Vascular Biology, Elastic Fibers, and Related Diseases
(Nautilus II)
Chair: Cecilia Giachelli

Abstract #	*Time*	
	2:00	**Vascular Matrix Calcification;** Cecilia Giachelli, *University of Washington*
		Selected Talks
#13	2:30	***ASMB Travel Award Winner*** **COL4A1 and COL4A2 mutations cause abnormal angiogenesis and genetically modifiable Cerebrovascular Diseases;** Marion Jeanne, *University of California, San Francisco (UCSF)*
#14	2:50	**Expression of V3 versican in arterial smooth muscle cells reprograms cell phenotypes by modulating TGF-β- and EGF-induced signaling pathways;** Inkyung Kang, *Benaroya Research Institute*
#15	3:10	**Extracellular regulation of BMP signaling by fibrillin microfibrils;** Alexander Wohl, *University of Cologne, Germany*

3:30 – 4:00 pm
Coffee Break
(Grande Foyer and Grande Ballroom A)

4:00 – 5:30 pm
Concurrent Sessions 5–8

CS5: Carbohydrate and Matrix Metabolism in Disease States
(Grande Ballroom C)
Chair: Marian Young

Abstract #	*Time*	
	4:00	**Key Roles of Proteoglycans and their Partners in Skeletal Homeostasis and Disease;** Marian Young, *NIH, NIDCR*
		Selected Talks
#16	4:30	**Patient fibroblast alpha-dystroglycan glycosylation studies: A versatile tool for identification of novel muscular dystrophy disease genes;** Tobias Willer, *Howard Hughes Medical Institute, University of Iowa*
#17	4:50	**Glycomic analysis of cell lineages derived from Salt-and-Pepper Syndrome iPS Cells;** Kazuhiro Aoki, *Complex Carbohydrate Research Center, University of Georgia*
#18	5:10	**An absence of ADAMTS5 significantly reduces degenerative changes in dystrophic skeletal muscle;** Chantal Coles, *Murdoch Childrens Research Institute*

CS6: Basement Membranes
(Nautilus I)
Chair: Taina Pihlajaniemi

Abstract #	*Time*	
	4:00	**Collagen XIII – A Novel Neuromuscular Junction Component;** Taina Pihlajaniemi, *University of Oulu*
		Selected Talks
#19	4:30	**Prolyl 3-hydroxylation of type IV collagen is obligatory to avoid early embryonic lethality in mice;** Elena Pokidysheva, *Shriners Hospital for Children*
#20	4:50	**Fibrillin-1 deficiency in cardiac basement membrane zone causes myopathy in Marfan Syndrome;** Jason Cook, *Mount Sinai School of Medicine*
#21	5:10	**Feasibility of Repairing GBM Defects in Alport Syndrome;** Jeffrey Miner, *Washington University School of Medicine*

CS7: Genetics and Gene Expression
(Grande Ballroom B)
Chair: Kelley Moremen

Abstract #	*Time*	
	4:00	**Regulation and Recombinant Production of Mammalian Glycosylation Enzymes;** Kelley Moremen, *University of Georgia*
		Selected Talks
#22	4:30	**Nervous system-specific sialylation in Drosophila;** Hilary Scott, *Texas A&M University*
#23	4:50	**Epigenetic Silencing of Cosmc in Leukocytes Expressing Tn Antigen;** Rongjuan Mi, *Emory University School of Medicine*
#24	5:10	**Macrophage Proteoglycans: Decrease in Sulfation Results in Accentuated Atherosclerosis;** Philip L.S.M. Gordts, *University of California, San Diego*

CS8: Mechanobiology and Biomedical Engineering
(Nautilus II)
Chair: Thomas Barker

Abstract #	*Time*	
	4:00	***ASMB Junior Investigator Awardee***
		Mechanochemical Signaling of the ECM; Thomas Barker, *Georgia Institute of Technology*
		Selected Talks
#25	4:30	**Matrix Elasticity and Cell Geometry Regulate Beta-catenin Signalling in Mesenchymal Stem Cells;** Silvia Goldoni, *Imperial College London*
#26	4:50	**Evaluating fibronectin conformation based on biochemical and mechanical cues;** Brant Hubbard, *Boston University*
#27	5:10	**Myoepithelial cells mediate the balance between cytoskeletal tension and extracellular stiffness during mouse submandibular salivary gland development;** Sarah Peters, *University at Albany, SUNY*

6:00 – 7:30 pm
SFG Award Lectures
(Grande Ballroom C)
Chair: Hudson Freeze

Abstract #	*Time*	
	6:15	**Rosalind Kornfeld Award Lecture**
		Some Face Lifting of NANA
		Werner Reutter, *Charité - Universitätsmedizin Berlin*
	6:45	**Karl Meyer Award Lecture**
		Recognizing Sugars
		Kurt Drickamer, *Imperial College*

6:00 – 8:00 pm
ASMB Award Lectures
(Grande Ballroom B)
Chairs: Jean Schwarzbauer and ISMB President
Join us for the presentation of the ASMB Junior and Senior Investigator awards and lecture presentations by the ASMB Senior and ISMB Distinguished Investigator Awardees.

Abstract #	*Time*	
	6:00	**ASMB Senior Investigator Award Collagen IV Glycoprotein: An Essential Innovation at the Dawn of Tissue Evolution;** Billy Hudson, *Vanderbilt University*
	6:45	**ISMB Distinguished Investigator Award**
		Sponsored by The International Society of Matrix Biology (ISMB)
		Decoding the Matrix; Richard Hynes, *Massachusetts Institute of Technology*
		ISMB Travel Awards
#28		Vanessa López-Alpuche, *University of Oulu, Oulu, Finland*
#29		Alessandro Malara, *University of Pavia, Pavia, Italy*
#30		Wei Xin, *University of Cologne, Cologne, Germany*

TUESDAY, NOVEMBER 13

7:00 am – 5:00 pm
Registration
(Grande Foyer)

7:30 – 8:30 am
Continental Breakfast
(Grande Foyer and Grande Ballroom A)

7:30 – 8:30 am
Career Mentoring Breakfast (*pre-registration required)
(Marina 5)

7:30 am – 6:00 pm
Exhibits
(Grande Ballroom A)

8:30 – 10:00 am
Plenary III: Biosynthesis, Secretion and Assembly
(Grande Ballroom B & C)
Chair: Robert Haltiwanger, *Stony Brook University*

Abstract #	*Time*	
	8:30	**Stabilizing Proteins by N-Glycosylating their Reverse Turns is also a Prescription for Increased N-Glycosylation Efficiency;** Jeffery Kelly, *The Scripps Research Institute*
	9:00	**Imaging the Secretory Pathway and its Dynamics;** Jennifer Lippincott-Schwartz, *NICHD*
	9:30	**Collagen Glycosylation;** Theirry Hennet, *University of Zurich*

10:00 – 10:30 am
Coffee Break
(Grande Foyer and Grande Ballroom A)

10:30 am – 12:00 pm
Plenary IV: Immunology and Inflammation
(Grande Ballroom A & B)
Chair: Michael Hawkeye Pierce, *University of Georgia*

Abstract #	*Time*	
	10:30	**The Lymphocyte Homing Cascade Updated**; Steve Rosen, *University of California, San Francisco*
	11:00	**Integrins and the Immune Response;** Yoji Shimizu, *University of Minnesota*
#31	11:30	**Regulation of Leukocyte Migration through Galectin-Glycan Interactions;** Linda Baum, *UCLA*

12:00 – 1:00 pm
National Academy of Sciences Luncheon – Future of Glycoscience
(Spinnaker) *pre-registration required

12:00 – 2:00 pm
Poster Session II Lunch
(Nautilus III, IV, V)

2:00 – 2:30 pm
ASMB Business Meeting
(Grande Ballroom C)
Chair: Jean Schwarzbauer

2:00 – 2:30 pm
SFG Business Meeting
(Grande Ballroom B)
Chair: Hudson Freeze

2:30 – 4:00 pm
Concurrent Sessions 9–12

CS9: Stem/Progenitor Cells and Their Environment
(*Grande Ballroom C*)
Chair: Hiroshi Nakato

Abstract #	*Time*	
	2:30	**Heparan Sulfate Proteoglycans in Gradients and Niche;** Hiroshi Nakato, *University of Minnesota*
		Selected Talks
#32	3:00	**ST6Gal-I sialyltransferase is highly upregulated in epithelial cancers, and correlates with stemness in both normal and cancer stem cell populations;** Amanda F. Swindall, *University of Alabama at Birmingham*
#33	3:20	**Substrate Stiffness Dependent Vinculin Activation Modulates Mechanosensitive Stem Cell Differentiation**; Andrew Holle, *UC San Diego*
#34	3:40	**Epithelial Stem Cell and ECM Dynamics in C. elegans Molting Cycles;** Alison Frand, *University of California, Los Angeles*

CS10: Biosynthesis, Protein Folding, Secretion, and Matrix Assembly
(*Nautilus I*)
Chair: Lance Wells

Abstract #	*Time*	
	2:30	**Remodeling of Glycans on Mammalian Cells,** Lance Wells, *University of Georgia*
		Selected Talks
#35	3:00	**Structural Basis of Procollagen Trimerization and Regulation of C-terminal Processing;** Jean-Marie Bourhis, *CNRS/University of Lyon 1, Lyon, France; University Joseph Fourier/EMBL/CNRS, Grenoble, France*
#36	3:20	**Glycosyltransferase-specific Golgi targeting mechanisms;** Armen Petrosyan, *University of Nebraska Medical Center*
#37	3:40	***ASMB Travel Award Winner*** **Fibronectin matrix assembly promotes mesenchymal cell condensation during chondrogenic differentiation;** Purva Singh, *Princeton University*

CS11: Cancer Microenvironment
(Grande Ballroom B)
Chair: Rick Cummings

Abstract #	*Time*	
#38	2:30	**Altered O-Glycan Expression in Tumor Glycoproteins: Genetics and Consequences;** Rick Cummings, *Emory University*
		Selected Talks
#39	3:00	**Heparanase promotes myeloma bone disease by regulating the bone microenvironment;** Jian Ruan, *Nanfang Hospital, China; University of Alabama at Birmingham*
#40	3:20	**Basement membrane localized tumor cells are protected from HER2-targeted therapy in vivo;** Jason Zoeller, *Harvard Medical School*
#41	3:40	***Tabor Award Winner*** **The Extracellular Matrix: Proteomic Signatures of Breast Cancer Progression and Metastasis;** Alexandra Naba, *MIT - Koch Institute for Integrative Cancer Research*

CS12: Glycomics/Chemical Biology I: Function
(*Nautilus II*)
Chair: Linda Hsieh-Wilson

Abstract #	*Time*	
	2:30	**Chemoenzymatic Tools for Glycan Detection and Their Applications to Biology;** Linda Hsieh-Wilson, *Caltech/Howard Hughes Medical Institute*
		Selected Talks
#42	3:00	**Competition between core-2 GlcNAc transferase and ST6GalNAc transferase regulates the synthesis of the leukocyte selectin-ligand on P-selectin glycoprotein ligand-1;** Sriram Neelamegham, *State University of New York*
#43	3:20	**Glycoengineering at the surface of living cell;** Nicolai Bovin, *Shemyakin Institute of Bioorganic Chemistry RAS, Moscow, Russia*
#44	3:40	***SFG Travel Award Winner*** **Engineering Carbohydrate Recognizing Biosensors via Computational Modeling and Directed Evolution**; Kausar N. Samli, *The University of Georgia*

4:00 – 4:30 pm
Coffee Break
(Grande Foyer and Grande Ballroom A)

4:30 – 6:00 pm
Concurrent Sessions 13–16

CS13: Development
(*Grande Ballroom C*)
Chair: Maurizio Pacifici

Abstract #	*Time*	
	4:30	**Matrix macromolecules in limb synovial joint development and function** Maurizio Pacifici, *Thomas Jefferson University*
		Selected Talks
#46	5:00	**Adamtsl2 modulates the ratio of fibrillin-1 to fibrillin-2 in tissue microfibrils in a mouse model for human geleophysic dysplasia;** Dirk Hubmacher, *Lerner Research Institute, Cleveland Clinic Foundation*
#47	5:20	***SFG Travel Award Winner*** **Altered Activity and Localization of Cathepsin K Underlies the Cartilage Defects in a Zebrafish Model of Mucolipidosis II;** Aaron Petrey, *University of Georgia*
#48	5:40	**Glycan glucuronylation is essential for female fertility in Drosophila melanogaster;** Marion Marshall, *Complex Carbohydrate Research Center, University of Georgia*

CS14: Fibrosis, Proteolysis, and Tissue Repair
(*Nautilus I*)
Chair: Paul Noble

Abstract #	*Time*	
	4:30	**Regulation of Pulmonary Fibrosis by Hyaluronan Synthase 2** Paul Noble, *Duke University*
		Selected Talks
#49	5:00	***ASMB Travel Award Winner*** **Granzyme B-mediated loss of decorin increases collagen fibril irregularity and susceptibility to aortic rupture in a mouse model of abdominal aortic aneurysm;** Lisa Ang, *University of British Columbia*
#50	5:20	***ASMB Travel Award Winner*** **Calreticulin is required for TGF-beta stimulation of extracellular matrix;** Kurt Zimmerman, *University of Alabama at Birmingham*
#51	5:40	**Restoring laminin polymerization by transgenic expression of αLNNd in skeletal muscle improves muscle integrity of laminin-α2-deficient mice;** Stephanie Crosson, *Robert Wood Johnson Medical School*

CS15: Glycomics/Chemical Biology II: Analytical Methods
(*Grande Ballroom B*)
Chair: Anne Dell

Abstract #	*Time*	
	4:30	**Glycomics and Glycoproteomics: status quo and quo vadis** Anne Dell, *Imperial College*
		Selected Talks
#52	5:00	***SFG Travel Award Winner*** **Detailed structural investigation of beta-glucans from yeast cell walls by electron-transfer dissociation;** Liang Han, *Boston University*
#53	5:20	***SFG Travel Award Winner*** **MS based insight in glycobiology: tyrosine kinase inhibitors alter the cell surface glycosylation patterns of lung cancer cell lines;** L. Renee Ruhaak, *University of California, Davis*
#54	5:40	**Il-6 Induction By Lipoteichoic Acids of the Immuno-Protective Lactococcus Lactis G121 is Tlr2 Independent;** Kathleen Fischer, *Division of Structural Biochemistry-Research Center Borstel*

CS16: Host-Pathogen Interactions
(*Nautilus II*)
Chair: Lora Hooper

Abstract #	*Time*	
	4:30	**Lectin-Mediated Defense of the Intestinal Epithelial Surface** Lora Hooper, *UT Southwestern*
		Selected Talks
#55	5:00	**Mucosal immunity mediated by antibody-mucin crosslinking;** Samuel Lai, *University of North Carolina,Chapel Hill*
#56	5:20	**A new model for T cell dependent immune activation by glycoconjugate vaccines;** Fikri Y. Avci, *Harvard Medical School*
#57	5:40	**Group B Streptococcus uses Sialic Acid Mimicry to Subvert Host Innate Immune Responses Through Engagement of Inhibitory Leukocyte Receptor Siglec-E;** Yung-Chi Chang, *Glycobiology Research and Training Center, University of California, San Diego*

7:00 – 10:00 pm
Banquet (ticketed event)
(Grande Ballroom C)

WEDNESDAY, NOVEMBER 14

7:00 am – 12:00 pm
Registration
(Grande Foyer)

7:30 – 8:30 am
Continental Breakfast
(Grande Foyer and Grande Ballroom A)

7:30 – 8:30 am
Special Guest Lecture: Jennifer Zeitzer, Director of Legislative Relations, FASEB Office of Public Affairs
Biomedical Research: A Perspective from Washington, DC
(*Grande Ballroom B & C)*

7:30 – 12:00 pm
Exhibits
(Grande Ballroom A)

8:30 – 10:00 am
Plenary V: Rare Glycosylation and Matrix Diseases
(Grande Ballroom B & C)
Chair: Elaine Davis, *McGill University*

Abstract #	*Time*	
	8:30	**The Physiological Function of Heparan Sulfate: Insights that can be gained from the study of multiple hereditary exostoses;** Yu Yamaguchi, *Sanford Burnham Medical Research Institute*
	9:00	**Matrix-Cell Interactions and Ascending Aortic Aneurysms;** Hiromi Yanigasawa, *University of Texas Southwestern Medical Center*
	9:30	**Exome Sequencing for Novel Disease Gene Discovery in Families with Rare Mendelian Phenotypes;** Sessions Cole, *Washington University, St. Louis*

10:00 – 10:30 am
Coffee Break
(Grande Foyer and Grande Ballroom A)

10:30 am – 12:00 pm
Plenary VI: Development and Morphogenesis
(Grande Ballroom B & C)
Chair: Roy Zent, *Vanderbilt Medical Center*

Abstract #	*Time*	
#45	11:00	**Roles for Glycans in Mammalian Development and Spermatogenesis;** Pamela Stanley, *Albert Einstein College of Medicine*
	11:30	**Protein Glycosylation in ECM formation and Organogenesis;** Kelly TenHagen, *NIH*
	12:00	**The Role of MMP-17 Ortholog in Embryonic Zebrafish Development;** Raman Ramachandran, *Children's Hospital of Wisconsin*

MONDAY, NOVEMBER 12

Poster Session 1:
12:00-2:00 pm
(Nautilus III, IV, V)
CS1: Matrix and Carbohydrate Immunology

Poster Topic
CS2: Matricellular Proteins

Poster Topic
CS3: Receptors, Signalnig, and Cytoplasmic Glycosylation

Poster Number *Abstract Number*

Poster Topic
CS4: Vascular Biology, Elastic Fibers, and Related Diseases

Poster Number *Abstract Number*

Poster Topic
CS5: Carbohydrate and Matrix Metabolism in Disease States

Poster Number *Abstract Number*

Poster Topic
CS6: Basement Membranes

Poster Number *Abstract Number*

Poster Topic
CS7: Genetics and Gene Expression

Poster Number *Abstract Number*

Poster Topic
CS8: Mechanobiology and Biomedical Engineering

Poster Number *Abstract Number*

Poster Topic
CS9: Stem/Progenitor Cells and their Environment

Poster Number *Abstract Number*

Poster Topic
CS10: Biosynthesis, Protein Folding, Secretion, and Matrix Assembly

Poster Number *Abstract Number*

Poster Topic
CS11: Cancer Microenvironment

Poster Number *Abstract Number*

Poster Topic
CS12: Glycomics/Chemical Biology I: Function

Poster Number *Abstract Number*

Poster Topic
CS13: Development

Poster Number *Abstract Number*

Poster Topic
CS14: Fibrosis, Proteolysis, and Tissue Repair

Poster Number *Abstract Number*

Poster Topic
CS15: Glycomics/Chemical Biology II: Analytical Methods

Poster Number *Abstract Number*

Poster Topic
CS16: Host-Pathogen Interactions

Poster Number *Abstract Number*

Tuesday, NOVEMBER 13

Poster Session 2:
12:00-2:00 pm *(Nautilus III, IV, V)*

Poster Topic
CS1: Matrix and Carbohydrate Immunology

Poster Number *Abstract Number*

Poster Topic
CS2: Matricellular Proteins

Poster Number *Abstract Number*

Poster Topic
CS3: Receptors, Signaling, and Cytoplasmic Glycosylation

Poster Number *Abstract Number*

Poster Topic
CS4: Vascular Biology, Elastic Fibers, and Related Diseases

Poster Number *Abstract Number*

Poster Topic
CS5: Carbohydrate and Matrix Metabolism in Disease States

Poster Number *Abstract Number*

Poster Topic
CS6: Basement Membranes

Poster Number *Abstract Number*

Poster Topic
CS7: Genetics and Gene Expression

Poster Number *Abstract Number*

Poster Topic
CS8: Mechanobiology and Biomedical Engineering

Poster Number *Abstract Number*

Poster Topic
CS9: Stem/Progenitor Cells and their Environment

Poster Number *Abstract Number*

Poster Topic
CS10: Biosynthesis, Protein Folding, Secretion, and Matrix Assembly

Poster Number *Abstract Number*

Poster Topic
CS11: Cancer Microenvironment

Poster Number *Abstract Number*

Poster Topic
CS12: Glycomics/Chemical Biology I: Function

Poster Number *Abstract Number*

Poster Topic
CS13: Development

Poster Number *Abstract Number*

Poster Topic
CS14: Fibrosis, Proteolysis, and Tissue Repair

Poster Number *Abstract Number*

Poster Topic
CS15: Glycomics/Chemical Biology II: Analytical Methods

Poster Number *Abstract Number*

Poster Topic
CS16: Host-Pathogen Interactions

Poster Number *Abstract Number*

(1) Siglec-Sialoglycan Binding Regulates cell–Cell Interactions

Ronald Schnaar
Johns Hopkins University School of Medicine

Vertebrate cell surfaces feature a rich glycan coat comprised of glycoproteins, glycolipids and proteoglycans. Glycoproteins and glycolipids are often terminated with sialic acid (*N*-acetylneuraminic acid, NeuAc), a favored component of larger glycan determinants involved in cell–cell recognition. Sialic acid-dependent binding proteins on one cell engage sialoglycans on an apposing cell to initiate cell–cell recognition. Siglecs—sialic-acid-binding immunoglobulin-like lectins—are a family of 14 sialic acid binding proteins in humans. They are expressed distinct sub-populations of blood cells, except Siglec-4 (myelin-associated glycoprotein, MAG), which is expressed only in the nervous system on the inner-most wrap of myelin, directly across from nerve cell axons. MAG binds preferentially to the "NeuAcα2-3Galβ1-3GalNAc" sequence prominent on two major sialoglycans widely distributed in the vertebrate brain - gangliosides GD1a and GT1b. Direct binding identifies GD1a and GT1b as MAG counter-receptors, and functional studies in vitro and in vivo implicate these gangliosides in maintaining myelin stability, protecting axons from toxic environmental insults and inhibiting axon regeneration after injury. GD1a and GT1b are synthesized by the transfer of sialic acid to the 3-hydroxyl of the terminal galactose of GM1 and GD1b respectively. There are six sialyltransferases in mice and humans that transfer sialic acid to the 3-hydroxyl of galactose, coded by *St3gal1* through *St3gal6*. We identified *St3gal2* and *St3gal3* as being responsible for GD1a and GT1b biosynthesis. *St3gal2*-null mice express half the GD1a/GT1b of normal mice and have normal sialoglycoprotein levels; *St3gal3*-null mice have normal GD1a/GT1b levels, but a 36% decrease in sialic acid on glycoproteins. *St3gal2*/*St3gal3*-double null mice are essentially devoid of GD1a/GT1b and have half the normal amount of sialic acid on glycoproteins. The double-null mice are growth retarded, have small brains, impaired reflexes and are short-lived. Although required for long-term stability and maintenance of axon-myelin interactions, temporarily blocking MAG-sialoglycan binding by delivery of sialidase enhances axon outgrowth, leading to improved outcomes after spinal cord injury. Knowledge of Siglec-4 and its sialoglycan ligands as both stabilizing and inhibitory molecules may provide opportunities to understand nervous system disorders and improve outcomes from traumatic nerve injury. Supported by NINDS grants NS037096 and NS057338.

(2) Siglecs in B cell Tolerance

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Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA, USA

Maintenance of peripheral B cell tolerance to self-antigens is still poorly understood, and of high interest with respect to the roles of B cells in autoimmune diseases. The B cell receptor is highly regulated by co-receptors that aid in distinguishing between self and non-self and minimize inappropriate activation to self-antigens. B cell siglecs, CD22 and Siglec-G/10, are unique among inhibitory co-receptors, which recognize sialic acid-containing glycans as self-ligands that are expressed on all mammalian cells. Previously we showed that multivalent presentation of high-affinity CD22 ligands in cis with a T-independent antigen can induce tolerance to B cells (Duong *et al.*, J. Exp. Med., 2010). To investigate the relevance to T-dependent antigens we developed a novel liposomal nanoparticle platform for presentation of both antigen and synthetic siglec ligands specific for CD22 or Siglec-G. Using this platform, robust B cell tolerance is achieved towards both T-independent and T-dependent antigens in mice. Tolerance is also achieved by presentation of antigens on cells expressing natural ligands of siglecs. Mechanistic studies demonstrate that tolerance induction is siglec-dependent and is the result of the induction of apoptosis of the antigen-specific B cells. These studies support the hypothesis that CD22 and Siglec-G/10 participate in maintaining peripheral B cell tolerance by recognition of sialic acid containing glycans as self. This method has potential for antigen-specific tolerization of B cells in a therapeutic setting. (NIH grants AI050143, AI099141, CA013889 and HFSP Fellowship LT001099/2010-L)

(3) Glycosylation of IgG by B cells is regulated by immune stimuli

Alison Mahan, Kendall Dionne, Todd Suscovich, Galit Alter
Ragon Institute of MGH, MIT and Harvard

The Fc region of IgG contains a single N-glycosylation site, which is known to be important for regulating the immune response through interaction with Fc receptors and complement molecules. Changes to the structure of these sugars can thus be very influential in determining the specific effector function of individual antibodies. While therapeutic antibodies can be designed with particular effector functions in vitro, little is known regarding how glycosylation is regulated in primary B cells. Purified B cells were stimulated with a variety of innate and adaptive stimuli. Quantitative RT-PCR was used to determine expression of genes known to be specifically involved in IgG *N*-glycan synthesis. Antibody purified from these supernatants was subjected to glycan analysis using capillary electrophoresis to identify changes in glycan structures after B cell stimulation. Additionally, mice were immunized against a haptenated antigen with an adjuvant based on the most interesting stimuli identified in the in vitro experiments described (TLR7/8 and TLR9 stimulation). Longitudinal plasma samples were collected and the IgG glycans of bulk and specific antibodies were analyzed by capillary electrophoresis on an ABI 3130Xl sequencer. We found that the expression of glycosylation genes is significantly impacted both by specific TLR stimulation alone and in combination with adaptive signals. Specifically, virus-derived stimuli that activate TLR 7, 8 or 9 can significantly decrease the expression of galactose adding enzyme B4GALT1, whereas TLR 9 stimulation in combination with CD40 stimulation decreases the expression of both sialic acid or GlcNac-adding enzymes ST6GAL1 and MGAT3, respectively. These changes in expression result in the production of antibodies

with Fc glycan structures with increased NK cells and monocyte-recruiting capabilities. In the mouse model, we found that there was an overall trend toward an inflammatory structure in the antigen-specific population of the conditions tested. Overall, these results are the first to show that the production of antibodies with specific effector functions can be regulated by external stimuli, including both innate and adaptive immune signals, suggesting that antibodies with specific, strong effector functions can be induced in vivo following vaccination.

(4) Identification of endogenous lung proteins bearing glycan counter-receptors formouse Siglec-F

Toshihiko Katoh·[1], Takumi Kiwamoto[2], Mary Brummet[2], Bruce Bochner[2], Michael Tiemeyer[1]
[1]Complex Carbohydrate Research Center, University of Georgia, Athens, GA; [2]The Johns Hopkins School of Medicine, Baltimore, MD

Lung inflammatory diseases (LID), such as asthma and chronic obstructive pulmonary disease (COPD), dramatically impact quality of life and affect large numbers of individuals. In both of these LIDs, overly exuberant myelocytic inflammatory responses in the lung are observed (eosinophils in asthma and neutrophils in COPD). Addressing the underlying mechanism of inflammation is a key to developing effective therapeutics. One or more members of the siglec family of carbohydrate binding proteins are expressed by myelocytes in LIDs. In particular, human Siglec-8 (mouse functional paralog Siglec-F) is expressed by eosinophils (as well as mast cells and basophils). Antibodies that cross-link Siglec-8/F induce apoptosis of eosinophils, indicating the potential for inducing therapeutic anti-inflammatory responses in asthma through siglec engagement. Glycan array screening has previously demonstrated that Siglec8/F binds the structural determinant known as 6'-sulfo-sialyl-Lewis X (6'-Su-SLe^x). However, the lung proteins that carry these determinants have not previously been identified. We have now identified Siglec-F-binding proteins from mouse tracheal epithelial cells (mTECs) and mouse lung tissue homogenates. Neuraminidase-sensitive binding of Siglec-F-Fc to proteins of MW 500 kD was detected for mTEC lysate and mTEC culture supernatants. In-gel tryptic digestion and subsequent LC-MS/MS analysis of materials pulled-down with Siglec-F-Fc identified a mucin as a candidate for a Siglec-F ligand carrier protein. Immunoprecipitation with an anti-mucin antibody and subsequent overlay analysis with Siglec-F-Fc confirmed that the mucin protein bears Siglec-F glycan ligands. We also characterized the *O*-linked glycan profile of the lung mucin protein using an optimized in-gel β-elimination protocol and nanospray ionization MS^n.

(5) Listeria monocytogenes subverts 2-*O*-sulfated HS domains in syndecan-1 topromote its infection

Rafael Aquino·[1], Jian Liu[2], Pyong Woo Park[1]
[1]Children's Hospital, Harvard Medical School; [2]University of North Carolina at Chapel Hill

Cell surface heparan sulfate proteoglycan (HSPG)-pathogen interactions are widely thought to promote the pathogenesis of infectious diseases. However, precisely how HSPGs promote infection and whether bacterial pathogens exploit specific HSPGs or HS structures remain to be determined. We examined the role of syndecans in a mouse model of *Listeria monocytogenes* (*Lm*) infection. *Lm* is a Gram-positive bacterial pathogen that has been suggested to exploit cell surface HSPGs for its attachment and internalization. The liver bacterial burden was significantly lower in syndecan-1 null (*Sdc1*$-/-$) mice comaped with those of wild-type (Wt), *Sdc3*$-/-$ and *Sdc4*$-/-$ mice infected with *Lm*, indicating that syndecan-1 is the HSPG that specifically promotes *Lm* infection. However, Wt and *Sdc1*$-/-$ mice showed a similar tissue bacterial burden at early times post-infection, and *Lm* adhesion and internalization in isolated Wt and *Sdc1*$-/-$ hepatocytes were also similar. Furthermore, addition of syndecan-1 or HS or syndecan-1 knockdown had little effect on *Lm* adhesion and internalization in cultured cells, indicating that syndecan-1 does not mediate these early steps of *Lm* infection. Instead, *Lm* infection induced syndecan-1 shedding in hepatocytes and significantly increased serum levels of syndecan-1 ectodomains in vivo in Wt mice. Importantly, the administration of purified syndecan-1 ectodomain or HS, but not CS or core protein, significantly enhanced *Lm* virulence in *Sdc1*$-/-$ mice. Among the selectively modified heparan compounds, heparin chemically desulfated at the *N*- or 6-*O*-position and heparosan chemoenzymatically sulfated at the *N*and 2-*O*-position significantly increased the susceptibility of *Sdc1*$-/-$ mice to listeriosis, whereas 2-*O*-desulfated heparin, *N*-sulfated heparosan and heparosan did not. These findings indicate for the first time that 2-*O*-sulfated domains in HS promote *Lm* infection in vivo. Our data suggest a new bacterial subversion mechanism where *Lm* induces syndecan-1 shedding and exploits specific HS modifications in syndecan-1 ectodomains to promote its pathogenesis.

(6) Thrombospondin-1 signaling through CD47 regulates cell and tissue radiosensitivity via autophagy

David Soto-Pantoja·[1], Thomas Miller[1], Michael Pendrak[1], William DeGraff[2], Camille Sullivan[1], Lisa Ridnour[2], Mones Abu-Asab[1], David Wink[2], Maria Tsokos[1], David Roberts[1]
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Accidental or therapeutic exposure to ionizing radiation has severe physiological consequences and can result in extensive cell death. We previously demonstrated that deficiency or thrombospondin-1 or its receptor CD47 results in profound cell and tissue protection against ischemic and radiation stress. Antagonists of CD47 or thrombospondin-1 enhance cell survival and preserve their proliferative capacity. However, the signaling pathways that mediate this cell autonomous radioprotection were unclear. We now report a marked increase in autophagy in irradiated T cells and endothelial cells lacking CD47. Irradiated T cells lacking CD47 exhibit significant increases in formation of autophagosomes comprising double membrane vesicles visualized by electron microscopy and numbers of MAP1A/1BLC3$^+$ puncta. Moreover, we observed significant increases in beclin-1, ATG5, ATG7 and a reduction in p62/sequestosome expression relative to irradiated wild-type T cells. Blockade of CD47 in combination with total body radiation similarly increased autophagy gene expression in mouse lung

while suppressing apoptosis in the same tissue. Pharmacological or siRNA-mediated inhibition of autophagy selectively sensitized CD47-deficient cells to radiation, indicating that enhanced autophagy is necessary for the prosurvival response to CD47 blockade. Moreover, re-expression of CD47 in CD47-deficient T cells sensitized these cells to death by ionizing radiation and reversed the increase in autophagic flux associated with survival. Therefore, CD47 deficiency confers cell survival through the activation of a protective autophagic flux, and CD47 blockade is a pharmacological route to modulate autophagy for tissue radioprotection.

(7) The search for proteins bound to C-mannosylated TSR-derived peptides involved in the regulation of TGF-β signaling in cultured fibroblasts

Yoshito Ihara[1], Midori Ikezaki[1], Yoko Inai[1], In-Sook Matsui[1], Eiji Muroi[2], Yukinao Shibukawa[3], Yoshinao Wada[3], Shino Manabe[4], Yukishige Ito[4]

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Thrombospondin type 1 repeat (TSR) is a functional module of proteins called TSR superfamily proteins (e.g. thrombospondin-1 (TSP-1), Fspondin, mindin, ADAMTS-like 1 etc.) and includes a conserved Trp-x-x-Trp motif, in which the first Trp residue is preferably modified by Cmannosylation. In terms of the structural characteristics and functions of TSP-1, the Trp-x-x-Trp motif in the TSR is believed to play an important role in the binding of TSP-1 with molecules such as heparin, fibronectin and TGF-β. However, the biological function of protein C-mannosylation in the TSR is not fully understood. To investigate whether C-mannosylation in the TSR plays functional roles in the cell, we examined the effect of synthesized C-mannosylated TSR-derived peptides (e.g. C-Man-Trp-Ser-Pro-Trp) on TGF-β signaling in NRK-49F fibroblast cells. We found that TGF-β-induced cell proliferation was suppressed by the C-mannosylated peptides, comaped with the peptides without mannose. In the presence of the C-mannosylated peptides, TGF-β-induced phosphorylation and nuclear accumulation of smad2 were suppressed, resulting in decrease in the expression of collagen type I. These results suggest that C-mannosylated TSR plays a functional role in the regulation of TGF-β signaling. We also searched specific binding partners for C-mannosylated peptides in the cells and isolated several candidate proteins. Peptide mass finger printing followed by a data base search suggested that the proteins are plectin, myosin I heavy chain, Hsc70 and vimentin. We will also demonstrate our results concerning the functional relevance of the target proteins to C-mannosylated peptides in the TGF-β-induced cell signaling.

(8) Collagen signaling promotes hypoxia induced tumor progression in murine modelsof pancreatic cancer

Lee Rivera, Juliet Carbon, Jason Toombs, Kristina Aguilera, Gaurab Chakrabarti, Jordan Braunfeld, Rolf Brekken

UT Southwestern Medical Center

Evidence that anti-angiogenic therapy can enhance local invasion of primary tumors and metastasis is mounting. However, the molecular mediators responsible for these events are unclear. We report that VEGF-blockade controlled primary pancreatic ductal adenocarcinoma (PDAC) growth but induced tumor hypoxia, which resulted in a mesenchymal primary tumor phenotype and liver dissemination. Further, VEGF-blockade enhanced collagen deposition and collagen signaling in primary PDAC. The increase in collagen deposition resulted from hypoxia induced TGFβ expression and was sensitive to TGFβ blockade. We propose that collagen signaling via discoidin domain receptors (DDR1/2) facilitates TGFβ-mediated epithelial to mesenchymal transition (EMT) of PDAC tumor cells. In vitro, collagen promoted PDAC EMT and protected PDAC cells from TGFß-induced apoptosis. The matricellular protein SPARC orchestrates collagen deposition and reduces collagen-cell interaction. We show that SPARC inhibits collagen binding to DDRs and that collagen itself can drive EMT in the absence of SPARC in vitro. To model the effect of enhanced collagen signaling in the absence of other alterations in vivo we assessed tumor growth in SPARC-deficient mice. Tumor growth was accelerated and more aggressive in the absence of SPARC in implant and genetic models of PDAC. These findings implicate that collagen signaling through DDRs participates in PDAC progression. To perturb tumor progression upstream of collagen signaling we evaluated HIF inhibition with metronomic doxorubicin, which blunted tumor growth and hypoxia induced EMT. Studies are currently ongoing to identify downstream mediators of DDR signaling in PDAC tumors. Our results implicate DDRs as attractive targets for inhibition in PDAC.

This work was supported by NIH RO1 CA118240 and the Effie Marie Cain Scholarship in Angiogenesis Research.

(9) Matrix-associated glycoprotein 2 (MAGP2) binds TGFβ superfamily ligands and MAGP2 loss-of-function has pleiotropic effects in vivo

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Changes in ECM protein expression and regulation affect cell survival, differentiation, senescence, and cell signaling, as well as growth factor bioavailability. It is therefore no surprise that mutations in proteins of the ECM are increasingly linked to human disease. Microfibrils are a major component of most ECM and are comprised of fibrillins and accessory proteins such as growth factor complexes, fibulins, and matrix-associated glycoproteins (MAGPs). The MAGPs (MAGP1 and MAGP2) are structurally related but evolutionarily divergent proteins that bind covalently to microfibrils. This combined with overlapping but distinct expression patterns suggest MAGPs may have shared as well as unique functions *in vivo*. Using mice with a targeted inactivation of Mfap5, the gene for MAGP2 protein, we demonstrate this is indeed the case. MAGP2-deficient mice (2KO) appear grossly

normal, are fertile, and have no reduction in life span. Cardiopulmonary development in 2KO animals is unremarkable. They are normotensive and have vascular compliance comparable to age-matched wild type mice. These data are indicative of normal, functional elastic fibers in 2KO mice. 2KO animals are neutropenic, which contrasts with monocytopenia described in MAGP1-deficient murine models. This suggests that MAGP1 and MAGP2 have discrete functions in hematopoiesis. 2KO mice have normal bone mass and loss of MAGP2 is able to rescue MAGP1-defient mice from osteopenia, demonstrating that MAGP2 and MAGP1 function at cross-purposes in the regulation of bone homeostasis. Further, DKO mice have larger aorta and carotid artery diameter at physiological pressures, and this enlargement increases with age. This indicates that MAGPs have overlapping functions in maintaining large vessel integrity. Finally, using recombinant protein we show MAGP2 homodimerization and binding of active TGFβ1, active TGFβ2, and BMP2. Together, these data demonstrate loss of MAGP2 expression *in vivo* has pleiotropic effects likely related to MAGP2's ability to regulate growth factor bioavailability.

(10) *O*-GlcNAc-regulated arginine methylation: a new paradigm in survival signaling

Albert Lee, Devin Miller, Roger Henry, Natasha Zachara
The Johns Hopkins University, School of Medicine

The modification of nuclear, mitochondrial and cytoplasmic proteins by *O*-linked β-*N*-acetylglucosamine (*O*-GlcNAc) has emerged as a novel regulator of cell survival and cardioprotection. Notably, the elevation of *O*-GlcNAc levels is protective in both in vivo and in vitro models of heat stress, oxidative stress, hypoxia, ischemia reperfusion injury and trauma hemorrhage. However, the mechanisms by which *O*-GlcNAc regulates protein function leading to enhanced cell survival have not been defined. In this study, we have used Stable Isotope Labeling of Amino Acids in Cell Culture (SILAC), in combination with an *O*-GlcNAc immunoprecipitation, to screen for proteins whose O-GlcNAcylation status changes in response to hydrogen peroxide treatment (a mimic of ischemia reperfusion injury). We identified >90 proteins that were putatively *O*-GlcNAc modified, including the protein arginine methyltransferases PRMT1 and PRMT 4 (also known as Carm1). Notably, when *O*-GlcNAc levels were lowered by overexpression of *O*-GlcNAcase or deletion of OGT, arginine methylation was increased on a subset of cellular proteins. These data suggest that *O*-GlcNAc may regulate cell survival by altering the activity of PRMT1 and PRMT4 and subsequent methylation of non-histone proteins. Interestingly, PRMT1 is thought to promote cell death by methylating proteins such as BAD and Foxo, preventing their phosphorylation by Akt. Consistent with a hypothesis in which *O*-GlcNAc promotes AKT phosphorylation and survival, the pro-apoptotic protein BAD is hypophosphorylated in the OGT null and this can be reversed by the inhibition of methyltransferases. The studies described highlight a potential molecular mechanism by which *O*-GlcNAc protects cells from oxidative damage via the tripartite interplay between *O*-GlcNAc, methylation and phosphorylation.

(11) Combating adaptation to stretch conditioning through prolonged activation of extracellular signal-regulated kinase

Justin Weinbaum, Jill Schmidt, Robert Tranquillo
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In developing an implantable tissue-engineered artery based on cellular remodeling of a fibrin scaffold, a key measure of success is high final collagen content. Cellular collagen synthesis is promoted by cyclic distension during the remodeling process but is limited by adaptation to the stretching stimulus (Syedain et al. 2010). Cellular adaptation to stretching is mediated by the deactivation of extracellular signal-regulated kinase (ERK); therefore, a chemical method for prolonging ERK activation should improve stretch-induced collagen production and accelerate the development of a strong bioartificial artery. The hypothesis of this study is that p38 mitogen-activated protein kinase (p38) activation by stretching inhibits ERK and that a chemical inhibitor of p38, SB 203580, will therefore lead to higher ERK activation and subsequent collagen production. Inhibition of p38 during cyclic stretching of cell monolayers increased ERK activation in a dose-responsive manner; one of the effective inhibitory doses, 5 μM, was selected for studies using cells in fibrin scaffolds. Pretreatment with the p38 inhibitor increased short-term ERK activation in biaxially stretched, but not static, fibrin-based tissue constructs. When constructs were exposed to 3 weeks of incremental amplitude cyclic stretching, p38 inhibition led to an increase in total collagen vs untreated controls. Future studies will investigate whether SB treatment will also lead to increased collagen content and superior mechanical strength in stretch-conditioned bioartificial arteries. In conclusion, we have found a chemical method to circumvent adaptation that may be useful in engineering tissues where collagen-endowed mechanical strength is a priority.

Reference

Syedain ZH, Weinberg JS, Tranquillo RT. 2008. Cyclic Distension of Fibrin-Based Tissue Constructs: Evidence of Adaptation During Growth of Engineered Connective Tissue. *Proc Natl Acad Sci USA*. 105(18): 6537–42.

(12) Recognition of hyaluronan by Siglec-9 blunts neutrophil activation: Molecularmimicry by group A *Streptococcus*

Ismael Secundino-Vélazquez, Xiaoxia Wang, K Markus Roupé, Ajit Varki, Victor Nizet
Glycobiology Research and Training Center, University of California, San Diego

Inhibitory CD33-related Siglecs (Sialic acid-binding Ig-like lectins) are a family of receptors expressed on innate immune cells that dampen immune response via engagement of sialylated glycans, which act as "self-associated molecular patterns" (SAMPs). During a screen for Siglec recognition of human pathogens, we found that group A *Streptococcus* (GAS) was recognized by Siglec-9, via its capsular hyaluronan (HA) polysaccharide. This interaction was blocked by soluble HA, but not by heparan sulfate or heparin. Both anti-CD44 and anti-Siglec-9 blocking Abs decreased HA binding to neutrophils. We therefore explored

recognition of HA by Siglec-9 and its impact on innate immune responses. We found prominent binding of high-molecular weigh HA to a Siglec-9-Fc chimera, significantly lower degrees of binding to Siglec-5- or -11-Fc, and no binding to Siglec-7-Fc. Recognition of HA by Siglec-9 was independent of an Arg residue in the V-set domain, which is critical for Sia binding. Blocking and non-blocking antibodies confirmed that Sia- and HA-binding sites are distinct. Interaction of Siglec-9 on normal human neutrophils with HA results in diminished formation of NETs [visualized by immunofluorescence using anti-human myeloperoxidase and DNA staining (DAPI), a decrease in oxidative burst (measured by FACS), blunting of IL-6 secretion and increase in IL-10 production (determined by ELISA)]. As the HA capsule of GAS is identical to mammalian HA, we hypothesized that engagement by Siglec-9 might suppress neutrophil activation. Indeed, the HA of an invasive disease-associated GAS serotype M1T1 strain promoted bacterial resistance to neutrophil killing. Thus, Siglec-9 is the first example of a Siglec that recognizes a glycan other than Sias, and HA may act as a SAMP in vivo, to dampen self-reactivity of innate immune cells. As with Sias on other bacteria, GAS capsular molecular mimicry of HA is a mechanism to suppress innate immune recognition/activation and may contribute to invasive infections in humans.

(13) COL4A1 and COL4A2 mutations cause abnormal angiogenesis and genetically modifiable cerebrovascular diseases

Marion Jeanne[1], Jeff Jorgensen[1], Cassandre Labelle-Dumais[1], Yi-Chinn Weng[1], W Berkeley Kauffman[1], Michelle de Leau[1], Steven M Greenberg[2], Jonathan Rosand[2], Jack Favor[3], Douglas B Gould[1]

[1]University of California, San Francisco (UCSF), CA, USA; [2]Massachusetts General Hospital Boston, MA, USA; [3]Helmholtz Zentrum Munchen, Neuherberg, Germany

Patients with mutations in the collagen type IV α 1 gene (*COL4A1*) suffer from a broad spectrum of highly penetrant cerebrovascular diseases (CVDs), including subclinical cerebral microbleeds, leukoaraiosis, infantile porencephaly and fatal intracerebral hemorrhage (ICH). COL4A1 associates with COL4A2 in heterotrimers before being secreted into the extracellular matrix where they form a network critical for basement membrane stability and function. We recently screened a cohort of patients with non-familial, spontaneous ICH. We identified novel *COL4A1* mutations, and importantly, we discovered the first *COL4A2* mutations causing hemorrhagic stroke. Notably, these data suggest that *COL4A1* and *COL4A2* mutations could contribute to 5–10% of non-traumatic, sporadic ICHs. We are now using cell biological and genetic approaches to identify the pathogenic mechanisms underlying the disease. Using a cell-based assay, we found that mutations impair COL4A1 and COL4A2 secretion. We showed that mutant COL4A1 or COL4A2 proteins accumulate within cells where they titrate normal COL4A1 and COL4A2 and can ultimately trigger ER stress and the unfolded protein response. We also found that the intracellular accumulation of COL4A1 and COL4A2 proteins and/or their absence in the vascular basement membrane cause abnormal angiogenesis and impair the maintenance of the vascular network. Indeed, mutant mice have tortuous blood vessels with abnormal branching and, develop age-related cerebrovascular lesions. Using cerebral magnetic resonance imaging and histological analysis, we determined that the hemorrhage localization and severity were dependent on the position and nature of the mutation in the Col4a1 or Col4a2 gene and also on their genetic context. Our data suggest that allelic heterogeneity and the presence of genetic modifiers contribute to the CVD heterogeneity in human patients. Our ongoing experiments to better understand the cell biology of *COL4A1* and *COL4A2* mutations and the mechanisms of genetic modification could lead to targeted therapeutics to reduce the risk of CVD.

(14) Expression of V3 versican in arterial smooth muscle cells reprograms cellphenotypes by modulating TGF-β-and EGF-induced signaling pathways

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Inflammatory response consisting of leukocyte sequestration and emigration is one of the key factors in the progression of cardiovascular disease. This is accompanied with a loss of elastic fibers which are extremely difficult to regenerate. Versican is a large chondroitin sulfate proteoglycan that is highly upregulated during vascular disease progression. We have previously shown that arterial smooth muscle cells (SMCs) expressing the non-chondroitin sulfate isoform of versican, V3, had significantly enhanced elastin expression and deposition while resisting monocyte/ macrophage adhesion and accumulation *in vitro* and *in vivo*. However, the mechanisms by which V3 expression promotes elastogenesis and resists inflammation were not clear. To address these mechanisms, we determined signaling pathways affected by expressing V3 in SMCs that are involved in tropoelastin expression and monocyte adhesion in the present study. Expression of V3 induced an upregulation of TGF-β receptor I (TGFBRI) and TGF-β target gene plasminogen activator inhibitor 1 (PAI-1), and when stimulated with TGF-β, enhanced smad2/3 phosphorylation. Inhibition of this pathway using a TGFBRI inhibitor, SB431542, abolished the upregulation of tropoelastin mRNA in V3-expressing SMCs. V3-expressing SMCs had reduced activation of Erk1/2 MAPK and NFkB when stimulated by EGF. V3-expressing SMCs also had reduced activation, expression, and nuclear accumulation of c-Jun, suggesting that V3 may affect activity of AP-1 transcription factor. These findings are consistent with previous reports that tropoelastin transcript levels are stabilized by signaling pathways mediated by TGF-β while antagonized by EGF-MAPK as well as AP-1 signaling. On the other hand, V3-expressing SMCs had reduced levels of inflammatory mediators such as CCL2, ICAM-1, and VCAM-1. This may be due to the reduction of NFkB, MAPK, and AP-1 activity in V3expressing SMCs. Our findings suggest that expressing V3 in SMCs reprograms cell phenotypes, promoting pro-elastogenic and anti-inflammatory pathways, which may provide novel therapeutic interventions for cardiovascular diseases.

(15) Extracellular regulation of BMP signaling by fibrillin microfibrils

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Our previous studies have helped to establish the novel concept that fibrillin containing microfibrils serve as important spatio-temporal extracellular scaffolds actively targeting and sequestering growth factors of the TGFβ superfamily. We could demonstrate that bone morphogenetic proteins (BMPs) form growth factor complexes with their processed prodomains which then target BMPs as prodomain/growth factor complexes to fibrillin microfibrils. However, the biological function of these interactions is unknown. Our goal is to test whether interaction with fibrillin confers latency to BMP growth factors and is required in vivo for appropriate regulation of BMP signaling. These experiments are crucial for understanding the pathomechanisms of the fibrillinopathies. Both in vitro and in vivo approaches were used. The molecular requirements for the BMP prodomain–fibrillin interaction were biochemically explored. Fibrillin-1 mutant mice (GT-8) carrying a dominant negative fibrillin-1 truncation mutation were analyzed. Biochemical data indicated that fibrillin-1 can confer latency to the BMP-7 prodomain–growth factor complex. In vivo analyses showed that the C-terminal truncation of fibrillin-1 in mice leads to increased fragmentation of elastic fibers in the skin and aorta in post-natal life accompanied with increased with canoncical and non-canonical BMP signaling in these tissues. Together, these data show for the first time that fibrillin is required to limit BMP activity. We hypothesize that this control over BMP activity is exerted through proper targeting of BMP complexes and also through sequestration of BMP activity by fibrillin. BMP signaling may be activated by proteases as cells respond to their mutant microfibril matrix environment.

(16) Patient fibroblast α-dystroglycan glycosylation studies: A versatile tool for identification of novel muscular dystrophy disease genes

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Walker-Warburg syndrome (WWS) is clinically defined as congenital muscular dystrophy accompanied by a variety of brain and eye malformations. It represents the most severe clinical phenotype in a spectrum of α-dystroglycan post-translational processing abnormalities, which share a defect in laminin binding glycan synthesis. Although six WWS causing genes have been described, only half of all patients can currently be diagnosed genetically. We now report that skin fibroblasts can be utilized to analyze the status of α-dystroglycan functional glycosylation, which is lost or reduced in dystroglycanopathy patient cells. To identify and validate the disease causing genes in dystroglycanopathy patients we developed a functional complementation assay based on adenoviral gene transfer into patient fibroblasts. We also examined cell fusion complementation assays using fibroblasts from undiagnosed WWS individuals and identified five novel complementation groups. Further evaluation of one group by linkage analysis and targeted sequencing identified recessive mutations in the isoprenoid synthase domain containing (*ISPD*) gene. Confirmation of the pathogenicity of the identified *ISPD* mutations was demonstrated by complementation of fibroblasts with wild-type ISPD. Finally, we provide conclusive genetic and biochemical evidence that recessive mutations in *ISPD* abolish the initial step in laminin binding glycan synthesis by disrupting dystroglycan *O*-mannosylation. Further studies are needed to determine how defects in ISPD influence protein *O*-mannosylation, as this is the first WWS gene without proposed glycosyltransferase activity and direct role in α-dystroglycan glycosylation. This establishes a novel mechanism for WWS pathophysiology.

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(17) Glycomic analysis of cell lineages derived from salt-and-pepper syndrome iPS cells

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Human disorders, such as Tay-Sachs, Gauchers, Fabry and the gangliosidoses, are associated with impaired degradation of glycosphingolipids (GSL), but the diversity of these lysosomal storage disorders is not matched by identified disorders involving GSL biosynthesis. Thus, excessive GSL seems to be more well tolerated than GSL loss in humans. We have identified a glycosylation-related human disorder that affects a GSL-specific biosynthetic enzyme, the sialyltransferase St3gal5 (GM3 synthase) which produces the simplest ganglioside, GM3. Patients with this mutation in the St3gal5 gene exhibit a complex range of phenotypes including profound intellectual disability, failure to thrive, early lethality, seizures and altered cutaneous pigmentation, leading to the name "Salt-and-Pepper" syndrome (S&PS). In contrast, knockout of the enzyme in mouse generated a viable, fertile animal with relatively mild phenotypic consequences. Thus, the value of the mouse is somewhat limited for investigating the impact of altered GM3 biosynthesis in humans. To further understand the contribution of GSL deficiency to the pathology of S&PS and, by extension, to gain greater insight into the normal function of complex gangliosides, we have investigated GSL expression changes in S&PS and normal fibroblasts. We have demonstrated that GM3 is absent from the GSL profile of S&PS patient fibroblasts and that compensatory changes are detected, particularly as increased sialylation of *N*- and *O*-linked glycans. Furthermore, we generated iPS cells from S&PS fibroblasts and differentiated these cells into neural crest cells, a cell type closely related to the disease phenotype. Glycomic and phenotypic analyses of these cell types, along with the characterization of gene knockdown in zebrafish, suggest that a defect in the regulation of neural apoptosis may underlie the major pathophysiology of S&PS.

(18) An absence of ADAMTS5 significantly reduces degenerative changes in dystrophic skeletal muscle

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Duchenne muscular dystrophy (DMD) is a devastating childhood disorder which is characterized by progressive muscle loss and the accumulation of connective tissue. Dampening the inflammatory response is a recognized way of reducing the devastating loss of skeletal muscle in DMD. We have recently shown that the expression of ADAMTS5 is dynamically regulated during skeletal muscle regeneration. This suggested a potential role for ADAMTS5mediated extracellular matrix remodeling in the muscular dystrophy phenotype. We tested this hypothesis by crossing mdx and Adamts5 knockout mice and saw a striking improvement in muscle pathology in mdx:Adamts5$^{-/-}$ mice. There were fewer necrotic fibers identified by IgG staining, and serum creatine kinase, used as a marker of muscle damage, was lower, indicating reduced pathological permeability of the muscle cell membrane. In addition, mdx:Adamts5$^{-/-}$ mice had fewer fibers with central nuclei, a marker of past muscle damage, degeneration and regeneration. Macrophage infiltration is reduced in mdx:Adamts5$^{-/-}$ muscle at 12 weeks of age (50% reduction). This suggests that inflammation is a major pathway modulated in mdx:Adamts5$^{-/-}$ muscle, and critically, is the first indication that ADAMTS5 activity may be proinflammatory. This strongly supports the hypothesis that inflammation and fibrosis is mediated or enhanced by a cleaved ADAMTS5 substratum. This indicates that our studies are likely to have implications well beyond DMD. Inhibiting ADAMTS5 may improve outcomes in many other muscular dystrophies where inflammation is increased and, even more broadly, in other conditions involving unregulated inflammation including wound healing, liver cirrhosis and cardiovascular fibrosis. These data demonstrate that ADAMTS5 activity is detrimental in mdx muscle and open up a new therapeutic approach for muscular dystrophy-inhibiting ADAMTS5.

(19) Prolyl 3-hydroxylation of type IV collagen is obligatory to avoid early embryoniclethality in mice

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120 Prolyl 3-hydroxylation is a crucial posttranslational modification of collagens which is accomplished by prolyl 3-hydroxylases (P3Hs). There are three members in this family of enzymes: P3H1, P3H2, and P3H3. Every family member is thought to work on specific collagen type. P3H1 predominantly modifies type I collagen. Deficiencies in P3H1 have been shown to cause recessive osteogenesis imperfecta. Previous studies have identified type IV collagen as a main substrate for P3H2. To investigate the role of prolyl 3hydroxylase 2 and the function of 3-hydroxyl groups in collagen IV, we created a P3H2 knockout mouse. We demonstrate that 3hydroxylation of collagen IV is required to avoid an aberrant interaction with the platelet specific glycoprotein VI (GPVI) resulting in platelet aggregation, thrombosis of the maternal blood and death of embryo at E6.5. Homozygous P3H2 null embryos die before E8.5. We show that lethal phenotype can be rescued by producing P3H2 nulls on a GPVI null background. Double nulls are viable and fertile. Thus 3-hydroxylation of collagen IV is indispensable for embryonic development. Our findings provide the first molecular function for the 3-hydroxylation in collagens. Preliminary characterization of P3H2/ GPVI double nulls shows abnormalities in basement membrane-rich tissues.

(20) Fibrillin-1 deficiency in cardiac basement membrane zone causes myopathy in Marfan Syndrome

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Although generally thought to be a secondary manifestation of MFS, ventricular dysfunction without evidence of valvular disease

has raised the possibility of an inherent myocardial insufficiency. Studies of mice with early onset of progressively severe MFS (*Fbn1*$^{mgR/mgR}$ mice) supported this hypothesis, thereby providing the first genetic evidence of a causal connection between normal cardiac function and integrity of the basement membrane zone (BMZ) around cardiomyocytes. Histological and physiological analyses demonstrated that *Fbn1*$^{mgR/mgR}$ mice display dilated cardiomyopathy (DCM) prior to the emergence of aortic aneurysm and valvular regurgitation. Moreover, DCM was evident in mice with *Fbn1* inactivation in cardiomyoblasts, but not in the forming aortic arch or cardiac valves. Loss of immunoreactive fibrillin-1 in the basement membrane zone (BMZ) of *Fbn1*-null cardiomyocytes, together with reduced phosphorylation of focal adhesion kinase (FAK) in the muscle, correlated myocardial insufficiency with impaired cell–matrix interactions that may in turn lead to persistent mechanical stress from the pericellular microenvironment. Consistent with angiotensin II type I receptor (AT1r) role as a cardiac mechanosensor, DCM was rescued in *Fbn1*$^{mgR/mgR}$ mice that were treated with the AT1r antagonist losartan or in *Fbn1*$^{mgR/mgR}$ mice that also lacked AT1r. No DCM improvement was instead noted in *Fbn1*$^{mgR/mgR}$ mice treated with telmizartan, an AT1r antagonist that principally targets angiotensin II-stimulated signaling. Collectively, these genetic and pharmacological data indicate that fibrillin-1 deposited in the BMZ of cardiomyocytes protects the mammalian heart against DCM by providing structural support for muscle function.

(21) Feasibility of repairing GBM defects in Alport syndrome

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The glomerular basement membrane (GBM) is a crucial component of the kidney's glomerular filtration barrier to plasma albumin. The GBM's major components are the collagen IV α3/4/5 network, laminin-521, nidogen-1 and -2 and agrin. Of these 9 components, mutations that affect 4 of them cause human kidney disease: *COL4A3*/*A4*/*A5* mutations cause Alport syndrome, and *LAMB2* mutations cause Pierson syndrome/congenital nephrotic syndrome. The existence of well-characterized mouse models makes these diseases especially attractive for investigating therapies. Several groups have explored the potential for cell-based therapies aimed at replacing the missing collagen IV network in Alport mice. Although the positive effects of bone marrow and other cell transplants or infusions on progression of kidney disease are promising, the fate of the infused cells and the reported effects of these procedures on the GBM's collagen IV network have been disparate, difficult to interpret and controversial. We have used podocyte-specific and ubiquitously expressed reverse tetracycline transactivator (rtTA) transgenic mice and a novel TetO7-regulated collagen IV α3 transgene in *Col4a3*$^{-/-}$ Alport mice to attempt to induce collagen IV network restoration either before or after GBM maturation. We have generated novel nephrin-rtTA-3G transgenic mice with improved podocyte-specific doxycycline-induced gene activation. Prenatal induction of Col4a3 expression by doxycycline in *Col4a3*$^{-/-}$ Alport mice decreased proteinuria and BUN and greatly extended life span by promoting collagen α3/4/5(IV) deposition and assembly in the GBM. Post-natal induction of Col4a3 expression at P14 also promoted collagen α3/4/5(IV) network assembly, with significant improvements in proteinuria, BUN and life span. Even induction starting as late as P21 improved kidney function by reducing BUN and extending life span. (i) Post-natal incorporation of collagen α3/4/5(IV) into the mutant Alport GBM is feasible. (ii) Even partial restoration of the collagen IV network slows progression to ESRD. The results of these feasibility studies have implications not only for treating human kidney disease, but also for our basic understanding of BM biology, GBM plasticity and cell–matrix interactions.

(22) Nervous system-specific sialylation in *Drosophila*

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Sialylation has numerous roles in the development and physiology of animals. These roles include regulation of such important processes as immune responses to pathogens, interactions between different cells in the nervous and immune systems, various cell adhesion, recognition and signaling events. While many of these roles have been extensively studied in mammalian organisms, molecular and genetic mechanisms underlying these roles often remain not well understood due to the complexity of mammalian glycosylation and limitations of available in vivo approaches. In our project, we focus on sialylation in Drosophila, a model organism with simplified glycosylation pathways. We previously characterized the function of DSiaT, a sole Drosophila sialyltransferase closely related to mammalian ST6Gal enzymes. DSiaT has a nervous system-specific expression and regulates neural transmission and synaptic development. Using genetic approaches, we employed a candidate-based screen to identify genes collaborating with DSiaT. Several genes involved in the regulation of neural excitability have been identified, including some voltage gated channel genes and genes involved in cell adhesion. Interestingly, we found genetic interactions between DSiaT and between *sialyltransferase* and *β1,4-N-acetylgalactosaminyltransferase-A* (GalNAcT-A). Our results indicated that they function in the same pathway and suggested that β4GalNAcT-A may participate in the biosynthesizes of *N*-glycans, including sialylated glycans. We also characterized interactions between DSiaT and CSAS, a Drosophila CMP-Sia synthetase. Our results provided evidence that CSAS is involved in the sialylation pathway in vivo. They also revealed that CSAS expression is tightly controlled in the CNS, which suggested that CSAS mediates a regulatory function within the pathway. We will discuss new data that elucidate the regulation and function of the sialylation pathway in the nervous system and shed light on the evolutionary relation between Drosophila sialylation and the sialylation in mammalian organisms. This project was supported by NIH/NS075534 to VP.

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(23) Epigenetic silencing of Cosmc in leukocytes expressing Tn antigen

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Cosmc is the specific molecular chaperone in the endoplasmic reticulum for T-synthase, a Golgi enzyme responsible for synthesizing the core 1 *O*-glycan Galβ1-3GalNAcα-Ser/Thr in glycoproteins. Dysfunctional *Cosmc*, and consequently inactive T-synthase, is associated with several human diseases where the abnormal Tn antigen (GalNAcα1-Ser/Thr) is expressed, but the molecular regulation of *Cosmc* expression, encoded by a single gene on Xq24, is poorly understood. Here, we report that epigenetic silencing of *Cosmc* leads to loss of *Cosmc* transcripts through hypermethylation of its promoter. Tn4 cells, an immortalized B cell line from a male patient with Tn-syndrome-like phenotype lack *Cosmc* transcripts, lose T-synthase activity and express the Tn antigen. Treatment of cells with 5-aza-2'-deoxycytidine causes restoration of *Cosmc* transcripts, restores T-synthase activity and reduces Tn antigen expression. Bisulfite sequencing shows that CG dinucleotides in the *Cosmc* core promoter are hypermethylated. Interestingly, several other X-linked genes are not silenced in Tn4 cells and we observed no correlation of DNA methyltransferases to aberrant methylation of *Cosmc* in Tn4 cells. Thus, hypermethylation of the *Cosmc* promoter in Tn4 cells is specific. Epigenetic silencing of *Cosmc* provides another mechanism for cells to express Tn antigen, which is important in understanding its aberrant expression in human diseases, including immunoglobulin A nephropathy and cancer.

(24) Macrophage Proteoglycans: Decrease in Sulfation Results in AccentuatedAtherosclerosis

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Atherogenesis initiates by retention of atherogenic lipoproteins within the vessel wall. Macrophage uptake of these atherogenic lipoproteins subsequently triggers the formation of foam cells and plaque deposition. In order to examine the role of macrophage heparan sulfate proteoglycans in atherogenesis and foam cell formation, we inactivated the biosynthetic gene GlcNAc N-deacetylase/ N-sulfotransferase 1 (*Ndst1*) selectively in macrophages by crossing *Ndst1$^{f/f}$* mice with *LysMCre^{+}* mice. When bred onto an *Ldlr$^{-/-}$* background and placed on an atherogenic diet, *Ndst1$^{f/f}$ LysMCre^{+} Ldlr$^{-/-}$* mice demonstrated increased atherosclerosis compared to *Ldlr$^{-/-}$*mice. Plaque analysis also revealed significantly increased macrophage content in lesions from *Ndst1$^{f/f}$ LysMCre^{+} Ldlr$^{-/-}$* mice. Diminished sulfation of HSPGs in bone marrow-derived macrophages (BMDMs) from Ndst1$^{f/f}$ LysMCre^{+} mice resulted in significantly increased expression of inflammatory genes such as *Ccl5, Ccl7, Ccl8 and Ccl12*. Aggregated LDL induced foam cell formation was increased compared to BMDMs from wild-type mice. Binding and uptake of aggregated LDL were not affected, but reduction of sulfation of heparan sulfate proteoglycans reduced HDL-mediated cholesterol efflux, suggesting that heparan sulfate participates in reverse cholesterol transport. These findings indicate that one or more heparan sulfate proteoglycans participate in macrophage inflammation and foam cell conversion, with severe adverse effects on cardiovascular disease when changes in heparan sulfate composition occur.

(25) Matrix elasticity and cell geometry regulate β-catenin signaling in mesenchymal stem cells

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Changes in tissue compliance and architecture cooperate with soluble factors in modulating cell behavior (Wang et al. 2009). For instance, the contribution of mechanical stimuli to cell migration and differentiation is pivotal during embryogenesis and the progression of pathologies such as cancer (Nelson et al. 2006) and fibrosis (MacKenna et al. 2000). Therefore, understanding the molecular mechanisms governing how cells translate physical cues into functional responses is necessary. The role of integrins and the cytoskeleton in cellular mechano-sensing has been demonstrated (Ingber and Tensegrity 2003); however, it is still unclear how these elicit cell-specific responses. β-catenin, a co-transcription factor essential during embryogenesis and adult tissue homeostasis, is a key regulator of bone development and repair (Day et al. 2005). In this study, we explore mechanical regulation of β-catenin signaling in mesenchymal stem cells (MSCs) and pre-osteoblasts to understand its role in osteogenic commitment. By using two established bio-engineering platforms, hydrogels with tunable mechanical properties (Engler et al. 2006) and micro-patterned surfaces (Chen 1997), we show for the first time that β-catenin signaling is modulated by changes in substratum stiffness and cell geometry. MSCs cultured for 24h on collagen I-coated polyacrylamide gels with osteoid matrix-like stiffness, as measured by atomic force microscopy, accumulate significantly more β-catenin in the nuclei comaped with cells grown on more complaint substratums. MSCs plated on triangular, square and circular collagen I or fibronectin islands, unexpectedly, showed 50% more nuclear β-catenin in round cells or square cells treated with the ROCK inhibitor Y-27632. Shape-dependent differences in βcatenin localization were evident only after 6h following adhesion and spreading and persisted up to 24 h. Moreover, the underlying mechanism was Wntindependent. Preliminary data suggested the involvement of plasma membrane lipid dynamics and caveolin-1 in β-catenin signaling regulated by cell geometry. These findings introduce novel mechanisms of β-catenin regulation in MSCs and are likely to have a broader biomedical impact and direct tissue engineering applications (Mager et al. 2011).

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(26) Evaluating fibronectin conformation based on biochemical and mechanical cues

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Fibronectin fibers are a prevalent component of the extracellular matrix during development and wound healing that have been shown to fold into non-equilibrium protein conformations in vitro. Two factors important in altering fibronectin conformation are allosteric regulators, such as heparan sulfate and mechanical strain. The complex glycosaminoglycans heparin and heparan sulfate have been shown to affect a region of Fn type III modules in the heparin 2 binding domain (modules IIIto III) that are important for binding vascular endothelial growth factor to fibronectin matrices. Here, we describe a technique for monitoring fibronectin conformation in its fibrillar state that can be used to evaluate conformational changes in the presence of allosteric regulators and mechanical strain. This approach is based on measuring the ratio of two fluorescent antibodies; one that is insensitive to fibronectin conformational changes and a second whose binding is altered or enhanced by non-equilibrium conformational changes. Immunohistochemistry of fibronectin fibers that were tuned to different conformations with a strain device or treated with heparin was used to identify an array of commercially available antibodies that demonstrate different affinities for Fn based on heparin exposure (A32, MAB1935), strain independence (C6F10, AHP08) and strain dependence (L8). Multiple antibodies with different conformation specificities allow fibronectin conformation to be monitored in biological samples by quantitatively measuring the intensity ratios of the antibodies in a sample. Future use of this technique will allow examination of fibronectin conformational dynamics during development, tissue injury and disease. The dual antibody intensity ratio will provide insight and a better overall understanding of the impact of heparan sulfate and mechanical strain on fibronectin conformation under physiological conditions. Supported in part by NIH R01 HL088572.

(27) Myoepithelial cells mediate the balance between cytoskeletal tension and extracellular stiffness during mouse submandibular salivary gland development

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Current research in mechanotransduction has focused primarily on isolated mesenchymal cells, but little research has attempted to determine the interplay between these cells and the epithelial tissue they support. The submandibular salivary gland (SMG) requires the support of its surrounding environment to develop into a functional organ. To examine mechanical contributions to SMG development, we cultured embryonic mouse SMG organ explants on polyacrylamide (PA) gels of varying stiffness. On gels with a Young's modulus of 0.5 kPa, the glands demonstrated high levels of branching with rounded proacinar structures that was significantly disrupted at 20 kPa by 96 h. Western analysis indicated a 2-fold drop in fibronectin in the glands grown on the 20 kPa gels, suggesting that the mesenchyme cells sensed the stiffness and altered their extracellular environment. By confocal imaging, the outer cuboidal cells (OCCs) in the proacinar buds expressed elevated levels of α-smooth muscle actin (aSMA), phosphorylated myosin light chain (pMLC) and Hic-5. Contrary to models that indicate a direct correlation between extracellular stiffness and contractility in fibroblasts, these putative myoepithelial cells expressed pMLC, aSMA and Hic-5 at the highest levels at 3 kPa, rather than at 20 kPa. The OCC population also shuffled Yes-associated protein (YAP), from the cytoplasm on the 0.5 kPa gels to the nucleus on the 3 kPa gels and increased nuclear YAP at the 20 kPa modulus, indicating a transcriptional response to increasing extracellular stiffness. At 20 kPa, the acinar population showed altered expression and localization of actin stress fibers, tight junction protein, zona occludens protein 1 (ZO1) and the water channel, Aquaporin 5, indicative of a more differentiated epithelium. These data demonstrate that the molecular relay stimulated by external stiffness in a complex organ system is distinct from that in isolated mesenchymal cells, which has significant implications for biomaterials design and tissue engineering.

(28) Collagen XVIII regulates hair follicle cycling and growth of skin tumors

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Collagen XVIII (ColXVIII) is a multidomain basement membrane (BM) proteoglycan with three tissue-specific isoforms. The longest

variant contains a cysteine-rich frizzled domain in its N terminus, which has been suggested to interfere with Wnt/β-catenin signaling in vitro. In the mouse skin, the long variants of ColXVIII are expressed in the BM surrounding the dermal papilla and in the bulge containing the stem cells that enable the cyclic growth of the hair follicles. Based on these and knowing that murine hair follicles can be used as an easily accessible model of various biological processes, including Wnt/β-catenin signaling, we decided to study the in vivo role of ColXVIII in the growth and cycling of hair follicles. We found that the genetic inactivation of all three variants led to an accelerated first hair cycle. Similar observation was made in mice specifically lacking the two long variants of ColXVIII, suggesting that specifically these isoforms have a role in hair follicle cycling. Our ongoing work focuses on hair follicle cycling in adult mice and on β-catenin signaling in our mutant mouse lines. Moreover, when subjected to a chemical mouse skin carcinogenesis model (DMBA-TPA), the mice lacking of ColXVIII expression developed significantly less skin papillomas than the wild-type controls apparently due to the increased sensitivity of the ColXVIII-deficient keratinocytes to carcinogen treatment, as we found a higher number of caspase-3-positive keratinocytes undergoing DMBA-induced apoptosis in the mutant mice by comparison with the controls. As skin tumors are known to arise from epidermal stem cells in the hair follicle and in the interfollicular epidermis, we hypothesize that ColXVIII in the hair follicles supports the viability of stem cells by a currently unknown mechanism. Taken together, our findings indicate that the longest frizzled variant of collagen XVIII has a role in modulating stem cell homeostasis/function in hair follicles.

(29) Fibronectin, laminin and type IV collagen are key components of the sinusoids-associated megakaryocyte niche within bone marrow

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Megakaryocytes (Mks) develop within the bone marrow environment by interacting with different extracellular matrices (ECMs) located at the bone and the vascular structure level. Recent evidences demonstrated that Mks contribute to the establishment of stem cell niches by regulating matrix deposition from environmental cells, releasing pro- or anti-angiogenic factors and ECM cross-linking enzymes and supporting fibronectin fibrillogenesis. In this work, we have analyzed the spatial distribution of Mks and ECMs by immunofluorescence in murine femur sections. We found that Mks were predominantly located in the femur diaphysis with only 20% of Mks within 50 μm from the endosteal surface and more than 80% of Mks located less than 50 μm from a sinusoid. Correlation between Mk distance from sinusoids and dimension suggested a gradient of maturing Mks towards the vascular niche. Next, we deciphered bone marrow ECM composition by western blotting and mapped the location *in situ* of different collagens (I, III, IV and VI) and glycoproteins (fibronectin, thrombospondin, laminin and von Willebrand factor). We found that all these proteins were differently located in the endosteal, arteriolar and sinusoidal districts supporting the concept that the regulation of hemopoiesis, in the bone marrow, may also depend from matrix distribution. Further, we showed, for the first time, that Mks were surrounded by a pericellular matrix mainly composed of fibronectin, laminin and type IV collagen. Interestingly, these three proteins were also demonstrated to promote thrombopoietin-dependent Mk differentiation in in vitro cultures of bone marrow hemopoietic progenitor cells (3.53 ± 0.86, 1.19 ± 0.11 and 1.40 ± 0.23 fold increase, respectively). Finally, fibronectin, laminin and type IV collagen were also demonstrated to be expressed and synthesized by differentiated Mks in vitro as demonstrated by PCR and western blotting analysis. All together, these results suggested that Mks are important ECM-producing bone marrow cells and that released ECMs support megakaryopoyesis and concur to the generation of bone marrow niches.

(30) Collagen II plays an important role in the differentiation of chondrocytes via regulating the Ihh/PTHrP axis

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Collagen II is the major fibril-forming collagen in cartilage. Mutations in the COL2A1 gene lead to osteochondrodysplasias with diverse phenotypes ranging from mild early-onset osteoarthritis to perinatal lethality in humans. The Col2A1-null mice die around birth with a phenotype resembling human achondrogenesis type II. In both cases, the lack of collagen II resulted in expression of collagen I which is not found in normal hyaline cartilage but could have a compensatory function to maintain tissue integrity. However, the misexpression of collagen I could affect the differentiation state of the chondrocytes. In this study, we performed siRNA knockdown of collagen II in primary mouse chondrocytes to mimick the in vivo situation and to analyze the differentiation in more details. We found that collagen II knocked down cells produced more collagen I and that COMP can associate with newly formed collagen I fibrils. However, other collagen II binding proteins like collagen IX and matrilin-3 can not be anchored to the matrix and are more secreted into the supernatant. At the same time, the expression pattern of collagen-binding integrins was altered. ITGA2 was diminished while ITGA1 was up-regulated. Further, the PTHrP expression was down-regulated, paralleled by an increased expression of the prehypertrophic and hypertrophic chondrocyte markers Ihh and collagen X which indicates that the differentiation of chondrocytes is accelerated. In conclusion, our study demonstrates an important role of collagen II in the regulation of the Ihh/PTHrP feedback loop and suggests a novel mechanism of chondrocyte maturation which is regulated by the cross-talk between the extracellular matrix and the cells via integrins.

(31) Regulation of leukocyte migration through galectin–glycan interactions

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Galectin–glycan interactions at the plasma membrane control a broad array of cellular processes, including cell development and differentiation, cell adhesion and migration, cell signaling and survival. While the range of cellular functions and the number of glycoprotein receptors involved in these functions are broad, a limited number of glycosyltransferase enzymes appear to control many of these galectin–glycan interactions. Galectins preferentially bind to lactosmaine sequences, with increased binding avidity for polylactosamine sequences, and as production of polylactosamine results from the actions of just a few glycosyltransferases, those enzymes are critical regulators of galectin effects on cells. While previous work has demonstrated that the GnTV enzyme, that modifies *N*-glycans, regulates galectin-3 interactions on the cell surface, our group has found that the core 2 GnT enzyme that modifies *O*-glycans regulates interactions of galectin-1, galectin-3 and galectin-9 on a variety of leukocytes, including T cells, B cells and dendritic cells. The roles of galectins in regulating migration of different types of leukocytes, and the role of core 2 *O*-glycans in these processes, will be discussed.

(32) ST6Gal-I sialyltransferase is highly up-regulated in epithelial cancers and correlates with stemness in both normal and cancer stem cell populations

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The golgi glycosyltransferase, ST6Gal-I, adds an α2-6-linked sialic acid to the *N*-glycans of selected receptors. Previously, we determined that the ST6Gal-I-mediated sialylation of the Fas death receptor in tumor cells confers an apoptosis-resistant phenotype. At the mRNA level, ST6Gal-I is reportedly overexpressed in numerous human cancers and correlates with metastasis and poor prognosis. However, the prior lack of effective antibodies precluded analyses of ST6Gal-I protein expression and tissue localization. In this study, we show ST6Gal-I protein to be highly up-regulated in several epithelial cancers. Tumors from 14/15 colon carcinoma patients have elevated ST6Gal-I compared with pair-matched normal tissues, and furthermore, Fas is hypersialylated in tumor specimens. Interestingly, ST6Gal-I protein expression in normal epithelium appears restricted to the stem cell compartment in colonic crypts and epidermis, with staining patterns similar to the established stem cell marker, ALDH1. Additionally, ST6Gal-I is highly expressed in induced pluripotent stem cells (iPSs), with no detectable expression in the fibroblasts from which iPS cells were derived. Given the apparent stem cell localization, we investigated whether ST6Gal-I is a novel biomarker for cancer stem cells (CSCs). A colon carcinoma cell line, SW948, selected for resistance to the chemotherapeutic drug, Irinotecan, was shown to have a greater proportion of CSCs compared with parental SW948 cells, measured by flow cytometric labeling of CSC markers, CD133 and ALDH1A1 activity (Aldefluor). Correspondingly, the chemoresistant cells exhibited marked ST6Gal-I up-regulation. We also evaluated the HD3 colon carcinoma cell line, in which ST6Gal-I was knocked down by RNAi. We found that the percentage of CD133/ALDH1A1-positive cells was significantly decreased in cells with ST6Gal-I knockdown, implicating a causal relationship between ST6Gal-I and CSC number. Collectively, these data suggest a role for ST6Gal-I in tumorigenesis and further point to ST6Gal-I as a possible regulator of the stem cell phenotype in both normal and cancer cell populations.

(33) Substratum stiffness-dependent vinculin activation modulates mechanosensitive stem cell differentiation

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Human mesenchymal stem cells (hMSCs) in extracellular matrices (ECM) with stiffnesses mirroring that of specific native tissues will differentiate spontaneously towards that tissue type, e.g. muscle stiffness induces myogenesis. While cell fate is regulated by cytoskeletal contractility and transmitted to the ECM via focal adhesions, a consensus on the necessary and sufficient signaling pathways for mechanosensing that converts biophysical forces into chemical signals to induce differentiation has yet to be reached. Here, we assess whether focal adhesion proteins may differentially initiate signaling cascades by acting as "molecular strain gauges", undergoing force-dependent conformational changes that create new binding sites in response to changes in matrix stiffness. One such strain gauge, talin, unfolds under physiological force to expose vinculin binding sites, suggesting that vinculin may play a role in mechanosensitive signaling. Small interfering RNA (siRNA) was used to achieve a 75% reduction in total and focal adhesion-localized vinculin levels, confirmed by western blot, qPCR and immunofluorescence. As a result of this knockdown, we observed an 80% decrease in matrix stiffness-induced MyoD, a muscle transcription factor. Moreover, knockdown impaired hMSCs' ability to sense mechanical differences between soft and stiff ECM, thus blocking stiffness-sensitive migration or "durotaxis. " To determine whether vinculin knockdown perturbed focal adhesion structure, altered adhesive properties or diminished cellular force generation, immunofluorescence on other focal adhesion proteins, a spinning disc adhesion assay and 3-D traction force microscopy were performed, respectively, with little data indicating global changes in focal adhesions. Reintroduction of vinculin domains into knocked-down cells indicated that domains containing a MAPK1 binding site are necessary for hMSC myogenesis. Furthermore, the chemical inhibition of MAPK1 was shown to be sufficient to abrogate MyoD expression. Together, these data suggest the first in situ evidence that force-sensitive focal adhesion proteins can activate stem cell differentiation signals.

(34) Epithelial Stem Cell and ECM Dynamics in *C. elegans* Molting Cycles

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Our research focuses on the molecular mechanisms that regulate the epithelial stem cell and the extracellular matrix (ECM) dynamics during animal development. In juvenile *C. elegans*, epithelial stem cells repeatedly switch between proliferative and quiescent states in phase with four periodic molts. Each molt involves the

removal and reconstruction of the collagen-rich cuticle and related cell–ECM attachments. *C. elegans* molt at regular 8 hr intervals, but the anticipated pacemaker had not been defined. Here, we show that inactivation of *lin-42*, which encodes a homolog of the human circadian clock gene and tumor suppressor *PERIOD*, results in arrhythmic molts associated with abnormal stem cell behaviors. Expression of *lin-42* oscillates in phase with the molting cycle. Further, expression of *lin-42* in epithelial stem cells is sufficient to restore the proper timing of molting cycles in *lin-42* mutants. These findings suggest that a LIN-42/PER-based oscillator drives the rhythmic cellular and animal behaviors critical for completion of the molts. Together, reiterated and stage-specific functions of LIN-42 may coordinate-specific molts with successive transitions in the temporal fates of epithelial stem cells. Certain genes regulated by the molting timer encode proteins that directly affect cell–ECM adhesion and ECM dynamics. These proteins include the fibrillin-like FBN-1 and members of the unconventional MLT-10 family. Mutations in *fbn-1* and *mlt-10* are associated with incomplete removal of preexisting cuticles, aberrant patterning of new cuticles and abnormal stem cell behaviors such as hyperplasia. We propose that FBN-1 and MLT 10 macromolecules serve as both structural and instructive components of matrices that are remade or remodeled during every molt, including the stem cell niche. Dysfunction of related molecules and pathways in humans may contribute to inherited disorders of connective tissue and the progression of carcinomas.

(35) Structural basis of procollagen trimerization and regulation of C-terminal processing

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The C-propeptides of fibrillar procollagens play crucial roles in tissue homeostasis and remodeling by controlling both the intracellular assembly of procollagen molecules and the extracellular assembly of collagen fibrils. Mutations in the C-propeptides affecting the intracellular molecular assembly are associated with several, often lethal, genetic disorders affecting bone, cartilage, blood vessels and skin. In addition, the proteolytic cleavage of the C-propeptides, generally carried out by the BMP-1/tolloid-like proteinases (BTPs), is the rate-limiting step in the extracellular fibril assembly. Excess fibril deposition is associated with several different types of fibrosis, leading causes of mortality and morbidity worldwide. Here, we report the first crystal structure of a C-propeptide domain, from human procollagen III. The trimeric form has the overall shape of a flower, consisting of a stalk, a base and three petals. The structure reveals an exquisite structural mechanism by which different genetic types of procollagen form trimeric molecules each with the correct chain composition. It also gives insights into the why some types of procollagen are homotrimers, while others are heterotrimers. In addition, when mapped on to the structure, the data show striking correlations between the sites of numerous disease-related missense mutations in different C-propeptide domains and the degree of phenotype severity. Concerning fibril formation, procollagen C-proteinase enhancers (PCPEs) are extracellular proteins that specifically increase the activity of BTPs, by up to 20-fold, during C-terminal processing of fibrillar procollagens. While the detailed mechanism of action of PCPEs remains to be elucidated, this is known to require strong binding to procollagen. We have recently shown that PCPE-1 binds exclusively, via its CUB1CUB2 region, to the procollagen III C-propeptide region (CPIII; Vadon-Le Goff 2011). We also report here the low-resolution structure of the CPIII/CUB1CUB2 complex, determined by small-angle X-ray scattering, showing CUB1CUB2 to bind to the stalk/base region of CPIII. Together with site-directed mutagenesis and kinetic analysis, these data give the first detailed insights into how PCPEs bind the procollagen molecule and activate BTPs. The results have broad implications for the understanding of genetic disorders of connective tissues and also for new therapeutic approaches against fibrosis.

Reference

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(36) Glycosyltransferase-specific Golgi targeting mechanisms

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Glycosylation of secreted and membrane-bound glycoproteins takes place primarily in the Golgi apparatus. Golgi glycosyltransferases are localized to the Golgi compartments according to the glycosylation steps in which they participate. However, the Golgi targeting mechanisms of Golgi glycosyltransferases are not clear. Herein, we investigate the ER-to-Golgi trafficking and Golgi docking of core 1 β3 galactosyltransferase (C1GalT1) and core 2 *N*-acetylglucosaminyltransferase M (C2GnT-M), which participate in the early steps of O-glycosylation. Employing living cells confocal fluorescence microscopy imaging to characterize the ER-to-Golgi transport of these two enzymes, we have found that they utilize distinct vesicular complexes (VCs) with an average diameter of 1.1 μm. This transport process is independent of ERGIC-53, COPI and COPII. Using siRNA technology to characterize the role of golgins (Giantin, GM130, GRASP65, p115) in the Golgi docking of these VCs, we have found that they use different mechanisms: C2GnT-M utilizes Giantin while C1GalT1 uses GM130-GRASP65 complex. However, in the absence of GRASP65, the C1GalT1-carrying VCs utilize the GM130-Giantin complex for Golgi docking. In addition, we have found that p115 is not involved in the golgins-dependent Golgi docking of C2GnT-M or C1GalT1 and GM130 and Giantin link with VCs via a p115-independent way. Thus, novel enzyme-specific Golgi targeting mechanisms are employed by glycosyltransferases and multiple Golgi docking strategies are utilized by C1GalT1. This

discovery is consistent with the important role C1GalT1 plays in the synthesis of mucin-type glycans. (The work is supported by grants from VA Merit Award 1I1BX000985, NIH 1R21HL097238 and Nebraska LB506).

(37) Fibronectin matrix assembly promotes mesenchymal cell condensation during chondrogenic differentiation

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Skeletal development requires temporal and spatial changes in the extracellular matrix (ECM) protein expression and assembly. During chondrogenesis, a cartilage-specific ECM composed of type II collagen, aggrecan and other cartilage proteins ultimately replaces the mesenchymal ECM. An early step in chondrogenesis is the up-regulation of fibronectin expression by condensing mesenchymal cells. This step is recapitulated in by mesenchymal stem cells (MSCs) as they differentiate into chondrocytes in high-density micromass or pellet culture. Addition of TGFβ3 induces MSCs to condense into a compact aggregate within 24 h accompanied by transient up-regulation of the condensation marker N-cadherin. Fibronectin expression increases during condensation, peaking by day 3 and remaining elevated through day 9; increased assembly of a fibronectin fibrillar matrix is also detected during this period. This early increase in fibronectin facilitates chondrogenic differentiation, since a reduction in fibronectin expression using siRNAs significantly impaired cell condensation. This defect can be rescued by addition of exogenous fibronectin. To determine whether fibronectin matrix assembly is required, differentiation was induced in the presence of a bacterial peptide (FUD) that blocks fibronectin-fibronectin interactions or a fibronectin RGD cell-binding fragment that inhibits integrin binding to fibronectin. Both treatments blocked condensation demonstrating that fibronectin matrix assembly is required for this early differentiation step. Mutations in the dystrophic dysplasia sulfate transporter (DTDST) cause chondrodysplasias. We previously showed that DTDST is required for fibronectin matrix assembly by tumor cells. DTDST knockdown in MSCs also blocked fibronectin matrix assembly and completely inhibited condensation. Our results show that blockade of fibronectin matrix assembly by differentiating chondrocytes either directly with FUD or indirectly by reducing sulfation of proteoglycans with DTDST knockdown inhibits condensation. These results show an essential role for fibronectin matrix assembly in facilitating cell condensation and raise the possibility that DTDST mutations cause chondrodysplasias at least in part through effects on fibronectin matrix assembly.

(38) Heparanase promotes myeloma bone disease by regulating the bone microenvironment

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Excessive bone destruction is a major cause of morbidity in multiple myeloma (MM) patients; however, mechanism(s) involved in the pathogenesis of MM-induced osteolysis are largely unknown. Heparanase, an enzyme that cleaves the heparan sulfate chains of proteoglycans, is up-regulated in a variety of human tumors, including MM. Using animal models of myeloma, we have previously discovered that tumor cells expressing high levels of heparanase (HPSE-high cells) dramatically enhance osteolysis locally within the tumors growing in the bone and can also stimulate osteolysis in the distal bones before the arrival of tumor cells at those sites, when comaped with tumor cells expressing HPSE-low cells. The goal of this study was to determine the mechanism by which heparanase promotes MM-induced osteolysis. Using molecular, biochemical, cellular and in vivo approaches, we show the following. (i) Myeloma cells with high levels of HPSE secrete substantially more RANKL and MMP-9 (two important regulators of bone resorption) compared with HPSE-low control cells. (ii) Conditioned medium from HPSE-high myeloma cells significantly enhanced the pro-osteoclast activity of osteoblast/bone marrow stromal cells, indirectly promoting osteoclastogenesis. (iii) Conditioned medium from HPSE-high cells inhibits osteoblast differentiation and mineralization of primary murine osteoblast precursors and promotes mesenchymal cell differentiation toward the adipocytes lineage. Taken together, enhanced heparanase expression in myeloma cells directly stimulates bone resorption, indirectly inhibits osteoblast differentiation and bone formation and promotes adipocyte differentiation in the bone microenvironment. These events result in the uncontrolled bone destruction that is the characteristic of MM. These data provide novel insight into the mechanisms driving myeloma bone disease and suggest that heparanase inhibitors are valid therapeutic targets for the treatment of MM.

(40) Basement membrane-localized tumor cells are protected from HER2-targeted therapy in vivo

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Drug resistance compromises the efficacy of HER2-targeted therapy. Results from our laboratory, and previous reports from others, indicate that tumor cell attachment to basement membrane (BM) and other extracellular matrix (ECM) proteins may confer drug resistance. We have discovered a differential drug response between the outer, matrix-attached cells and inner matrix-deprived cells comprising 3D tumor spheroids grown in reconstituted BM (Muranen 2012). The outer matrix-attached cells are resistant to

multiple drug therapies due to the up-regulation of a multi-factor survival program including anti-apoptotic proteins and growth factor receptors. To address whether these observations are relevant in vivo, we utilized a previously described model of human-in-mouse HER2+ ductal carcinoma in situ. The tumors are characterized by organized nests of non-invasive cells confined within a BM surrounded by ECM. A close examination of the tumor architecture revealed that cells closest to the BM, and nearest to the vasculature, display a striking insensitivity to lapatinib. Tumor RPPA (reverse phase protein array) profile analysis revealed a lapatinib-induced adaptive response program characterized by the up-regulation of prosurvival BCL2-family proteins. Evaluation of BCL2 in matched SUM225 tumor sections revealed selective up-regulation within the BM-localized tumor cells. Under 3D culture, lapatinib combined with the BCL2 antagonist ABT737 resulted in greater SUM225 tumor cell synthetic lethality comaped with single-agent treatment. We evaluated the translational relevance of our preclinical observations within the context of HER2+ clinical disease. Patient biopsy samples collected as part of an ongoing clinical trial were assayed for BCL2 before and after short-term HER2-targeted therapy. BCL2 was clearly up-regulated post-treatment in a subset of patient samples and largely correlated with a poor response to treatment. Our results suggest that resistant populations may be a source of residual disease post-therapy; therefore, identifying and characterizing these cells will be crucial to the prevention of disease recurrence in the clinic.

Reference

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(41) The extracellular matrix: proteomic signatures of breast cancer progression and metastasis

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The extracellular matrix (ECM) is a complex meshwork of cross-linked proteins providing both biophysical and biochemical cues that are major regulators of cell migration, differentiation, survival etc. Many studies have shown that alterations in ECM composition affect tumor progression and metastasis formation. However, until recently, no studies have systematically addressed the question of tumor ECM composition in vivo. To address this challenge, we have developed an innovative proteomic strategy to characterize the composition of the in vivo ECM and have shown that we can reproducibly identify 100+ ECM proteins in any given tissue (Naba et al. 2012). A challenging question in the field of tumor biology is to understand the origin of the tumor ECM: do the ECM proteins arise from the tumor or from the stromal cells? To address this question, we used our proteomics approach to characterizing the ECM of poorly metastatic MDA-MB-231 human breast tumor orthotopically transplanted in the mouse mammary fat pad. The sequence of a human protein is indeed different enough from the murine sequence to allow the distinction between the two by mass spectrometry. The results of our study show that both tumor and stromal cells contribute to the production of the tumor matrix. In parallel, we conducted the same analysis on closely related but highly metastatic tumors (LM2 tumors) and have shown that the tumors of different metastatic abilities differ in both the tumor- and the stroma-derived ECM components. In particular, we identified a subset of ECM proteins up-regulated in the highly metastatic tumors and exclusively by the tumor cells and not the stroma. Furthermore, we have shown that many of the proteins identified have a causal role in metastatic dissemination as knocking down their expression in tumor cells by shRNA strongly inhibited metastasis formation (manuscript in preparation). Future work will focus on understanding the mechanisms by which the ECM proteins identified in this differential proteomic screen contribute to breast cancer progression and metastatic dissemination.

Reference

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(42) Competition between core-2 GlcNAc transferase and ST6GalNAc transferase regulates the synthesis of the leukocyte selectin-ligand on P-selectin glycoprotein ligand-1

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The binding of adhesion molecules belonging to the selectin family to carbohydrate ligands expressed on leukocytes regulates inflammation. Among the ligands, a single *O*-linked glycan located at the N terminus of the leukocyte surface antigen P-selectin glycoprotein ligand-1 (PSGL-1, CD162) is important, since it binds all selectins (L-, E- and P-selectin) with high affinity under fluid shear conditions. Determination of the precise glycan (s) at this site is complicated due to the presence of a high density of proline residues and 71 additional potential O-glycosylation sites on this mucinous protein. To overcome these limitations, we developed a family of PSGL-1 variants or peptide probes (PSGLpp) that have a single *O*-glycan. These were purified from both HEK293T cells overexpressing fucosyltransferase-VII and HL-60 human promyelocytes. Ultra-high-sensitive mass spectrometry (MALDI-MS and MS/MS) analysis of these probes revealed the *O*-glycan distribution at the N terminus of PSGL-1. Besides confirming the presence of the sialyl Lewis-X glycan (sLe^X) antigen, the analysis also revealed the presence of a disialylated T-antigen (Neu5Acα2,3Galβ1,3[Neu5Acα2,6]GalNAc) at this site. To determine the significance of the latter glycan, HL-60 were transduced to overexpress either ST6GalNAc-1, -2 or -4, enzymes that preferentially act on glycoproteins. Among these, ST6GalNAc-2 co-localized with core-2 GlcNAc transferase (C2GnT-1) suggesting that the two enzymes may compete for the common substratum, Galβ1,3GalNAc. In agreement with this, the

overexpression of ST6GalNAc-2 abrogated cell surface sLe^X and CLA expression. It also reduced the number of rolling cells on L- and P-selectin bearing substrata by 85% in a microfluidic-based flow assay. ST6GalNAc2 overexpression also reduced leukocyte adhesion to platelet P-selectin in a suspension-based cell adhesion assay. Overall, the study provides the first detailed distribution of *O*-glycans at the N terminus of PSGL-1. It suggests that a competition between ST6GalNAcTs and C2GnT-I may play a role in regulating leukocyte selectin-ligand recognition.

(43) Glycoengineering at the surface of living cell

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Modification of cell glycolandscape with the help of gycohydrolases, glycosyltransferases or inhibitors of biosynthesis is known to be a low-specificity uncontrollable methodology. In contrast, self-insertion of glycolipids into cell membrane is mild, controllable and specific process. We synthesized [1] glycolipid-like constructs, which have "designer" spacer between a synthetically friendly lipid (as a rule, dioleoylphosphatidylethanolamine) and a glycan head. Using a variety of different glycans ranging from disaccharides to hyaluronic polymer, together with different lengths and high hydrophilicity spacers, we created a series of synthetic glycolipid constructs. Contacting these molecules with live cells gave modified cells, with controlled glycan density and/or altered biological function. Notably, rigid-type spacers allow us to distance the glycan head from lipid bilayer at a certain distance in interval 1–10 nm; a number of examples illustrates dramatic change of reactivity for these differently presented glyco-ligands. All the constructs have been designed to disperse as a solution directly in organic solvents free water, saline and biological media. Like natural glycolipids, all synthetic analogs have the ability to self-insert into cell membranes, their structure can be fully controlled and they can be designed to carry glycans not naturally occurring as glycolipids, for example hyaluronic acid. All the constructs can be easily attached to live cells in a controlled and precise manner making them powerful tools for studying and manipulation of glycoconjugates at the cell surface. In this presentation, we give examples of biological activity/applications including the functioning in vivo.

Reference

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(44) Engineering Carbohydrate Recognizing Biosensors via Computational Modelingand Directed Evolution

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Carbohydrate recognition is an integral part of normal biological processes. It is critical for host-pathogen interactions, biological development and increasingly important for disease-state biomarker detection. Due to the importance of carbohydrate recognition and variation in host glycosylation, glycans are obvious targets for detection, diagnostic and therapeutic applications. Not only do glycans serve as important disease biomarkers, but they also impact the pharmacological properties of therapeutic biologics. For example, glycan heterogeneity can impact batch-to-batch consistency, immunogenicity, pharmacokinetics, activity and clearance. Given that more than two thirds of therapeutic biologics are glycosylated recombinant proteins, new tools for glycosylation analysis during bioprocess monitoring are required. There is an urgent need for biosensors with defined carbohydrate specificity that can be used to interrogate biological samples to identify abnormal glycosylation states in cancer as well as the production of glycosylated therapeutics biologics. Reported here is the development of a novel class of lectin-like carbohydrate recognizing biomolecules engineered from carbohydrate-processing enzymes, which are called Lectenz®. By employing an interdisciplinary strategy that merges computational modeling and directed evolution, *N*-glycan binding Lectenz® biomolecules have been developed. The design of Lectenz® is initiated in silico to determine optimal carbohydrate-enzyme interactions using molecular dynamics simulations. In silico structure/function analyses are validated by generating focused biocombinatorial libraries for in vitro directed evolution, selection and downstream characterization of Lectenz® candidates. Surface Plasmon Resonance is utilized to determine binding kinetics. The production of Lectenz® shares many similarities with that of in vitro antibody selection, namely the selection of high-affinity mutants from a pool of clones based on a well-defined protein scaffold. They have several potential advantages over plant lectins and antibodies, including precise definition of specificity, ease of preparation in a monovalent form and (for human homologs) reduced chance of in vivo toxicity. Lectenz® biomolecules may be utilized as recognition elements in biosensors and multiplexed bead-based assays for monitoring of glycosylation states in glycoproteins and glycopeptides of interest, a key requirement for bioprocess monitoring and disease related biomarker detection.

(45) Roles for glycans in development and spermatogenesis

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Functions for *N*- and *O*-glycans during development have been determined by gene targeting of key glycosyltransferase genes. The inactivation of *Pofut1* (POFUT1), which transfers *O*-fucose to certain epidermal growth factor-like (EGF) repeats and is essential for canonical Notch signaling, or *Mgat1* (GlcNAcT-I), which transfers GlcNAc to initiate hybrid and complex *N*-glycan synthesis, or *C1galt1* (T-synthase), which transfers Gal to generate core 1 and 2 mucin *O*-glycans, leads to embryonic death at mid-gestation. Deletion of the same glycosyltransferase genes in oocytes to generate maternal/zygotic mutant embryos also results in embryonic death at the same stage. Therefore, complex *N*-glycans, core 1-derived *O*-glycans,

O-fucose glycans and Notch signaling are not required for embryogenesis, implantation or gastrulation. We have now investigated roles for these glycosyltransferases in spermatogenesis using mice carrying floxed *C1galt1*, *Pofut1*, *Notch1* or *Mgat1* alleles and testis-specific Cre recombinase transgenes, including Stra8-iCre expressed in spermatogonia, Sycp1-Cre expressed in spermatocytes, Prm1-Cre expressed in spermatids and AMH-Cre expressed in Sertoli cells. Only males in which the synthesis of complex and hybrid *N*-glycans was blocked by deletion of *Mgat1* in spermatogonia exhibited disrupted spermatogenesis and were infertile. Therefore, core 1 and 2 *O*-glycans, *O*-fucose glycans, NOTCH1 and Notch signaling are dispensable for spermatogenesis. The stage at which spermatogenesis is affected, and the nature of germ cell defects in *Mgat1*[F/F]: Stra8-*iCre* males will be presented. Histological analyses and immunohistochemistry reveal no apparent defects in Sertoli cells, spermatogonia or spermatocytes, but round and elongated spermatids form multinucleated giant cells or symplasts. In addition, we are investigating functions of a physiological, testis-specific inhibitor of MGAT1 termed GlcNAcT-I inhibitory protein (GnT1IP). Membrane-bound GnT1IP-L is expressed almost exclusively in testicular germ cells, and expression is tightly regulated during spermatogenesis. Our working hypothesis is that high-mannose *N*-glycans induced in spermatocytes by increased expression of GnT1IP-L are important for germ/Sertoli cell interactions, whereas the reappearance of complex *N*-glycans in spermatids due to reduced inhibition of MGAT1 is necessary for spermatid/Sertoli cell interactions. Supported by NIH grants RO1 CA 30645, RO1 CA 36434 and RO1 CA 95022 to PS.

(46) Adamtsl2 modulates the ratio of fibrillin-1 to fibrillin-2 in tissue microfibrils in a mouse model for human geleophysic dysplasia

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Geleophysic dysplasia (GD) is a rare human genetic disorder presenting with short stature, short distal limbs, joint contractures and thick skin. High morbidity, and frequently, juvenile mortality results from cardiac valvular and tracheo-pulmonary anomalies. Mutations in the extracellular proteins ADAMTSL2 or fibrillin-1 (FBN1) lead to recessive or dominant GD, respectively. FBN1 assembles in microfibrils which modulate extracellular TGFβ and BMP signaling. Excess TGFβ signaling was described in cells derived from GD patients and we showed previously that recombinant ADAMTSL2 directly bound to latent transforming growth factor-β binding protein (LTBP)-1 and FBN1. The *Adamtsl2* gene was targeted in mice by insertion of an IRES-*lacZneomycin* cassette. β-gal staining showed focal *Adamtsl2* expression in the heart, lung and in specific connective tissues of the limbs. Deletion of *Adamtsl2* resulted in neonatal lethality due to a ventricular septal defect in the heart and airway occlusion. Increased FBN2 immunostaining was seen in the extracellular matrix at the crest of the non-closed ventricular septum and around bronchi. Surprisingly, neonatal death in Adamtsl2 −/− mice was prevented by additional deletion of one allele of *Fbn1*. Moreover, the postnatal growth of *Adamtsl2* −/−; *Fbn1* +/− mice was severely restricted and these mice died by 14 days of age. *Adamtsl2* −/−; *Fbn1* +/− mice walk on tiptoes and have joint contractures. Their skin is tight, and histology revealed a thickened stratum corneum combined with reduced fat in the hypodermis, which is traversed by collagen extending from the dermis to the hypodermal muscle. In summary, ADAMTSL2 may be part of a previously undefined mechanism that locally modulates the ratio of FBN1 to FBN2 in tissue microfibrils. *Adamtsl2* −/−; *Fbn1* +/− mice present with many of the major manifestations seen in GD and disclose a crucial functional relationship between ADAMTSL2 and FBN1.

(47) Altered activity and localization of cathepsin K underlies the cartilage defects in a zebrafish model of mucolipidosis II

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The severe disorder, mucolipidosis II (ML-II; I-cell disease), is caused by defects in mannose 6-phosphate (M6P) biosynthesis. Patients exhibit multiple developmental defects including skeletal, craniofacial and joint abnormalities. Utilizing an ML-II zebrafish model, we previously showed that the cartilage defects are associated with altered chondrocyte differentiation, elevated levels of type II collagen and increased and sustained activity of several proteases including cathepsin K (Ctsk). Inhibition of Ctsk by genetic and pharmacological means ameliorated multiple aspects of the craniofacial phenotypes. Further, we have demonstrated that the elevated activity of Ctsk is due to enhanced proteolytic processing of the enzyme proform into highly active mature forms. We hypothesize that this enhanced proteolytic processing correlates with the loss of M6P and Ctsk hypersecretion from cells. To test this hypothesis, we first analyzed the mannose phosphorylation status and the activity of zebrafish cathepsins D (Ctsd) and K in WT and ML-II embryos. In WT embryos, Ctsd was only marginally M6P modified, whereas Ctsk was extensively modified. In ML-II, the mannose phosphorylation of both cathepsins was reduced, with the majority of excessive Ctsk activity lacking M6P. To address whether unmodified Ctsk is in fact hypersecreted from the cell, we isolated WT and ML-II zebrafish chondrocytes and found that the intracellular activity of Ctsk but not Ctsd is substantially reduced in ML-II cells, consistent with cathepsin-specific hypersecretion. Immunohistochemical staining for Ctsk and Ctsd confirmed a unique extracellular localization of Ctsk within ML-II cartilage. To assess the direct contribution of Ctsk to the pathology of ML-II, we overexpressed Ctsk in WT embryos, which enhanced enzyme activation, lead to an increase in activity lacking M6P and resulted in craniofacial abnormalities, each reminiscent of the biochemical and phenotypic aspects of ML-II. These findings further support a central role for Ctsk in the cartilage pathogenesis of this disease.

(48) Glycan glucuronylation is essential for female fertility in *Drosophila melanogaster*

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Glycoprotein and glycolipid glycans isolated from *Drosophila* tissues are frequently terminated with an acidic monosaccharide. In contrast to the predominance of sialic acid found on vertebrate glycans, *Drosophila* glycans are much more likely to be capped with glucuronic acid (GlcA). Not only do the most abundant glycosphingolipid glycans bear GlcA, but three of the major *O*-linked glycans in *Drosophila* are also either branched or capped with GlcA. One of the major *O*-linked structures is of special interest because it is built on an *O*-linked Fuc core (GlcNAc-β3-(GlcA-β4-)Fuc-*O*-Ser/Thr), suggesting that it may contribute to Notch function. In order to better understand the potential functions of glycan glucuronylation in *Drosophila*, we have screened candidate glucuronyltransferase (GlcAT) genes for their ability to synthesize the structures that we have detected in embryos. One of these candidates, designated GlcAT-S based on its similarity to the vertebrate enzyme of the same name, was found to transfer GlcA to core 1 disaccharide in vitro. Furthermore, analysis of the *O*-linked glycans produced in embryos with reduced GlcAT-S (hypomorphic P-insertion mutant and RNAi knockdown line) demonstrated reduced glucuronylation of core 1 disaccharide and reduced abundance of the glucuronylated *O*-Fuc structure. We generated a new GlcAT-S mutant by the mobilization of a P-insertion into the first intron of the GlcAT-S gene. The resulting P-excision mutant, designated GlcAT-S^{cms}, appeared to be nominally wild-type upon initial characterization. However, when the excision mutant was crossed into a *w*+ background, the resulting GlcAT-S^{cms} homozygous females failed to lay eggs. The *w* (*white*) gene encodes an ABC transporter that contributes to the production of eye pigments and may also impact levels of GDP-Fuc. Further analysis of the *w* + ; the GlcAT-S^{cms} infertility phenotype demonstrated that egg development arrests around stage 6/7 in association with over-proliferation of follicle cells, a phenotype previously linked to altered Notch signaling.

(49) Granzyme B-mediated loss of decorin increases collagen fibril irregularity and susceptibility to aortic rupture in a mouse model of abdominal aortic aneurysm

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Abdominal aortic aneurysm (AAA) is an age-related disease caused by progressive degradation of the extracellular matrix within the vessel wall. Although aortic dilation leading to rupture can be fatal, effective pharmacological interventions aimed at halting AAA progression are not yet available. We have previously demonstrated that knocking out the serine protease granzyme B (GZMB) reduces incidence and severity of AAA in mice in a perforin-independent manner. GZMB is known for its role in apoptosis, but also accumulates extracellularly during inflammation and can cleave the extracellular matrix (ECM) components such as decorin and fibrillin-1. We hypothesized that GZMB contributes to AAA development through the degradation of vascular ECM and that the inhibition of extracellular GZMB will reduce incidence and severity of AAA progression. To induce AAA, apoE−/− mice were implanted with an osmotic minipump that released angiotensin II for 28 days. Animals were injected with GZMB inhibitor, serpin A3N (4–120 μg/kg) or anti-GZMB neutralizing antibody (1 mg/kg) prior to implantation. Effects of GZMB degradation on decorin-mediated collagen fibrillogenesis in vitro were assessed. A significant dose-dependent reduction in the frequency of aneurismal rupture was observed in mice that received serpin or antibody treatment. Pre-incubation with serpin also prevented decorin cleavage by GZMB in vitro. Reduced GZMB and a corresponding reduction in loss of adventitial decorin were observed in serpin-treated mice, while collagen density was increased. Adventitial collagen from serpin-treated mice exhibited significantly higher fiber density and reduced fibril size irregularity when evaluated by multiphoton confocal microscopy and transmission electron microscopy. Furthermore, GZMB was elevated in human aortic aneurysm and reduction in collagen density correlates with severity of aneurysm progression. Extracellular inhibition of GZMB prevents degradation of decorin and promotes beneficial remodeling of collagen leading to higher tensile strength and resistance to rupture in aortic aneurysm.

(50) Calreticulin is required for TGF-β stimulation of the extracellular matrix

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Calreticulin is an endoplasmic reticulum (ER) stress response protein which functions as a protein chaperone and the regulator of ER calcium and downstream calcium signaling. ER stress is an emerging factor in fibrotic disease and calreticulin expression is up-regulated in conditions of ER stress and in organs affected by fibrotic sequelae of chronic diseases, such as diabetic nephropathy and atherosclerosis. Previously, we showed that ER calreticulin regulates type I collagen transcript, ER trafficking, secretion and processing into the extracellular matrix (ECM). To determine the role of calreticulin in the regulation of ECM protein expression in fibrotic disease, we asked whether calreticulin modified cellular responses to the major pro-fibrotic cytokine, TGF-β. Wild-type and calreticulin null mouse embryonic fibroblasts (MEFs) were stimulated with TGF-β, and levels of type I collagen and fibronectin mRNA and protein were examined. Wild-type MEFs significantly increased collagen and fibronectin transcript and protein upon treatment with TGF-β. However, TGF-β treatment of calreticulin null MEFs failed to stimulate collagen or fibronectin production, despite apparently normal Smad phosphorylation and nuclear

translocation. Correspondingly, fibroblasts expressing increased levels of calreticulin showed the enhanced production of collagen and fibronectin transcript and protein upon treatment with TGF-β. Calreticulin regulation of calcium release is important for TGF-β stimulation of ECM, since calcium release was deficient in calreticulin null MEFs upon TGF-β treatment. Furthermore, TGF-β induced NFAT reporter activity and nuclear translocation of NFAT, a calcium-calcineurin-dependent transcription factor that regulates collagen and fibronectin transcription, in wild-type but not in calreticulin null MEFs. In addition, TGF-β stimulation of collagen and fibronectin transcription in wild-type MEFs can be blocked with NFAT inhibitors. Taken together, these data identify calreticulin as an important regulator of TGF-β-dependent ECM production and suggest a novel link between ER stress and fibrotic disease through calreticulin.

(51) Restoring laminin polymerization by transgenic expression of αLNNd in skeletal muscle improves muscle integrity of laminin-α2-deficient mice

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Laminin-α2-deficient congenital muscular dystrophy (MDC1A) is a genetic disorder characterized by severe muscle weakness, respiratory and peripheral nerve myelination defects. Laminin-211, the primary α2 containing laminin in skeletal muscle basement membranes, mediates key molecular bonds that stabilize the underlying sarcolemma. Absence or truncation of the laminin-α2 chain results in weakening of the sarcolemmal basement membrane increasing susceptibility to contraction induced muscle damage. In vivo mouse models for MDC1A include dy3K (α2-null) and dy2J (splice-donor defect resulting in an internal LN domain truncation). The LN domains of laminin are essential for laminin polymerization and its loss (dy2J) is associated with decreased laminin accumulation in muscle and nerve. When laminin-211 is absent, laminin-411, a non-polymerizing and poor receptor binding laminin, ineffectively compensates in muscle and nerve. To restore polymerization, our lab developed a chimeric linker protein, αLNNd, composed of the α1 N-terminal domains (LN-LEa) fused to the nidogen G2-G3 domains for laminin binding. αLNNd can restore polymerization to recombinant laminin-111 with an α N-terminal deletion and increase basement membrane assembly on cultured Schwann cells and C2C12 myotubes. αLNNd has been transgenically expressed in the dy3K and dy2J mouse models of MDC1A. In dy3K, αLNNd prolonged mouse lifespan and increased accumulation of basement membrane components by immunofluorescence, with some improvements in muscle histology. A significant amelioration of the dystrophy was seen in dy2J, with a major reduction in fibrosis, increased regularity in muscle fiber area and decreased regeneration. This study demonstrates that αLNNd, by restoring laminin polymerization in vivo, can significantly ameliorate laminin-α2-deficiency in mice, furthering its potential as a therapeutic agent alone or in combination with miniagrin.

(52) Detailed structural investigation of β-glucans from yeast cell walls by electron-transfer dissociation

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The polysaccharides from cell walls of yeast and other fungi determine the cell shape, cell division and growth of the organism. There are three main groups of polysaccharides: polymers of mannose covalently linked to peptides (mannoproteins, 40%), polymers of glucose (β-glucans, 60%) and polymers of *N*-acetylglucosamine (chitin, 2%). β-Glucans may have two subtypes based on the predominant linkages between their monosaccharide residues, -β-1,3-glucans and β-1,6-glucans. Traditionally, cell wall polysaccharide composition needs to be determined through a series of complex and time-consuming steps. Various enzymatic digestions are classic methods to differentiate the linkages, but these may provide ambiguous results. We developed a simple and efficient method to determine the detailed structures of these glycans, using electron transfer dissociation (ETD). β-Glucans were released from cell walls and permethylated. The magnesium-adducted glycans were analyzed on a Bruker AmaZon quadrupole ion trap and the Solarix FT mass spectrometer. The fragmentation technique most commonly employed for the structural identification of glycans is collision-induced dissociation (CID). However, CID generates product ions primarily by the rupture of glycosidic bonds and this provides information only on the weights and sequences of monomer residues. β-1,3- and β-1,6-glucans thus cannot be distinguished in terms of their compositions. By comparison, ETD generates both glycosidic bond cleavage and cross-ring cleavage ions. The cross-ring cleavages clarified the different linkage types of these glucans. The utilization of Solarix Qh-FT-ICR and ETD-CID MS^3 techniques made it possible to verify peak assignments and to distinguish the overlapping peaks. ETD will decrease the workload and will allow the rapid differentiation between β-1,3- and β-1,6-glucans. The results indicate that ETD can also contribute to confident structural analysis of other oligosaccharides from a wide variety of biological samples; examples of such applications will be provided. This research is supported by NIH grants P41 RR10888/GM104603 and S10 RR025082. The authors are grateful to J Samuelson, PW Robbins and E Motari for helpful discussions and for providing the samples and to Bruker Daltonics for loan of the AmaZon instrument.

(53) MS-based insight in glycobiology: tyrosine kinase inhibitors alter the cell surface glycosylation patterns of lung cancer cell lines

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Glycans play important roles in cancer biology and metastasis, especially in cell–cell and cell–matrix interactions. Recently, several studies reported altered glycan patterns in blood, as well as tissue samples from different types of cancer. There is, however, not

much known regarding the role of glycosylation in cancer treatment. Mutations of the epidermal growth factor receptor (EGFR) are important in lung cancer cell survival and currently two drugs are on the market that target EGFR phosphorylation and inhibit its downstream pathways. Using cell-based assays, these compounds were shown to cause EGFR-dependent apoptosis, however, the role of glycans in this process remains unknown. In this study, a mass spectrometric approach was used to investigate the effects of two tyrosine kinase inhibiting drugs on the cell surface glycosylation of lung cancer cell lines A549 and H1650. Cell membranes were isolated and *N*-glycans were released using PNGase F. Mass spectrometric analysis of the glycans was performed using nLC-chip-(Q)-TOF-MS with a graphitic carbon stationary phase. This method was previously successfully applied for serum and was shown to enable separation of *N*-glycan isomers. First, we developed a dedicated *N*-glycan library for the cell surface glycans from the two cell lines using retention time, accurate mass and CID fragmentation using a Q-TOF instrument. We will report our glycan library, currently consisting of 150 entries, of which 50 *N*-glycans from the cell surface are fully characterized. To show how glycosylation changes with treatment of lung cancer, glycosylation of lung cancer cell lines A549 (EGFR WT) and H1650 (EGFR mutant), treated with three different concentrations of dasatinib, a c-Src inhibitor and erlotinib, a TKI inhibitor for 72 hours was analyzed. Cells were lysed and both whole cell lysates and cell surface fractions were prepared. Altered cell surface *N*-glycan patterns observed after treatment of the cells will be reported.

(54) IL-6 induction by lipoteichoic acids of the immuno-protective lactococcus lactis G121 IS TLR2 independent

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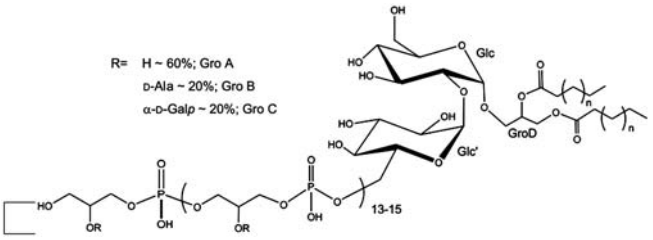
Epidemiological studies proved the traditional farming way of life to prevent children from atopic sensitizations (1, 2). *L. lactis* G121 was identified from Bavarian barnyards and chosen as a Gram-positive representative due to its abundance and antibodies against it found in the sera of farm children. In an ovalbumin-sensitized mouse model of asthma, this bacterium resulted in a diminished asthma phenotype as shown, for example, by less numbers of eosinophils in the BAL (3). The overall aim of our study is the determination of bacterial structures and immune receptors as well as signaling pathways involved in allergy protection. Therefore, lipoteichoic acid (LTA), one main cell envelope polymer, was extracted with *n*-BuOH at 20–22°C. Subsequently, HIC on an octyl-sepharose column yielded the pure LTA. Analytical chemistry, ESI FT-ICR-MS as well as 1D and 2D NMR spectroscopy identified a 1,3-poly(glycerol phosphate) backbone with α-D-Ala and α-D-Gal*p* decorations at O-2 of glycerol. Only HF cleavage (48%, 4°C, 48 h) and hydrazinolysis (37°C, 1 h) revealed the linker to be α-D-Glc*p*-(1 → 2)-α-D-Glc*p*-(1 → 3)-Gro. In fatty acid analyses, 14:0, 16:1, 16:0, 18:1 and Δ-19:0 were detected, and six different LTA species could be verified by ESI-MS. The LTA led to a release of IL-6 in human MNC indicating relevance for the humoral immunity. HEK293 cells transiently transfected with TLR2, TLR4, NOD1 or NOD2 were incubated with LTA and it could be clearly demonstrated that none of these receptors was able to confer responsiveness (4). Hence, future experiments will hopefully identify the underlying receptor(s) and pathway(s). The LTA structure is summarized as:



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(55) Mucosal immunity mediated by antibody-mucin cross-linking

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Mucus, a highly viscoelastic gel composed of a matrix of densely glycosylated mucins, serves as the first line of defense against pathogens. To infect or to persist at the mucosa, pathogens must penetrate the mucus layer. Nevertheless, the real-time mobility of many pathogens in human mucus, and the mechanisms by which mucus excludes most commensal and pathogenic microbes, remain not well understood. We engineered muco-inert nanoprobes to determine the pore sizes in fresh undiluted human cervicovaginal mucus (CVM) from volunteers with healthy, lactobacillus-dominated vaginal microflora. We found that the pores in CVM are markedly larger than mammalian viruses (average 340 +/− 70 nm) (Lai et al. 2010). The native microstructure alone cannot trap viruses, as reflected by the rapid mobility of herpes simplex virus (HSV; d 180 nm) and HIV (d 110 nm) in pH-neutralized CVM (Lai et al. 2009). Despite its large pores, we found that mucus can leverage secreted antibodies (Ab), including IgG, to effectively trap pathogens in the mucus gel. Ab diffuse rapidly through mucus, retarded only slightly by transient, low-affinity bonds with mucins. We hypothesize that this feature allows pathogen-specific IgG to diffuse to and accumulate on pathogens, eventually forming sufficient low-affinity cross-links with mucus to permanently trap otherwise rapidly diffusing virions or

highly motile pathogens. Consistent with this hypothesis, we found HSV-1 is effectively immobilized in pH-neutralized CVM samples with modest levels of native HSV-1-specific IgG, but not in samples with low-endogenous anti-HSV-1 IgG. Addition of purified IgG trapped HSV-1 to CVM even at levels well below neutralizing titers also led to HSV-1 trapping. The IgG-mucin interactions are mediated in part by the glycosylation of the IgG. These observations suggest that secreted Ab possess a novel, glycan-mediated "muco-adhesion" effector function that represents an exceptionally potent biophysical mechanism of mucosal immunity.

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(56) A new model for T cell-dependent immune activation by glycoconjugate vaccines

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Capsular polysaccharides are found on the outermost surfaces of many pathogenic bacteria. Each bacterial pathogen possesses a polysaccharide with unique structure that is distinctively recognized by our immune cells. Because these polysaccharides are located on the surface of pathogens, they are easily accessible by the immune cells and therefore are essential vaccine candidates. To induce polysaccharide-specific professional immune response (e.g. T cell mediated B cell response), these polysaccharides are conjugated with carrier proteins and the conjugation products are called glycoconjugate vaccines. Glycoconjugate vaccines provide enormous health benefits against bacterial pathogens, yet the conjugation of polysaccharides to proteins has often been a "black art", with poorly defined chemistry resulting in inadequately characterized conjugates. Especially, problematic has been the lack of appreciation of the cellular mechanisms by which the immune system processes these complex antigens. In a series of experiments, we have revised the model that describes how protein-polysaccharide conjugate vaccines interact with the adaptive arm of the immune system. We demonstrated that, in antigen-presenting cells, a carbohydrate epitope is generated upon endolysosomal processing of group B streptococcal type III polysaccharide (GBSIII) coupled to a carrier protein. In conjunction with a carrier protein–derived peptide, this carbohydrate epitope binds to major histocompatibility class II (MHCII) and stimulates carbohydrate-specific CD4+ T-cell clones. Moreover, based on our mechanistic findings, we designed and synthesized a model vaccine that is substantially more immunogenic than a currently available vaccine.

(57) Group B streptococcus uses sialic acid mimicry to subvert host innate immune responses through engagement of inhibitory leukocyte receptor Siglec-E

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Group B *Streptococcus* (GBS) is a Gram-positive encapsulated bacterium that colonizes the vagina of up to 30% of healthy women. This bacterium typically causes serious infection in newborns, the elderly and immunocompromized populations and is the most common agent of neonatal bacterial meningitis in the USA, Europe and Asia. All GBS capsular polysaccharides (CPS) are capped with sialic acids (Sia), and these terminal Neu5Acα2-3Galβ1 motifs have been shown to reduce complement deposition and phagocytosis, as well as dampening neutrophil bactericidal activity by engaging the inhibitor lectin receptor Siglec-9 (Sialic acid-binding immunoglobulin-like lectin-9). To date, the role of Siglecs in the innate immune response to Sia-expressing pathogens has not been studied in vivo. In this study, we utilize *Siglece*-null mice deficient in Siglec-E, a murine functional paralog of human Siglec-9, being similarly expressed on innate immune cells of the myelomonocytic lineage. We show that like human Siglec-9, murine Siglec-E also binds to GBS in a Sia-specific fashion. In keeping with this, GBS triggered stronger NF-κB and MAPK activation in Siglec-E-deficient macrophages, leading to higher proinflammatory cytokine secretion. Moreover, Siglec-E-deficient macrophages also exhibited better phagocytic and bactericidal activity against GBS. Following intravenous GBS challenge, Siglec-E-deficient mice showed increased proinflammatory (IL-6) and decreased anti-inflammatory (IL-10) cytokine production, reduced bacterial blood counts and reduced invasion of GBS into the central nervous system. These data show that GBS Sia mimicry can subvert host innate immune responses through engagement of an inhibitory Siglec on leukocytes. Development of the antibodies or small molecules to disrupt GBS and Siglec interaction may provide an additive therapeutic option to augment host immune responses against the pathogen, perhaps in synergy with conventional antibiotic therapy.

(58) ST6Gal-I prevents macrophage apoptosis via sialylation of TNF-R1

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Monocytes/macrophages play a central role in innate immunity and autoimmune pathologies. These cells have distinct lifespans, influenced by inflammatory stimuli and cell differentiation status. In this investigation, a standard model system was employed to study monocyte behavior: treatment of U937 monocytes with the PKC activator, PMA. This treatment induces macrophage differentiation and ultimately, apoptosis. In addition, we have shown that PMA stimulates down-regulation of the ST6Gal-I sialyltransferase, leading a loss in α2-6 sialylation on the TNF-R1 death receptor. To better understand the ST6Gal-I function, we generated U937 cells with constitutively-expressed ST6Gal-I that cannot be down-regulated by PMA. These studies revealed that PMA induces apoptosis in parental and empty vector cells, but not in cells constitutively expressing ST6Gal-I. PMA-induced apoptosis is reportedly mediated through increased synthesis and secretion of TNF-α, which then binds to TNF-R1 to signal cell death. Accordingly, we directly examined the effects of variant sialylation

on TNF-R1 signaling. Forced overexpression of ST6Gal-I, leading to the hypersialylation of TNF-R1, protected U937 cells against TNF-α induced apoptosis, whereas ST6Gal-I knockdown conversely sensitized cells to apoptosis by TNF-α. To elucidate physiologic relevance, we generated transgenic mice with constitutive ST6Gal-I expression. These mice were injected with thioglycollate and peritoneal macrophages were harvested. When compared with wild-type mice, ST6Gal-I transgenics had greater numbers of peritoneal macrophages, consistent with enhanced cell survival and ST6Gal-I-expressing macrophages treated with TNF-α exhibited reduced apoptosis. In more recent studies, we are exploring the hypothesis that the sialylation of TNF-R1 serves as a switching mechanism to divert TNF-α-induced signaling away from apoptosis and toward cell survival pathways. It has long been appreciated that TNF-α can stimulate either cell survival/proliferation or apoptosis, depending on cellular context; however, the mechanisms involved are poorly-understood. New preliminary results suggest that TNF-α treatment of U937 cells with α2-6-sialylated TNF-R1 receptors causes the activation of MAPK and NFkB pathways, consistent with a putative switching mechanism controlling downstream signaling. Taken together these results suggest that ST6Gal-I activity tunes the response of monocytes/macrophages to TNF-α by controlling the function of TNF-R1.

(59) Natural occurrence of *N*-glycolyl-hexosamines in chondroitin/dermatan sulfates in mammalian tissues, derived from the metabolism of *N*-glycolylneuraminic acid

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Mammalian glycans commonly contain two major sialic acids *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid (Neu5Gc). Humans cannot synthesize Neu5Gc but incorporate this xeno-autoantigen from dietary sources (1, 2). We recently found that mammalian cells metabolize Neu5Gc to glucosamine 6-phosphate and glycolate, with *N*-glycolylglucosamine (GlcNGc) as an intermediate (3). Parallel studies found UDP-GlcNGc and UDP-GalNGc (UDP-*N*-glycolylgalactosamine) in cells supplemented with GlcNGc (4). UDP-GalNGc and UDP-GlcNGc serve as substratums for assembly of many major glycan classes in mammalian cells supplemented with GalNGc (5). We have now considered the possibility that Neu5Gc turnover in mammalian cells could be a natural source for glycan structures harboring GalNGc or GlcNGc. Indeed, mammalian cells cultured in [^{3}H*glycolyl*] Neu5Gc incorporated radioactivity into the glycosaminoglycan fraction, which was sensitive to chondroitinase ABC treatment. We next studied mammalian muscle (red meat, a tissue type rich in Neu5Gc) for the presence of naturally occurring GalNGc-containing chondroitin/dermatan sulfate, resulting from cellular Neu5Gc turnover. Mass spectrometry indeed demonstrated these novel GalNGc-containing chondroitin/dermatan sulfate structures. While the natural abundance of such structures is low, the possibility that they may mediate novel functions is being explored. The Neu5Gc breakdown products GalNGc and GlcNGc could also potentially be xeno-autoantigens in humans. A search for human anti-GalNGc and anti-GlcNGc antibodies is therefore ongoing.

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(60) Tonic for T-cells: hyaluronan promotes STAT5 signaling in the absence of IL-2

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How T-cells persist in peripheral tissues in the absence of high-dose IL-2 is critical for immune memory and regulation and is a fundamental, unanswered question in immunology. We have identified a non-traditional pathway for IL-2R signaling involving hyaluronan (HA), a matrix polysaccharide. We find that CD44, the HA receptor, physically interacts with the IL-2R complex. Cross-linking of CD44 by HA results in STAT5 phosphorylation (pSTAT5) that is rapid (5 min) and additive with IL-2-mediated pSTAT5. This effect is inhibited by neutralizing Ab-directed against CD122 or CD132 but not by cyclosporine-A, rIL-2Rα or lipid raft inhibitors. Given that CD44 has only a modest cytoplasmic tail without signaling capabilities, these data support a direct role for CD44 in IL-2R complex formation and signaling. Further, IL-2-mediated pSTAT5 and basal pSTAT5 are enhanced in T-cells from CD44 −/− mice and these animals have pronounced lymphocytosis, indicating that CD44 is a constitutive negative regulator of STAT5. We propose that T-cell interactions with HA relieve the CD44-mediated negative regulation of IL-2R complex formation and that antibody-mediated cross-linking is equivalent to HA interactions in relieving this negative block. HA thereby provides tonic signals that sustain T-cells in the absence of high-dose IL-2. In support of this, CD44 cross-linking can sustain CD4 + GFP/Foxp3+ regulatory T-cells (Treg) cultured in the presence of cyclosporine-A and in the absence of IL-2 while treatments that inhibit HA production impair Treg stability. These findings may explain how T-cells and Treg persist in peripheral tissues and lead to innovative strategies to influence immune memory and tolerance.

(61) Role of galectin-3 in exosome biogenesis

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Exosomes are small vesicles (50–100 nm) secreted by different cell types. The exosomal secretion pathway is primed by

intraluminal vesicles (ILVs) budding into the late endosomes to form multivesicular bodies (MVBs), which then fuse with the plasma membrane resulting in release of ILVs to the extracellular space. The exosomal component proteins include the ESCRT (endosomal sorting complex required for transport) family members (Alix, Tsg101), tetraspanins family members (CD9, CD63, CD81), membrane trafficking members (Rab5, Rab7, Rab11b) and galectin-3. Exosomes function in cell-to-cell communication, antigen presentation, reticulocyte differentiation and tumor progression. Galectin-3 has been identified as a component of dendritic cell (DC)-derived exosomes by proteomic analysis and DC-derived exosomes have been demonstrated to be capable of stimulating T cell activation. As galectin-3 can be associated with Alix in a number of cell types, we hypothesize that galectin-3 may participate in exosome biogenesis through interacting with Alix. Immunofluorescence staining showed that galectin-3 is colocalized with Alix and CD63, which is an MVB marker. Immuno-EM and immunoblotting analyses showed that galectin-3 is expressed on the surface as well as inside of DC-derived exosomes. By comparing exosomes collected from bone marrow-derived (BM) DCs, we further discovered that gal3 −/− BMDCs secreted more exosomes than gal3 +/+ BMDCs. Immunoblotting analysis of the protein composition indicated exosomes from gal3 −/− BMDCs contained different levels of protein components comaped with those from gal3 +/+ BMDCs. These results suggest that galectin-3 may play a role in regulating exosome biogenesis.*Keywords:* exosome / dendritic cell / galectin-3 / ESCRTs

(62) Carbohydrate-specific interaction of mammalian pancreatic α-amylases with glycoproteins on intestinal mucosa regulates glucose as similation for homeostasis

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Pancreatic α-amylase plays a role in the digestion of starch to produce maltooligosaccharides in the intestine, which are further processed by the exoenzymes located at the brush-border membrane (BBM) of enterocytes. We previously reported that porcine pancreatic α-amylase (PPA) binds to the *N*-glycans of glycoproteins[1]. To clarify the biological functions of the carbohydrate binding of pancreatic α-amylase, whether other pancreaticα-amylases share the common activity and their biological ligands were elucidated in this study.

Recombinant human pancreatic α-amylase (recHPA) was expressed in yeast and purified by single-step affinity chromatography. RecHPA exhibited almost the same carbohydrate-binding specificity as PPA. Exogenous PPA was localized at the BBM in porcine duodenum by immunohistochemistry. The localization was inhibited by mannan but not galactan, indicating that PPA binds carbohydrate-specifically to BBM. The glycoprotein ligands for PPA were identified by combining purification of ligands using a PPA-Sepharose column, PAGE and LC/MS/MS. The identified ligands for PPA in BBM included sucrase-isomaltase (SI) and Na^+/glucose-cotransporter 1 (SGLT1), which function to assimilate glucose. Using BBM vesicles, we found the functional changes in PPA and its ligands by the *N*-glycan-specific interaction. The starch-degrading activity of PPA and maltose-degrading activity of SI were remarkably enhanced, while Glc uptake by SGLT1 was almost completely inhibited at high, but physiologically possible PPA concentrations.

The results indicate that mammalian pancreatic α-amylases share a common carbohydrate-binding activity and carbohydrate-specifically bind to the intestinal mucosa. Interaction with *N*-glycans in the BBM enhanced PPA and SI to produce much Glc on the one hand and to inhibit Glc absorption by enterocytes via SGLT1 in order to prevent a rapid increase in blood sugar on the other. Therefore, α-amylase plays a key role in regulating Glc assimilation for blood homeostasis.[2]

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(63) Soluble polysialylated NCAM-110 produced by lung epithelial cells acts as a novel player of innate immune system

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In the mammalian system, post-translational modification of glycoproteins by polysialic acid (polySia) serves to dynamically modulate biological processes during development as well as in the adult organism. Yet, the role of polySia in innate immunity has not been addressed. Here, NCAM-140-attached polySia chains with more than 40 sialic acid residues were detected in adult human lungs and primarily located in the late *trans*-Golgi apparatus of bronchial epithelial cells. Using a cell culture model, interleukin (IL)-1β or lipopolysaccharide (LPS) were found to efficiently induce ectodomain shedding of this glycoprotein resulting in the release of soluble polySia-NCAM-110. Likewise, during chronic obstructive pulmonary disease (COPD) that comes along with elevated IL-1β, increased levels of shedded polySia-NCAM-110 were seen in diseased human lung tissue. Soluble polySia-NCAM-110 was

demonstrated to directly interact with extracellular histones as well as DNA/histone-network-containing "neutrophil extracellular trap" (NET), thereby inhibiting their cytotoxic activity. Thus, shedding of polySiaNCAM-110 may provide a novel host-protective mechanism to reduce lung tissue disruption during inflammatory processes. Consequently, the induction of polysialylation or the direct administration of polySia might be useful strategies to combat diseases with strong exaggerated NET formation-like sepsis, systemic lupus erythematosus, cystic fibrosis or lupus nephritis.

(64) Attenuation of type 3 α2,3 sialyltransferase (St3gal3) is associated with enhancement of eosinophilic allergic airway inflammation in mice

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In a previous study, type 3 α2,3 sialyltransferase (St3gal3) was required for constitutive Siglec-F lung epithelial ligand synthesis (Am J Respir Cell Mol Biol. 2011;44:238–243). Although Siglec-F is a pro-apoptotic receptor on mouse eosinophils, little is known about the contribution of its natural lung ligand to modulating eosinophilic airway inflammation. We hypothesized that attenuation of St3gal3 would decrease the Siglec-F lung ligand synthesis and this would be associated with enhanced eosinophilic airway inflammation in mice. C57BL6 wild-type (WT) mice and St3gal3 heterozygous deficient ($St3gal3^{+/-}$) mice were used. To induce eosinophilic airway inflammation, mice (8–10-week-old) were intraperitoneally sensitized with 50 μg ovalbumin (OVA) in 1 mg alum on days 0 and 14, then challenged intranasally with 20 μg OVA on days 17, 19 and 21. Control mice were injected and challenged with PBS. All mice were sacrificed on day 22. Lung tissue and lavage samples were used for western blotting (Siglec-F-Fc), histologic (Giemsa) and cytologic analyses. Although western blotting of lung tissue homogenate with Siglec-F-Fc detected high-molecular weight bands in both WT and $St3gal3^{+/-}$ mice, bands in $St3gal3^{+/-}$ mice were weaker than those in WT mice. Lung lavage total cell numbers were significantly increased in both WT and $St3gal3^{+/-}$ mice after OVA sensitization and challenge. This increase was due to eosinophil and unexpectedly lymphocyte numbers that were 4- and 2-fold higher, respectively, in OVA sensitized and challenged $St3gal3^{+/-}$ mice comaped with WT mice. Infiltrating peribronchial eosinophils were significantly increased in OVA challenged $St3gal3^{+/-}$ mice comaped with WT mice. Neither eosinophils nor lymphocytes were increased in blood after OVA challenge. $St3gal3^{+/-}$ mice have reduced levels of Siglec-F ligands and more severe eosinophilic and lymphocytic inflammation comaped with WT mice. Reduced intrapulmonary Siglec-F ligands may contribute to this result.

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(65) Characterization of the FLDNF glycan epitope in *Schistosoma manonsi* and a semi-synthetic approach for heterologous expression

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Schistosomiasis is a debilitating intravascular parasitic disease caused by the helminth *Schistosoma mansoni* and related species. It is a major global health problem with over 200 million people currently infected and 500 million at risk due to insufficient diagnostics, treatments and lack of a vaccine. Previous vaccine platforms, which have been largely focused on protein antigens, have failed, due to our limited knowledge of which antigens should be targeted to induce a robust and protective immune response. *Schistosoma mansoni* has a complex and poorly understood tissue architecture and glycoconjugate profile, and there is evidence that immunodominant antigens are glycans. The majority of antigenic and immunogenic epitopes are contained in complex glycans, which can contain unique linkages and high levels of fucosylation. We hypothesize that specific glycans and their presentation are associated with protective immunity and that by defining the relationship between the glycome and the "glycogenome", we can decipher the structures and functions of parasite glycans in adaptive and innate immune responses. In this regard, our studies have focused on the glycan FLDNF [(Fucα1-3)GalNAcβ1-4(Fucα1-3) GlcNAc-R], a unique and antigenic fucosylated structure specific to *S. mansoni*. Our specific goals include characterization of FLDNF as a major glycan antigenic determinant, and the identification of the enzymes responsible for synthesizing FLDNF-containing glycans. Our innovative approaches employ heterologous expression of parasite genes in mammalian cells and identification of newly generated antigens using specific mono- and polyclonal antibodies to glycan antigens. Identification of these genes will allow us to study the structure and function of important carbohydrate antigens. These studies will lead to the first expression of a parasitic glycome and help us to better understand the significance and breadth of the immune response to glycan antigens.

(66) FUT2 genotypes of the premature infant is associated with distinct microbiota

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In intestinal mucosa, initial bacterial exposure induces transcription of the *FUT*2 gene, resulting in a more fucosylated mucosa, a putatively enriched niche for further colonization. Almost 25% of many human populations are homozygous recessive for *FUT*2,

and the adult intestinal microbial communities of these non-secretors differ from those of secretors. Factors that mediate the ontogeny of these distinct microbiota have not been investigated. This study tests the hypothesis that the early microbiota of the premature infant is influenced by FUT2 genotype of the infant and the mother. *FUT2* polymorphism was determined as the 428G > A mutation in 130 samples from 47 preterm infants and their mothers, enrolled from level III neonatal intensive care units in Cincinnati, OH. Infant stools were prospectively collected from 4 to 23 days of life from infants who survived hospitalization free of NEC or sepsis. The 16S rDNA bacterial gene was amplified followed by 454 FLX Titanium pyrosequencing of the V3-V5 region. Infants <29 weeks GA had different microbial communities than infants ≥29 weeks GA. Microbiota of infants <29 weeks GA differed between those who were *FUT2* recessive (non-secretor genotype) and those with an active *FUT*2 allele ($P = 0.02$): Non-secretors had higher relative abundances of Proteobacteria ($P < 0.001$), and OTUs of Klebsiella ($P < 0.001$), Serratia ($P < 0.001$) and Enterobacteriaceae ($P < 0.001$). Secretors had higher relative abundances of Firmicutes ($P < 0.001$) and an OTU of Staphylococcus ($P = 0.002$). Non-secretor infants and mothers may, in the early neonate, select for differing pioneering species during early gut colonization. This non-secretor microbial phenotype may mediate the strong relationship between *FUT*2 polymorphism and risk of necrotizing enterocolitis and could be a factor in the pathobiology of other conditions of unknown etiology. Supported by NICHD (HD059140 and HD013021) and NIAID (AI075563 and Contract HHSN272200900018C).

(67) Expression and antigenicity of tumor-associated Neu5Gc-containing *O*-glycans in human carcinomas

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Aberrant glycosylation related to altered expression of glycosyltransferases is prevalent on cancer cells and frequently involves variations in patterns of sialylation. In humans, this altered sialylation can also result from metabolic incorporation of dietary *N*-glycolylneuraminic acid (Neu5Gc), which differs from the human sialic acid *N*-acetylneuraminic acid (Neu5Ac) by one oxygen atom (Varki 2010). Neu5Gc-containing glycan epitopes are immunogenic in humans, generating a polyclonal antibody repertoire that is highly variable among individuals (Padler-Karavani et al. 2008). Studies in *Cmah* null mouse models in a human-like genetic deficiency of Neu5Gc show that such anti-Neu5Gc antibodies can either promote tumor growth at low doses or cause tumor regression when administered in excess (Hedlund et al. 2008; Padler-Karavani et al. 2011). Circulating antibodies against Neu5Gcα2-6GalNAcα1-*O*-Ser/Thr (anti-Neu5Gc-Sialyl-Tn IgG) appear to be novel carcinoma biomarkers (Padler-Karavani et al. 2011). This neo-sialoglycan resembles the well-known carcinoma-associated biomarker Sialyl-Tn, except that Neu5Ac is replaced with dietary Neu5Gc. However, direct evidence for expression of this epitope on human carcinomas is lacking. Here, we used immunohistochemistry with two highly specific antibodies to characterize the extent of co-expression of Neu5Gcglycan epitopes and Neu5Ac-Sialyl-Tn on human breast, prostate, ovary, colon and lung carcinomas, revealing some co-staining, particularly in mucinous carcinomas. We also generated MUC1-Fc that is heavily glycosylated with Neu5Gc-*O*-glycans (including Neu5Gc-Sialyl-Tn) and used it for affinity purification of human antibodies reacting with such glycans. These affinity-purified antibodies detected expression of Neu5Gc-*O*-glycans on human mucinous carcinomas by immunohistochemistry. The presence of Neu5Gc in *O*-glycans from the positively-stained tissues was confirmed by a mass spectrometry approach, including direct evidence for the presence of Neu5Gc-Sialyl-Tn.

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(68) Antigen mimicry by mimivirus collagens

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The giant Mimivirus harbors a linear dsDNA genome of 1.2 Mbp comprising 1,018 open reading frames of which 979 encode putative proteins. Mimivirus codes for genes like aminoacyl-tRNA synthetases, elongation factors, several DNA repair enzymes and topoisomerases among others which have not been identified in a virus so far. Furthermore, Mimivirus expresses seven collagen-like proteins during infection of its host *Acanthamoeba polyphaga.* At least two of these collagens are present on the surface of the virus as demonstrated by surface biotinylation analysis. We have recently shown that Mimivirus also expresses a bi-functional enzyme capable of hydroxylating and glycosylating lysine residues on collagen. Considering the structural similarity of Mimivirus collagen with animal collagen, we have investigated the potential of Mimivirus proteins to trigger a collagen-induced arthritis response in mice. Intradermal immunization of DBA mice with Mimivirus proteins resulted in formation of IgG antibodies recognizing mouse collagen type II. This response was accompanied by inflammation of joints as monitored by histology and in vivo imaging monitoring disease-associated proteases MMPs and cathepsins. This work demonstrates that exposure to Mimivirus collagen can break immune tolerance and induce auto-immunity in mice.

(69) The involvement of sialylation mediated by ST3GAL-I or ST3GAL-IV in pathogenesis of polymicrobial infection

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The reduction in neutrophil migration to the focus of infection is a central event that correlates with mortality in sepsis. Neutrophil

migration is mediated by the interaction of a variety of sialylated glycoproteins, especially by glycoconjugates that contains SLeX oligossacharide, such as α-1 acid glycoprotein (AGP). These sialoglycoconjugates can attach to adhesion molecules and block adhesion of neutrophil to endothelial cells, therefore decreasing neutrophil migration. In this context, our group has demonstrated that the AGP, inhibits neutrophil migration to the focus of infection during sepsis. Thus, understanding the mechanisms that control the synthesis of sialylated glycoconjugates and/or soluble glycoproteins could be of very impact in sepsis and other systemic inflammatory conditions. The objective of this study was to investigate the role of α2,3 sialyltransferase-I (ST3Gal-I) and -IV (ST3Gal-IV) in severe sepsis induced by cecal ligation and puncture model. Our results showed that ST3Gal-I- or ST3Gal-IV-deficient mice exhibited higher survival rate and higher neutrophil migration to the focus of infection when comaped with their wild-type littermates. We do not observe differences in the levels of pro-inflammatory cytokines such as TNF-α, CXCL2 and CXCL1in the local of infection or in serum as well. Corroborating with these results, bacterial content in these compartments were similar in both mice strains. Interestingly, we observed a high percent of neutrophils expressing the AGP receptor, Siglec-F, in ST3Gal-I deficient mice when comaped with the wild type, suggesting a compensatory mechanism to control neutrophil migration in the absence of ST3Gal-1. However, the mechanisms involved in the survival rate improvement during sepsis of the ST3Gal-I- or ST3Gal-IV-deficient mice are under investigation.

(70) Galectin-1 differentially regulates migration of immunogenic and tolerogenic dendritic cells across lymphatic endothelium

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Dendritic cells (DCs) migrate from inflamed tissues across lymphatic endothelium into lymphatic vasculature and traffic to regional lymph nodes where immune responses are initiated. However, the processes that regulate DC tissue egress and migration across the lymphatic endothelium are ill defined. Our goal is to understand the roles of glycan–lectin interactions in tissue egress of immunogenic and tolerogenic human DCs (iDCs and tDCs). Galectin-1 (gal-1) is highly expressed by human lymphatic endothelial cells (LECs). Using a migration assay that combines the extracellular matrix (ECM) with LECs, we found that addition of galectin-1 to ECM selectively inhibits migration of iDCs through the ECM and across LECs, while tDCs are not affected by the presence of galectin-1. However, the decreased expression of gal-1 by LECs overlying the ECM decreased tDC migration, indicating that gal-1 expression by LECs contributes to basal tDC migration. We identified the major gal-1 counterreceptor on human DCs as the cell surface mucin CD43. Gal-1 binds CD43 on both iDCs and tDCs, although CD43 expressed on iDCs displays more core 2 *O*-glycans than CD43 on tDCs and binds more gal-1. Treatment of iDCs with benzyl-α-GalNAc to block core 2-O glycan elongation abrogated the inhibitory effect of gal-1 on iDC migration, indicating that core 2 *O*-glycans are important for this effect. The mechanism by which gal-1 binding to CD43 retards iDC migration is currently being investigated. In lymphedema, fluid and immune cells accumulate in lymphedematous tissue, so that DCs are "stuck" in affected tissue and exacerbate inflammation. We determined that gal-1 is highly expressed in human lymphedema tissue samples, and we are currently analyzing the role of galectin-1 in tissue egress of DCs and lymphedema progression in an in vivo surgical mouse model.

(71) Comparison of Siglec-8 and Siglec-9 specificities for defined glycans and human lung counter-receptors

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Siglec-8 and Siglec-9, members of the sialic acid binding immunoglobulin-like lectin family, are immune inhibitory receptors expressed selectively on subsets of inflammatory cells that may suppress inflammation in asthma and COPD (chronic obstructive pulmonary disease) respectively. To explore the counter-receptors of Siglec-8 and Siglec-9 on human lung, we transiently expressed their extracellular domains fused with human IgG Fc in HEK293T cells. These were tested for avidity and glycan specificity using ELISA to glycolipids and neoglycolipids. Siglec-8-Fc binding specificity was very narrow binding only to 6'-sulfo-sialylLacNAc (NeuAc α2-3 (6-SO3)Galβ1-4 GlcNAc), whereas the Siglec-9-Fc bound to several α2-3-terminated glycans, including 6-sulfosialylLacNAc (NeuAc α2-3Galβ1-4 (6-SO3)GlcNAc), GT1b and GD1a. To search for endogenous counter-receptors in human lung, the same siglec chimeras were used in blot overlay assays of glycoproteins extracted from different human lung tissue compartments and cells. Siglec-8-Fc bound to a narrow set of counter-receptors in extracts of human lung tissue. Among the several lung fractions, Siglec-8-Fc bound robustly and in a sialic acid-dependent manner to a high-molecular weight component extracted from fresh human bronchial scrapings, but not to components in extracts from lung parenchyma, blood vessels or cultured human airway epithelial cells (grown at an air-liquid interface). In contrast, Siglec-9-Fc had broader specificity, binding to different molecular weight components in extracts of lung parenchyma, blood vessels, cultured airway epithelial cells, as well as bronchial scrapings. Siglec-8 and Siglec-9 bound to components in the bronchial scrapings that were of different molecular weights. Characterization of Siglec-8 and Siglec-9 counter-receptors will be important in the study of cell–cell interactions in lung inflammatory diseases. Supported by the Lung Inflammatory Disease Program of Excellence in Glycoscience (LID-PEG), NHLBI HL107151.

(72) SPARC as a regulator of collagen signaling in pancreatic cancer

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The extracellular matrix (ECM) is a principal component of pancreatic ductal adenocarcinoma (PDAC) and is rich in fibrillar

collagens, which provide structural support and enhance tumor cell survival and chemoresistance. Collagen expression and deposition is a complex process that is orchestrated in part by the matricellular protein SPARC. We propose that SPARC controls collagen binding to the cell surface and reduces collagen-mediated signaling. Discoidin domain receptors (DDR1, DDR2) are two homologous receptor tyrosine kinases that serve as receptors for collagens. DDRs have been implicated in regulating cell proliferation, migration, adhesion, ECM remodeling and response to growth factors. The fact that SPARC and DDRs share the same collagen binding site suggests that SPARC perturbs collagen interaction with DDRs. We propose that tumor cell invasion is enhanced in PDAC due to the progressive loss of SPARC expression by PDAC tumor cells, which results in increased ligation and the activation of DDRs. This is supported by studies that have demonstrated that growth of PDAC tumors is significantly more aggressive in "SPARC-deficient" animals. We have also recently discovered that nilotinib, a high-specificity DDR inhibitor, can reduce tumor growth selectively in SPARC-deficient animals. Therefore, we hypothesize that SPARC reduces DDR activation and that loss of SPARC by pancreatic tumor cells enhances DDR-mediated signaling, which promotes tumor progression. In this study, we have established that SPARC can interfere with collagen binding to DDR1/2. Additionally, we have demonstrated that collagen stimulation of primary murine PDAC cells alters the expression of DDR1/2 and expression of epithelial and mesenchymal markers. We propose that targeting DDR signaling has the potential to directly affect tumor cell viability and that inhibition of DDRs will improve response of PDAC to standard chemotherapy. Furthermore, we suggest that SPARC, by inhibiting collagen receptor signaling, blunts PDAC tumor progression.

(73) Post-natal analysis of the 200 and 125–150 kDa thrombospondin-2 immunoreactive isoforms in murine skeletal tissues

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The matricellular protein, thrombospondin-2 (TSP2) is highly expressed in the developing skeleton, it is a component of the bone matrix and it promotes osteoblast differentiation in vitro. We recently reported that two distinct species (200 and 125–150 kDa relative molecular weights) of TSP2 are present in the epiphyseal–metaphyseal region of femurs harvested from 1- and 2-month-old mice. Here, we have extended these observations to include tissue harvested from 1-day and 18-day-old mice. Similar to bones harvested from 4-week-old mice, whole femurs harvested from 18-day-old animals displayed both 200 and 125–150 kDa TSP2 immunoreactive isoforms ($n = 2$). Conversely, only the full-length 200 kDa TSP2 immunoreactive species was detected in long bones, as well as in calvaria, dissected from 1-day-old mice ($n = 9$). Calvaria cultured for 6 and 10 days with ascorbate and β-glycerolphosphate to promote mineralization also only displayed the 200 kDa TSP2 species ($n = 4$–5 per time point). We hypothesize that the appearance of the lower molecular weight species of TSP2 in the epiphyseal-metaphyseal region happens in conjunction with the post-natal formation of secondary centers of ossification in the long bones of mice.

(74) Localization of growth factor binding within the N-terminal region of MAGP-1

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Microfibril-associated glycoprotein-1 (MAGP-1) is a matricellular component of fibrillin microfibrils. Although MAGP-1 protein is abundant in the microfibril, equimolar with the fibrillins, loss of the *MFAP-2* locus encoding MAGP-1 does not result in structural defects in the microfibril. We have previously shown full-length MAGP-1 binds tightly to active TGFβ-1 and BMP-7 with affinity constants in the low-nM range. Furthermore, many of the phenotypes associated with the loss of MAGP-1 are consistent with dysregulation of the TGFβ/BMP signaling system. Thus, the interaction of MAGP-1 with active growth factors may be a key to understanding the biological role of MAGP-1. The work presented here further defines the growth factor binding domain of MAGP-1. Using SPR analysis to measure the kinetics of the interaction of soluble TGFβ-1 to the surface of chips covalently coated with purified MAGP-1 domains, we localized a single binding site to the N terminus of MAGP-1 within domains 3 and 4. This growth factor binding site is spatially distinct from matrix binding previously localized to domains 7 and 8. Further localization was accomplished using inhibition of TGFβ-1 binding to a chip coated with full-length MAGP-1 by synthetic peptides from domains 3 and 4. The smallest peptide capable of inhibiting TGFβ-1 binding to full-lenght MAGP was 19 residues in length representing amino acids 17–35 from the mature protein. This peptide also inhibited BMP-2 binding to full-length MAGP-1. To assess functional consequences of the MAGP-1 interaction with TGFβ-1, we pre-mixed TGFβ-1 with MAGP-1 then added the complex to TGFβ-1 reporter cells. These reporter cells secrete easily measured soluble alkaline phosphatase in response to TGFβ stimulation. Excess MAGP-1 did not inhibit TGFβ-1 activity measured by these reporter cells. Thus, a function of the MAGP-1 TGFβ/BMP interaction may include sequestration and/or storage of active growth factor in the microfibrillar matrix.

(75) Expression, biosynthetic control and function of LacdiNAc/phospho-LacdiNAc on ECM glycoproteins

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N,*N*'-Diacetyllacosamine (LacdiNAc or LDN) has been found as a terminal modification of *N* and *O*-linked glycans on a restricted series of proteins, like neuropeptide hormones, carbonic anhydrase or glycodelin (*N*-linked) and proopiomelanocortin or a couple of extracellular matrix-related proteins (O-linked). This comparatively small number of proteins suggests activity control of the β4-specific GalNAc transferases (T3 or T4) by a substratum-specific peptidic determinant. While a cis-located peptide was described for the control of *N*-linked LDN formation (Miller et al.

2008), we could provide evidence for a distinct peptidic determinant in the control of LDN on ZP3 located downstream of the three O-glycosylation sites. On the other hand, a proteomic approach based on affinity-isolated glycoproteins from HEK-293 cells revealed numerous other hitherto unidentified LDN-positive O-glycoproteins and throws doubts on the concept of biosynthetic control by substratum-specific peptidic determinants in this context. The modification of ZP3 with phosphorylated LDN (Breloy et al. 2012) may be of particular biological relevance, since earlier studies have shown that this glycoprotein serves as the primary sperm receptor and that the binding of spermatozoa to ZP3 is primarily regulated by glycan side chains. The structure of the *O*-linked chains, however, that were claimed to be involved in the fertilization process, was quite different, including terminal disaccharides Galα1-3Gal, Gal β1-4GlcNAc and GalNAcβ1-4GlcNAc. It has been controversially discussed whether sulfation may be important in human sperm–ZP3 interactions similar to those involving sulfate on high-affinity ligands for selectins. In the same manner, also phosphorylated glycans, in particular phosphorylated LDN, could play a role in the regulation of sperm–egg binding or in the fertilization process in general. Binding studies on ZP3 truncation constructs expressing phospho-LDN, enzymatically dephosphorylated LDN or biosynthetic LDN precursors are currently perfomed to reveal insight into potential regulatory mechanisms.

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(76) Characterization of a recombinant HA binding protein: TSG6dHep-Fc

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TSG6 (Tumor necrosis factor-Stimulated Gene-6) protein is a 30kDa secreted hyaluronan (HA)-binding glycoprotein composed of an N-terminal

HA-binding LINK module and a C-terminal CUB module. The HA-binding LINK module is involved in extracellular matrix stability and cell migration. and thus plays critical roles in inflammation and tissue remodeling during physiological and pathological processes. It is also important for the formation and remodeling of HA-rich pericellular coats and ECM. The interaction of the TSG6 LINK module with HA as well as its HA binding features are well characterized. Its unique structure, GAG binding property and size make it an attractive candidate as an HA binding probe for in vitro and potentially in vivo detection of HA in cells and tissues. Here, we describe the successful production of a unique, recombinant HA binding protein by fusion of the TSG6 LINK module mutated in its heparin-binding site and the Fc portion of human IgG1 (TSG6dHep-Fc) in CHO mammalian cells. The GAG binding proprieties of the purified protein were evaluated by direct binding and competitive binding assays. Our results indicate a highly specific binding of TSG6dHep-Fc to HA and to a lesser extent to Chondroitin Sulfate A, with no binding to Heparin and Chondroitin Sulfate C. Biotinylation of TSG6dHep-Fc resulted in similar HA binding affinity and specificity. Furthermore, the biotinylated TSG6dHep-Fc showed very similar GAG binding pattern when compared to that of commercially available biotinylated HA-binding protein. Similarly, the histochemical detection of HA in cells and formalinfixed, paraffin-embedded tissue sections, using biotinylated TSG6dHep-Fc resulted in hyaluronidase-sensitive staining patterns that were comparable to those obtained with commercially available biotinylated HA-binding protein. Taken together, our results suggest that TSG6dHepFc is a sensitive and specific tool for the detection of HA by biochemical and histochemical methods and possibly in other assay formats.

(77) BSA-loaded extracellular matrix gel for sustain drug delivery

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An extracellular matrix has been widely investigated as a biologic material for the treatment of disease. Through this investigation a wide range of biomedical products based on the extracellular matrix (ECM) have been developed. Among several ECM, the small intestine submucosa (SIS) and porcine cartilage powder (PCP) have the advantage of mimicking many features of native tissues. We describe the preparation of SIS and PCP solution as injectable formulation of model BSA drug and the assessment of in vivo release of BSA.

The BSA.FITC.loaded SIS and PCP solution was injected subcutaneously into the dorsum of rats. For the detection of BSA-FITC, an aliquot of blood was drawn from the tail vein of each rat at specified times. The concentrations of BSA-FITC in plasma were determined by fluorescence. The ECM suspension containing BSA-FITC were injected subcutaneously into the left dorsum of a nude mouse and fluorescence images of the mouse were collected. In addition, we characterized the host tissue response to determine the biocompatibility of injected ECM gels.

The SIS and PCP suspension easily entrapped BSA in pharmaceutical formulations and they formed gel formation in vivo. The plasma BSA-FITC concentrations released from rats with a BSA-FITC-loaded ECM gel exhibited a sustained-release profile, producing detectable levels of BSA-FITC in plasma over 1 week. The real-time molecular imaging of fluorescent images was sustained over 1 week after injection of a BSA-FITC-loaded ECM suspension. In addition, ECM gels provoked little inflammatory response in histological analysis.

In conclusion, we confirmed availability of SIS and PCP formulations, showing that BSA-loaded SIS and PCP gels are effective for sustain release of the BSA.

(78) α-Dystroglycan O-mannosylation influences the subsequent addition of GalNAc by UDP-GalNAc polypeptide *N*-acetylgalactosaminyltransferases

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α-Dystroglycan (a-DG) is the extracellular component of the dystrophin glycoprotein complex that links the cytoskeleton to the extracellular matrix in a number of cell types and is important in the organization of a variety of tissues. The complex glycosylation of a-DG, comprising *O*-Man and *O*-GalNAc glycans, as well as N-glycosylation is critical to its function, with *O*-Man glycans being directly implicated in the interaction with laminin. The distribution of *O*-glycan forms has been defined through mapping in considerable detail, providing an opportunity to explore how glycosylation sites are chosen and regulated. While a-DG is the only glycoprotein where O-Man glycosylation has been explicitly characterized, compelling evidence exists for other examples, and it serves as the paradigm molecule for this post-translational modification. In all likelihoods, the two forms of O-glycosylation coexist in the others. With the knowledge that the *O*-Man modifications occur first, followed by *O*-GalNAc and glycan elongation and with information on the specific positions occupied by these in the mucin-like regions of a-DG, we examined the ability of a number of UDP-GalNAc polypeptide *N*-acetylgalactosaminyltransferase (ppGalNAc-T) isoforms to modify glycopeptide substrata that emulated O-Man glycosylation patterns in a-DG. Two segments were chosen, each with clusters of four sites for glycosylation. Using recombinant forms of ppGalNAc-T1, -T2, -T3, -T4, -T5, -T7, -T10, -T11 and -T16, the ppGalNAc-T1, -T3 and -T5 isoforms were found to be active on the glycopeptide substrata. The presence and the position of *O*-Man sites controlled whether *O*-GalNAc modifications were allowed and the sites to which they were directed. The products of the enzyme transformations in vitro emulated the patterns identified in tryptic fragments from natural material. For the three isoforms we found active, RT–PCR on muscle shows that the Galnt1 gene is the only one expressed to significant levels, suggesting that this isoform is the one contributing to a-DG modification.

(79) Characterization of the substratum specificity of mammalian heparanase using the aglycomic approach

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Heparan sulfate (HS) glycosaminoglycans are complex acidic polysaccharides that interact with numerous protein ligands and receptors involved in diverse biological processes. Heparanase is an endo-β-D-glucuronidase that catalyzes the partial depolymerization of HS chains and plays a central role in the remodeling of the HS containing the extracellular matrix and basement membranes. The activity of heparanase directly correlates with the metastatic potential of tumor-derived cells. It is therefore of great interest to study the substratum specificity of heparanase for the drug-development purpose. In our study, we took a glycomic approach to profile the structure features of the heparanase cleaved products. We treated HS substrata from different sources with heparanase to generate heparanase-cleaved fragments, which were subsequently subjected to partial depolymerization by bacterial heparin lyase III to generate oligosaccharides. Since the serial action of heparanase and heparin lyase III will create a series of odd-numbered oligosaccharides, which carry information about the heparanase cleavage sites, we hypothesize that profiling of those oligosaccharides will reveal the substratum specificity of heparanase. Indeed, compared with the sample without heparanase pretreatment, LC/MS profiling of the oligosaccharides produced by the serial digestion demonstrates a dramatic increase in the amount of odd-numbered oligosaccharides. The most abundant compositions of the trisaccharides were found to be [1,1,1,0,1], [1,1,1,0,2] and [1,1,1,1,1] (ΔHexA, HexA, GlcN, Ac, SO_3) for the non-reducing-end side of the heparanase cleavage site and [0,1,2,0,3] and [0,1,2,0,4] for the reducing-end side. This profile pattern is consistent across the different HS substrata from different sources, confirming our hypothesis that it represents the substratum specificities of heparanase. We further studied the detailed structures of the most abundant trisaccharides by tandem MS. The structural features of those trisaccharides are in good agreement with previous studies on the substratum specificities of heparanase using other approaches.

(80) Expression of matrix components in liver cells: Studies in human hepatocytes (HepG2) and hepatic stellate cells (LX-2)

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Liver fibrosis occurs as a consequence of a sustained wound healing response; however. the cells involved in this process are still controversial. Quiescent hepatic stellate cells (HSCs) are known to be activated during liver fibrosis and are the main extracellular matrix (ECM)-producing cells in this process. There is also evidence to suggest that hepatocytes contribute to ECM production through epithelial-mesenchymal transition. The aim of this project is to investigate the ECM components, particularly proteoglycans and glycosaminoglycans, produced by model human HSCs and hepatocyte cell lines, LX-2 and HepG2, to provide further insight into the role of these cells in liver fibrosis. HepG2 and LX-2 cells were cultured and probed for the presence of perlecan, type XVIII collagen, heparan sulfate (HS), chondroitin sulfate (CS) and laminin. Perlecan was detected extracellularly in both cell types; however, LX-2 cells demonstrated a microfibrillar structure localized in the extracellular matrix. Type XVIII collagen was shown to be extracellular for HepG2 cells and present intracellularly for LX-2. HS and CS staining was pericellular for HepG2, while intracellular for LX-2 with additional pericellular HS staining and extracellular CS staining. HepG2 cells express B chain, α1 chain and LAMA1 laminins as extracellular stains, while LX-2 only express B chain laminins as intracellular components. In addition, HepG2 cells expressed human serum albumin, while LX-2 cells did not. The presence of these matrix components in LX-2 cells suggests their importance in liver fibrosis through the secretion of proteins into the surrounding environment in response to liver damage. Perlecan and laminin were found to be co-localized in HepG2 cells, however, did not co-localize

with either HS or CS, suggesting an interaction between these two proteins to influence cell differentiation, migration and adhesion for fibrosis. Conditioned media from both cell lines were enriched for proteoglycans by anion exchange chromatography and were found to contain perlecan, HS and CS; however, the concentration was higher in LX-2. Future studies will involve co-culture experiments to determine any changes in ECM component production and secretion.

(81) Retention of D469del-COMP causes necroptotic cell death of PSACH chondrocytes

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The D469del mutation in cartilage oligomeric matrix protein (COMP) causes pseudoachondroplasia (PSACH). PSACH is a severe disproportionate short-limb dwarfism associated with joint laxity and early onset osteoarthritis. PSACH growth plate chondrocytes show a unique intracellular pathology of retention of mutant COMP and premature death. To gain a better understanding of these underlying mechanisms causing chondrocyte death, we examined D469del-COMP activation of the unfolded protein response (UPR) and cell death pathways. Using an inducible expression system, we examined the effects of D469del-COMP retention after 4 days of mRNA expression and 5 days without the inducing agent in rat chondrosarcoma cells. Retention of D469del-COMP stimulated *Chop* and *Gadd34* triggering the reactivation of protein translation that exacerbated intracellular retention. High levels of *Nox4* and ER stress inducible *Ero1β* generated ROS causing oxidative stress. Increased expression of *Gadd* genes and the presence of γH2AX indicated that there was DNA damage. The presence of cleaved-apoptosis inducing factor (tAIF) and the absence of activated caspases indicate that the retention of D469del-COMP triggers cell death in chondrocytes by necroptosis which is a caspase-independent programed necrosis. In our transgenic mouse model of PSACH, that expresses D469del-COMP in chondrocytes, the retention of D469del-COMP also causes the activation of the UPR, oxidative stress and DNA damage resulting in premature death of growth plate chondrocytes by necroptosis. In addition, the increased expression of genes such as interleukins, chemokines, eosinophil-associated enzymes and metalloproteinases in the knee joints, suggest an inflammation process in these mice. Altogether, these results suggest a model in which D469del-COMP expression induces persistent ER stress, oxidative stress, inflammation and DNA damage, thus priming chondrocytes for necroptosis. This defines for the first time the precise mechanisms underlying D469del-COMP pathology in PSACH and suggests that oxidative stress and AIF may be promising therapeutic targets.

(82) Reduction in mutant COMP ER retention and cell death by drug therapy

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Cartilage oligomeric matrix protein (COMP), a secreted glycoprotein synthesized by chondrocytes, regulates proliferation and type II collagen assembly. Mutations in the COMP gene cause pseudoachondroplasia (PSACH). PSACH is a severe dwarfing condition associated with short limbs, protrusion of sternum and normal skull with attractive face. PSACH chondrocytes accumulate COMP and other ECM proteins in the ER which results in premature cell death. Using an inducible system to express D469del-COMP in mice, we have generated a in vivo model that replicates the critical cellular and clinical features of PSACH. One week in mouse development roughly translates into 2 years of human development, and D469del-COMP mice are smaller at 1 week of age which correlates with PSACH diagnosis at 2–3 years. Consistent with PSACH, both hind and fore limbs were reduced in the D469del-COMP mice and limb reduction was progressive. In the D469del-COMP mice, the skull and snout length was reduced comaped with controls and this may equate to the attractive face associated with PSACH. Immunostaining, transcriptome analysis and qRT–PCR suggest a molecular model in which D469del-COMP triggers apoptosis signaling during the first postnatal week. By 3 weeks, when most chondrocytes are retaining D469del-COMP, inflammation, oxidative stress and DNA damage contribute to chondrocyte cell death by necroptosis. Importantly, when CHOP was eliminated, D469del-COMP intracellular retention and premature chondrocyte cell death was drastically decreased. This indicates that UPR and ER stress signaling through CHOP plays a significant role in PSACH pathologic processes. Post-natal valproate and PBA therapy reduces intracellular retention of D469del-COMP and chondrocyte death in the growth plate. Taken together, this work suggests that there may be an optimal window prior to the induction of significant D469del-COMP retention in which the PSACH pathology could be reduced by psychotropic drugs or therapies that limit mutant COMP expression.

(84) Increased Expression of Connective Tissue Growth Factor (CTGF/CCN2) Mediates Collective Cell Migration in Skin Epithelial Cells

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Injury to the epidermis triggers an organized series of cellular and molecular events to restore the integrity of the damaged tissue. Activated skin epithelial cells at the wound edge contact dermal matrix and show a different profile of gene expression, which regulate migratory cell phenotype. Using expression arrays, we found that CCN2 (connective tissue growth factor/CTGF) was markedly up-regulated in keratinocytes migrating on type I collagen. CCN2 is known to promote fibrosis, mostly through its effect on fibroblasts. Although it is expressed by keratinocytes in skin, its function in the epidermis or other epithelial cells is unknown. To assess if CCN2 influences re-epithelialization, we utilized recombinant protein or manipulated its expression by adenovirus transduction and investigated its role in matrix deposition, migration, proliferation, adhesion and differentiation of primary human keratinocytes. Our studies demonstrated that CCN2 increased

collective cell migration, but not single cell migration, the former is the migratory phenotype of cells at the wound edge. This effect was seen only in cells overexpressing CCN2, but not in the presence of exogenous CCN2. We did not observe any significant effect for CCN2 in other functions that we tested. Our findings suggest that up-regulation of CCN2 in epithelial cells facilitates cell migration, and upon secretion it has more a paracrine function through its effect on other cells types, mostly fibroblast present at the wound site.

(85) A biodegradable, injectable, small intestine submucosa hydrogel with an adjustable therapeutic window

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Over the past few decades, several injectable in situ-forming gel systems have been developed for various biomedical applications, such as drug or cell delivery carriers. Small intestine submucosa (SIS), derived from the submucosal layer of porcine intestine, consists of types I and III collagens, small amounts of types IV, V and VI collagens and several biological factors. SIS was suspended with an aqueous solution consisting of acetic acid and pepsin, followed by freeze-drying to yield the final SIS powders. The SIS powder was dispersed in PBS to yield the desired concentrations of SIS. The SIS solution was cross-linked by ethyl-3-(3 dimethylaminopropyl) carbodiimide (EDC) at different concentration. The cross-linked SIS solution injected to rat through subcutaneous injection. The formed SIS gel showed an interconnecting three dimensional network structure. In addition, the injected SIS gels maintained its shape at the injection site for 4 weeks. However, the size of the excised SIS gels gradually decreased over timescale after subcutaneous injection into rats. The degradation rate showed the dependence on the EDC concentration. In conclusion, we prepared successfully the in situ formed SIS gels with cross-linking agents. Also, the SIS gels may serve as a minimally invasive in situ forming gel system with an adjustable in vivo biodegradable window.

(86) Expression of pro- and mature forms of decorin and biglycan in costal cartilage

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Small leucine rich proteoglycans (SLRPs) are small proteoglycans that are highly expressed in a wide variety of tissues. Decorin and biglycan are prominent members of the SLRP family and regulation of ECM growth, growth factor sequestration, and fibrillogenesis are important functions. Biglycan and decorin are homologous but with divergent patterns of expression. Null mutations lead to abnormal collagen architecture in mice suggesting a mechanistic role for these proteins in skeletal disorders. Decorin and biglycan have different isoforms, the pro and mature forms that show differential expression. Proforms have a 14 amino acid N-terminal pro-peptide that is cleaved in the mature forms. The abundance of pro-forms of both SLRPs is tissue and age-dependent. Costal cartilage is a type of hyaline cartilage connecting the bony ribs to the sternum. Structurally, costal cartilage has been shown to consist of straw-like structures running parallel to the length of the tissue. Gene expression in this tissue shows high levels of decorin comaped with biglycan. The complex arrangement of fibers observed in costal cartilage and the role of decorin and biglycan in these structures has not been explored. In this study, we investigated the presence and distribution of the different isoforms of decorin and biglycan, hypothesizing that both pro-forms will be present in our juvenile samples. This was achieved by immunohistochemistry and western blots of costal cartilage from teenage patients with pectus carinatum and an age-matched control. Our results show the presence of the mature form of decorin and the pro form of biglycan in the inter-territorial matrix in patient and control samples. Proforms of decorin and biglycan are

(87) BSA-FITC loaded small intestine submucosa hydrogels for in vivo delivery

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The aim of this study was to explore the potential utility of an injectable small intestine submucosa (SIS) as a depot for bovine serum albumin (BSA) model proteins. The SIS fine powder was prepared by the porcine jejunum and exhibited a size range of 10–20 μm. The SIS powder was swelled in a biological solution. The SIS solution easily entrapped BSA in pharmaceutical formulations at room temperature. The SIS solution gelled in the rat through subcutaneous injection. The formed SIS gel showed an interconnecting three dimensional network SIS structure to penetrate through it and maintained its shape at the injection site for the full experimental observation period. The amount of BSA-FITC released from the SIS gel was determined in rat plasma and monitored by real-time in vivo molecular imaging. The data indicated the sustained release of BSA-FITC for 20 days in vivo. In addition, SIS gel provoked little inflammatory response. Collectively, this work show that an SIS gel can act as an injectable drug depot. Our in situ gel-forming SIS system may provide numerous benefits as a minimally invasive therapeutics depot and as a useful experimental platform for testing the sustained in vivo pharmacological performance of protein drugs.

(88) Biochemical and biological characterization of fibulin-7

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Fibulin-7, the latest member of the fibulin ECM proteins, also called TM14, contains a unique sushi domain at the N terminus and a fibulin module at the C terminus. Its biochemical and biological functions are still unknown. By using northern blot and

immunohistochemistry, we found that fibulin-7 was highly expressed in the outer medulla of the kidney and transitional epithelium of the ureter and bladder in mouse. Our preliminary in vitro analysis using human umbilical vein endothelial cells (HUVEC) revealed that fibulin-7-mediated cell adhesion and this activity was blocked by α2, α5 or β1 integrin antibodies. Moreover, fibulin-7 was shown to interact with laminins-411 and -332 by solid-phase-binding assay. Fibulin-7 was present on the cell surface and released into the culture supernatant by treatment with exogenous heparin, suggesting that fibulin-7 may bind to the heparin-containing molecule(s) on the cell surface. To determine the cell surface binding site of fibulin-7, we used various forms of fibulin-7 mutants, including the N-terminal deletion and sushi deletion, as well as two C-terminal deletion mutants. Whereas the amount of mutant proteins released into the media were increased by exogenous heparin in sushi and C-terminal deletion mutants, the N-terminal deletion mutant abolished heparin-induced secretion. These results indicate that the N-terminal region of fibulin-7 is important for cell surface binding and fibulin-7 may modulate the function of other ECM proteins on the cell surface. We are currently identifying the binding sites for integrins and laminins using domain deletion mutant proteins.

(89) Purification and bioactivity of glycolipids from fish brain

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The purification methods and bioactivity of glycolipids from tuna fish brain were studied. The lipids of tuna fish brain were extracted with chloroform:methanol (2:1, V/V) including glycolipids and phospholipids. The total carbohydrate content in the lipids was 1.5%. The lipids were separated by the thin layer chromatography (TLC) and purified by silica gel column chromatography with chloroform: methanol including cephalin, lecithin, lipositol and unknown lipids. One of the unknown lipids was preliminarily identified as galactolipid by HPLC. The bioactivity of purified galactolipid from TLC was investigated. It was found that the growth of Hela and 7721 cells culture was suppressed by galactolipid, which suggested the potentially anticancer activity.*Keywords:* glycolipid / galactolipid / purification / anticancer activity

(90) Human microfibril-associated protein 4 is highly expressed in the heart and systemic levels are increased with acute myocardial infarction

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Microfibril-associated protein 4 (MFAP4), a matricellular-like protein, is reported to be present in the systemic circulation and previously suggested as a biomarker for cirrhotic disease. In the present study, we sought to characterize the expression profile of MFAP4 in human tissues. Moreover, we aimed to determine the potential of MFAP4 in the systemic circulation to serve as a biomarker for disease processes in patients with cardiovascular diseases. Immunohistochemical studies demonstrated high levels of MFAP4 mainly at sites rich in elastic fibers. Quantitative real-time PCR demonstrated that the relative MFAP4 mRNA expression was higher in the heart, lung and intestine than in other elastic tissues. AlphaLISA technique determines MFAP4 in serum samples from 30 patients with acute myocardial infarction (AMI) patients, 30 patient with ischemic heart disease (IHD) waiting for coronary bypass operation and 60 control age- and gender-matched patients. Systemic MFAP4 levels were significantly increased in patients with AMI comaped with control subjects ($P < 0.05$) and patients with IHD ($P < 0.01$). Systemic MFAP4 concentrations positively correlated with other extracellular proteins such as fibulin-1 ($r = 0.572$; $P = 0.007$), osteoprotegerin ($r = 0.619$; $P = 0.002$) and osteopontine ($r = 0.646$; $P = 0.001$) in AMI patients. We investigated the gene expression of MFAP4 in various human tissues and documented that MFAP4 was mainly located to elastic fibers and highly expressed in the heart, lung and intestine. Moreover, we have provided the first data demonstrating MFAP4 as a potential biomarker for AMI.

(91) Neu1 sialidase and matrix metalloproteinase-9 (MMP-9) cross-talk in alliance with neuromedin-B GPCR receptor is essential for ligand-induced intracellular TOLL-like receptor-7 and -9 activation and cellular signaling

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Toll-like receptors (TLRs) are essential sensors of microbial attack, and they orchestrate the innate immune response against many micro-organisms. Endosomal TLRs recognize nucleic acids from different pathogens and elicit pro-inflammatory responses. The signaling pathways of these mammalian TLRs are well characterized, but the initial molecular mechanisms activated following ligand interactions with their receptors remain poorly defined. Here, we report a novel signaling paradigm initiated by binding of specific TLR-7 and -9 ligands (Imiquimod and CpG) to potentiate G protein-coupled receptor and matrix metalloproteinase-9 (MMP9) activation to induce Neu1 sialidase. Central to this process is that Neu1–MMP9 complex is bound to TLR7 and TLR9 in naive and ligand stimulated macrophage cells as revealed by co-immunoprecipitation and colocalization assays. Using NFκB-dependent secreted alkaline phosphatase (SEAP) analysis, ligand-induced TLR7 and TLR9 activation was significantly inhibited by Tamiflu, MMP9 inhibitor and BIM-23127 (a specific NMBR inhibitor). Mal-2 lectin (*Maackia amurensis* agglutinin) bound to immunoprecipitated TLR-7 and -9 in cell lysates from naive but not TLR ligand stimulated RAW-blue macrophage cells, indicating the removal of the α-2,3 sialic acid residues from the stimulated receptor ectodomain. Tamiflu, MMP9 inhibitor and BIM-23127 blocked MyD88 recruitment to the ligand stimulated TLR-7 and -9 receptors. This study uncovers the molecular

mechanism involved in ligand-induced intracellular TLR activation. These findings may provide the future development of therapeutic drugs targeting intracellular TLR activation and signaling for disease modification. Research supported by NSERC to M.S. and CIHR dectoral award to S.A.

(92) NEU1 sialidase and matrix metalloproteinase-9 cross-talk in alliance with insulin receptors is an essential molecular signaling platform for insulin-induced receptor activation

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Insulin and insulin growth factor-1 receptors are covalently linked heterodimers of αβ-subunits on the cell membrane in the absence of insulin binding. The insulin receptor signaling pathways are initially triggered by insulin binding to the extracellular portion of α-subunits followed by the interaction of β-subunits and ATP. The parameter(s) controlling the insulin-induced conformational change of the receptor and activation remains unknown. Here, we report a membrane receptor signaling platform initiated by insulin binding to its receptor to induce Neu1 in live HTC-IR (rat hepatoma cells overexpressing the human insulin receptor), HTC-WT, HaCaT (human keratinocyte cell line) and MiaPaCa-2 (human pancreatic carcinoma) cell lines but not in type 1 sialidosis human fibroblast cells. Tamiflu, galardin and piperazine (broad-range MMP inhibitors), MMP9 inhibitor and anti-Neu1 antibody blocked Neu1 activity associated with insulin-stimulated live cells. Tamiflu blocked insulin-induced insulin receptor substratum-1 phosphorylation. Microscopy colocalization and co-immunoprecipitation analyses reveal that Neu1 and MMP-9 form a complex with naïve and insulin-treated receptors. These findings uncover a molecular organizational signaling platform of a novel Neu1 and MMP-9 cross-talk in alliance with insulin receptors. It proposes that insulin binding to the receptor induces MMP9 to activate Neu1 which hydrolyzes α-2,3 sialic acid in removing steric hindrance to generate a functional receptor. A complete understanding of the IR structure, activation and the role of sialic acids in the signaling pathways may provide a therapeutic strategy in the prevention of different diseases such as diabetes mellitus and cancer. Research supported by a NSERC grant to M. R.S. and King Abdullah Scholarship to F.A. from the Ministry of Higher Education, Saudi Arabia.

(93) The immunomodulatory activities exerted by recombinant forms of ArtinM depends on the protein quaternary structure

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ArtinM is an immunomodulatory lectin that induces Th1-balanced immunity, initiated by recognition of *N*-glycans of the TLR2 on the surface of phagocytes. ArtinM acts on macrophages and neutrophils, through the binding to *N*-glycans attached to TLR2 and CXCR2, respectively. Because these abilities make ArtinM a good candidate for pharmaceutical applications, we decided to obtain recombinant ArtinM in a large scale. Recombinant ArtinM expressed in *Escherichia coli* (bArtinM) and *S. cerevisiae* (yArtinM) were affinity purified on D-mannose columns and analyzed by glycoarray, which showed that all recombinant forms of ArtinM, similarly to the native lectin (jArtinM) obtained from *Artocarpus heterophyllus* seeds, bind specifically to a set of glycans containing the trimannoside that constitutes the *N*-glycans core. In terms of biological activities, jArtinM and yArtinM, but not bArtinM, induced macrophages to produce high levels of pro-inflammatory mediators, such as IL-6, IL-12, TNF-α and NO, an effect that was reduced in $TLR2^{-/-}$ macrophages. Neutrophil haptotaxis was assayed in vitro, providing cell migration of 129.2 ± 35.54 neutrophils/field (yArtinM), 59 ± 21.45 neutrophils/field (bArtinM) and 94 ± 32 neutrophils/field (jArtinM). Because the primary structures of the native and recombinant forms of ArtinM are coincident, we have investigated if the quaternary structure of these molecules could account for the observed differences in their biological activities. Gel filtration analysis has revealed molecular masses that indicate that bArtinM is a monomer and yArtinM is a dimer, contrasting with the tetrameric organization of jArtinM. The reported results support our postulation that the lectin oligomerization is required for exerting the ArtinM biological activities. Further biological and structural characterization of ArtinM recombinant forms are in progress in order to validate, or not, the pharmaceutical application of this immunomodulatory lectin.

(94) The role of CD14 in the ArtinM activity of inducing TLR2-dependent inflammatory response and IL-10 production

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ArtinM is a D-mannose binding lectin from *Artocarpus heterophyllus* that interacts with *N*-glycans of TLR2 expressed on the surface of antigen presenting cells (APCs). This binding triggers NF-kB-dependent cell signaling and IL-12 production and leads to the development of Th1 immunity, which confers protection against intracellular pathogens. The co-receptor CD14, an *N*-glycosylated GPI-anchored protein, has been associated with TLR2 pathway and can be considered a possible target for ArtinM recognition. Our aim is to investigate the participation of this co-receptor in the TLR2-mediated response to ArtinM stimulus. First, we observed that, in comparison to unstimulated cells, ArtinM stimulated macrophages showed an augmented population of $TLR2^{+}/CD14^{+}$ cells. Also, ArtinM-stimulated macrophages produced higher amounts of cytokines, such as IFN-γ (6-fold increased), IL-12 (4-fold increased) and TNF-α (20-fold increased), compatible with the Th1 immunity known to be induced by the lectin. Curiously, the production of IL-10 and IL-6 was also augmented, 115- and 1000-fold, respectively. The induced pro-inflammatory response was showed to be CD14-dependent, since ArtinM-stimulated macrophages from $CD14^{-/-}$ mice failed to produce the mentioned cytokines. Our data suggest that the effects of ArtinM on the innate immunity are highly dependent on CD14 expression. The occurrence of direct interaction ArtinM with CD14 glycans is under investigation.

(95) Sperm Sialome and Sexual Selection

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Spermatozoa face the unique task of shuttling a haploid genome towards the ovum. As allogeneic cells, they face many threats along the female reproductive tract. Female cellular and humoral immunity in the uterus exert strong (million fold) selection on sperm in mammals, allowing just a few out of each million of inseminated spermatozoa to reach the fallopian tubes. The mammalian sperm glycocalyx includes copious decoration with tens of millions of terminal sialic acid molecules. These are acquired during spermatogenesis, epididymal maturation and from seminal fluid components during ejaculation. Sperm sialic acids act as self-associated molecular patterns (SAMPs) promoting tolerance by female immune factors. The uterus recruits large number of female immune cells immediately after mating (leukocytic reaction). In addition, sperm face female complement and antibodies in uterine secretions. It has been known that sialic acid decoration of sperm changes during capacitation, a necessary process before sperm can bind and fertilize the egg. We show that the dynamic remodeling of the sperm sialome is mediated by two different sperm sialidases and results in shedding of free sialic acid (monosaccharides). Results from experimental inhibition of sperm sialidases indicate that his remodeling is necessary for successful capacitation and sperm signaling, without which sperm cannot bind the zona pellucida of the egg. Lack of sialidase activity and mismatches between sperm sialic acids and female immune response can affect fertility in mouse model systems and possibly in humans.

(96) Optimized expression of *O*-GlcNAcylated proteins in *E. coli*

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O-GlcNAc (*O*-linked-β-*N*-acetlyglucosamine) glycosylation, *O*-GlcNAcylation, is post-translational modification resulting in the addition of β-*N* -acetlyglucosamine to serine and/or threonine residues (Lamarre-Vincent and Hsieh-Wilson 2003). This modification is dynamically regulated by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA) (Lamarre-Vincent and Hsieh-Wilson 2003). Disregulation of the *O*-GlcNAc modification has been implicated in the development of diseases such as diabetes, cancer and Alzheimer's (Mi et al. 2011). The function of *O*-GlcNAc as well as its subproteome is not yet understood (Lubas et al.). Over 600 *O*-GlcNAc modified proteins have been identified (Love et al. 2010). However, one major protein of interest is cAMP response element binding 1 (CREB1). CREB1 is an essential protein in gene transcription (Lamarre-Vincent and Hsieh-Wilson 2003). It plays a key role in many functions of the human brain such as learning and long-term memory. CREB1 is glycoslyated in vivo by *O*-GlcNAc transferase (OGT) (Lamarre-Vincent and Hsieh-Wilson 2003) . This native, post-translational modification occurs in the Q2 domain of the protein (Lamarre-Vincent and Hsieh-Wilson 2003). Transcriptional activity is repressed when *O*-GlcNac is covalently bound to CREB1 because it inhibits CREB1's association with the coactivator, TATA-associated factor (TAFII130) (Lamarre-Vincent and Hsieh-Wilson 2003) . Four constructs of CREB1 were cloned into pET28b vectors and expressed in *E. coli* using LB media. These constructs were also expressed using minimal media with ^{15}N-ammonium chloride to produce isotopically labeled protein for NMR analysis. In order to glycosylate the protein, CREB1 and OGT were co-transformed and expressed in BL21(DE3), Tuner and Rosetta *E. coli* strains. The resulting protein and glycoprotein were purified by Ni-affinity chromatography. Glycoslyation of CREB1 was confirmed by lectin blotting using fluorescein isothiocyanate (FITC) conjugated wheat germ agglutinin (WGA). Glycosylated and unmodified CREB1 protein, from the same expression, were separated by WGA affinity chromatography. Results indicate a greater tendency for glycosylated CREB1 to aggregate, form inclusion bodies and degrade by proteolysis. In light of these finding, OGT has been sequence optimized for *E.coli* and expressed in pET28a containing a T7 tag. Once the expression of OGT has been optimized it will be coexpressed w a soluble protein in minimal media with isotopes to produce soluble isotopically labeled glycoprotein.

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(97) The *Caenorhabditis elegans* innate immune response to *S. aureus* is modulated by *O*-GlcNAc transferase

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The dynamic post-translational *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) modification of serine and threonine residues plays a critical role in cell signaling and is implicated in many human diseases. Its addition to proteins is governed by the enzyme *O*-GlcNAc transferase (OGT), while *O*-GlcNAcase regulates its removal. Data from our lab reveal that the dysregulation of *O*-GlcNAc results in the dysregulation of immune-responsive genes. Moreover, literature data demonstrate that OGT physically interacts with evolutionarily conserved proteins key for the innate immune response, including p38 MAPK (PMK). Given these findings, we hypothesize that *O*-GlcNAc "fine-tunes" the immune response. Utilizing the bacteriovore *Caenorhabditis elegans*, we explored whether *O*-GlcNAc cycling mutants mounted effective immune responses to pathogenic *S. aureus* and *P. aeruginosa*. Our results demonstrated that *O*-GlcNAc cycling mutants maintain a lifespan similar to wild-type (WT) when fed P. aeruginosa but loss of OGT yielded short-lived (immunocomprimised) animals upon S. aureus exposure. Interestingly, *S. aureus*-fed PMK null animals in the OGT null background have mean lifespans over 20%

shorter than the individual null alleles, suggesting synergy between these two pathways. These longevity data are bolstered by our whole-genome transcriptional profiling of *O*-GlcNAc cycling mutants after pathogen exposure: different bacteria illicit not only differential pathogen sensitivity but they also yield vastly different transcriptional changes in loss-of-function animals. Together, our data suggest that *O*-GlcNAc plays a novel, critical role for the innate immune response to *S. aureus* and the modification's addition may be a key component for immune regulation in concert with the PMK pathway. Additionally, with differing longevity and transcriptional dysregulation in pathogen-fed animals, our data support a complicated mechanism for both recognition and response to bacterial infection. Our findings provide the first insight into *O*-GlcNAc cycling's role in the immune response and highlight that our knowledge of signaling pathways involved in innate immunity is likely incomplete.

(98) Crosstalk between O-glcnacylation and phosphorylation insignaling and transcription

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N-acetylglucosamine monosaccharides on Ser(Thr) residues of nuclear and cytoplasmic proteins serves as a nutrient/stress sensor to regulate signaling, transcription and cellular metabolism. Phospho- and glycomic approaches have shown that an increase in global O-GlcNAcylation affects phospho-site occupancy at nearly every actively cycling site. Glycomic/Phosphoproteomic analyses show that cross-talk between site-specific phosphorylation and O-GlcNAcylation is extensive. Many kinases are both modified and regulated by O-GlcNAcylation, e.g. the major sensor of cellular energy state, AMPK is O-GlcNAcylated. AMPK and *O*-GlcNAc transferase share many substrata and the two systems directly interact. In skeletal muscle, AMPK regulates the nuclear localization of *O*-GlcNAc transferase. Major signaling cascades (e.g. CDK1, aurora kinase, polo kinase) that regulate cell division are strikingly affected by a small change in O-GlcNAcylation. *O*-GlcNAc is part of the histone code, but some of the *O*-GlcNAc residues are at sites interacting with DNA in the nucleosome, not in the histone tails. Multiple core ribosome proteins are modified by *O*-GlcNAc, which plays a role in ribosome biogenesis and assembly. *O*-GlcNAc cycling is strikingly elevated in most forms of cancer. Excessive O-GlcNAcylation of regulatory proteins appears to underlie "glucose toxicity" associated with diabetes. In the brain, reduced O-GlcNAcylation of many proteins is associated with neurodegenerative disease. Thus, O-GlcNAcylation modulates transcription and many signaling cascades to "tune" them to be highly responsive to nutrients and stress. Supported by NIH (R01CA42486, R01DK61671, N01-HV-00240, P01HL107153, R24DK084949) and the Patrick C. Walsh Prostate Cancer Research Fund. G.H. receives a share of royalty received by the university on sales of the CTD 110.6 antibody, which are managed by JHU.

(99) Reduced renal proteoglycans results in tubule epithelium changes and protein uria

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Proteoglycan(s) assembly is crucial to cellular homeostasis. The initial assembly of proteoglycans on the core protein requires the transfer of a xylose to a designated serine within the core protein. The enzyme responsible for this is xylosyltransferase and exists in two isoforms. Deficiency of Xylosyltransferase 2 results in reduced tissue proteoglycans leading to liver cystic disease and renal abnormalities similar too animal models of and patients with polycystic kidney disease (PKD). Overall our findings suggest that reduced proteoglycans may have a genetic modifying role in inherited polycystic kidney disease (PKD) the fourth leading cause of renal failure in the United States. Xylosyltransferase 2 (XylT2) is ubiquitously expressed in many organs including the kidney. In our XylT2 knockout mice (Xylt2-/mice), considerable xylosyltransferase activity remains in the kidney due to Xylosyltransferase 1 activity. However, despite this remaining activity, glomerular basement membrane (GBM) changes, fibrosis and tubule dilation still occur. Functionally these changes result in increased blood urea nitrogen and proteinuria. The proteinuria is of considerable interest since PKD patients that develop proteinuria have a much poorer long-term prognosis. Our analyses in the Xylt2$-/-$mice suggests the etiology of the proteinuria, although considered to be at least in part due to the GBM changes, arises as a result of a tubule protein reabsorption defect. The mechanistic role of reduced proteoglycans in this tubule defect is investigated.

(100) C-type llectin CLEC5A is critical for the pathogenesis of flaviviral infections

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CLEC5A/MDL-1, a member of the myeloid C-type lectin family expressed on macrophages and neutrophils, is critical for dengue virus (DV)-induced hemorrhagic fever and shock syndrome in *Stat1*$^{-/-}$ mice and ConA-treated wild-type mice. However, whether CLEC5A is involved in the pathogenesis of viral encephalitis has not yet been investigated. To investigate the role of CLEC5A to regulate JEV-induced neuroinflammation, antagonistic antiCLEC5A mAb and CLEC5A-deficient mice were generated. We find that Japanese encephalitis virus (JEV) directly interacts with CLEC5A and induces DAP12 phosphorylation in macrophages. In addition, JEV activates macrophages to secrete proinflammatory cytokines and chemokines, which are dramatically reduced in JEV-infected *Clec5a*$^{-/-}$ macrophages. Although blockade of CLEC5A cannot inhibit JEV infection of neurons and astrocytes, antiCLEC5A mAb inhibits JEV-induced proinflammatory

cytokine release from microglia and prevents bystander damage to neuronal cells. Moreover, JEV causes blood-brain barrier (BBB) disintegrity and lethality in STAT1-deficient (*Stat1* $^{-/-}$) mice, whereas the peripheral administration of anti-CLEC5A mAb reduces infiltration of virus-harboring leukocytes into the central nervous system (CNS), restores BBB integrity, attenuates neuroinflammation and protects mice from JEV-induced lethality. Moreover, all surviving mice develop protective humoral and cellular immunity against JEV infection. These observations demonstrate the critical role of CLEC5A in the pathogenesis of Japanese encephalitis and identify CLEC5A as a target for the development of new treatments to reduce virus-induced brain damage.

(101) Neolectins: controlling lectin valency and effect on cellular internalization

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The ability of lectins to specifically recognize glycoconjugates on the surface of cells makes them a useful tool in biomedical diagnosis associated with the change of glycosylation (inflammation, cancer etc.). However, only a limited number of lectins are available, limiting their use in biotechnology and research. RSL is a fucose-binding lectin from the bacterium *Ralstonia solanacearum* that adopts a β-propeller fold formed by trimer association and presenting six binding sites.[1] RSL has been chosen as the paradigm for designing neolectins, since it is easy to produce and has a high affinity for fucose and present six symmetrical binding sites. Alteration of the symmetry of the β-propeller architecture is used to produce neo-RSLs (nRSLs) with controlled valency in order to improve diversity in the development of technological tools and to understand the endocytosis mechanism. Several mutants have been produced in order to modify the valency of RSL. The R17A nRSL stochiometry has been reduced to three residues per β-propeller, whereas the affinity for monosaccharides is not altered. Characterization of the binding property of R17A nRSL to fucose by surface plasmon resonance spectroscopy shows only limited variations in the kinetic parameters comaped with wild-type RSL. However, when the neolectin is tested on HeLa cells, it shows slower kinetics of endocytosis, demonstrating a strong effect of the degree of multivalency on membrane dynamics and internalization.

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(102) *O*-GlcNAc participates in the cellular stress-response by a time-dependent manner

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O-GlcNAc (*O*-linked β- *N*-acetylglucosamine) is a reversible, dynamic post-translational modification on the Ser/Thr residues of proteins, similar to phosphorylation. Recently, several studies demonstrated that increasing *O*-GlcNAc levels prior to stress can prevent some of the deleterious effects and it was also shown that stress itself might elevate cellular *O*-GlcNAc levels. Our present aim was to study the dynamic progress of *O*-GlcNAc levels over short (0–4 h) and medium terms (24–72 h) following a prompt, modest stress. We used two fairly distinct models: oxidative stress (H_2O_2) in a neuronal cell line (SH-SY5Y) and sub-lethal brain electroshock on anesthetized Wistar rats. Following stress we collected cells and brain-tissue samples at similar times (0, 30 and 60 min, 2 and 4 h and 1, 2 and 3 days). We measured OGT (*O*-GlcNAc transferase) mRNA levels, protein *O*-GlcNAc levels by western blot and by immunfluorescence staining in both SH-SY5Y and rat brain tissue. We also determined the rate of apoptosis in SH-SY5Y by flow cytometry after propidium iodide/Annexin-V-FITC staining. We found remarkably similar *O*-GlcNAc pattern in both models: OGT as well as *O*-GlcNAc significantly increased and peaked between 2 and 4 h following stress. In the cell culture model, the initial *O*-GlcNAc elevation coincided with the highest rate of apoptotic events. In brain-tissue slides, we observed the highest nuclear accumulation of *O*-GlcNAc proteins 2 h after stress. After 24 h, *O*-GlcNAc levels almost returned to the basal level and medium-term *O*-GlcNAc levels remained relatively stable in both models. Our data suggest that the elevation of *O*-GlcNAc is a general, conserved response to a stress situation. Following short-term stress, *O*-GlcNAc levels immediately increase but after a short period, return to normal levels. The correct timing of this dynamic cellular response is probably crucial to avoid unneccessary cellular events (e.g. Ca^{2+}-overload) to happen. This work was supported by Hungarian Fund OTKA (73591 and 78480).

(103) Site Mapping of the *O*-GlcNAc Post-Translational Modification using Affinity Chromatography and Mass Spectroscopy

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The *O*-linked addition of *N*-acetylglucosamine on serine or threonine residues is one of several post-translational modifications that proteins may undergo. A multitutde of cytoplasmic and nuclear proteins are modified in this way, however the vast majority of *O*-GlcNAc sites on any given protein are unknown. Several proteins of interest have been glycosylated in vivo using *E. coli* and verified to possess one or more *O*-GlcNAc sites based on western blot analysis. The proteins were digested and the peptides were subjected to the modified BEMAD reaction according to Wells *et al.* In this method, peptides undergo β-elimination and then Michael addition of biotin pentylamine, which tags peptides at their glycosylation sites (Wells et al. 2002). The mixture is enriched selectively for biotinylated peptides using affinity chromatography and is analyzed using MALDI-MS. The sites of modification and a comparison to predicted sites will be discussed.

Reference

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(104) Silencing α1,3-fucosyltransferases in human leukocytes reveals a role for FUT9 during E-selectin-mediated cell adhesion

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Leukocyte adhesion during inflammation is initiated by the binding of sialofucosylated carbohydrates expressed on leukocytes to endothelial E-/P-selectin. While the glycosyltransferases (glycoTs) constructing selectin ligands have largely been identified using mouse knockouts, accumulating evidence suggests that important differences may exist between humans and mice. To address this, we developed a systematic lentivirus-based shRNA delivery workflow to create human leukocytic HL-60 cell lines that lack up to three glycoTs. Using this, the contributions of all three myeloid α1,3-fucosyltransferases (FUT4, FUT7 and FUT9) to selectin-ligand biosynthesis were evaluated. The cell adhesion properties of these modified cells to L-, E- and P-selectin under hydrodynamic shear conditions were comaped with bone marrow-derived neutrophils from $Fut4^{-/-}Fut7^{-/-}$ dual knockout mice. Results demonstrate that predominantly FUT7 and, to a lesser extent, FUT4 form the selectin ligand at the N terminus of human leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) in humans and mice. Greater than 90% reduction in leukocyte interaction was observed in human $FUT4^{-}FUT7^{-}$dual knockdowns on P-/L-selectin substrata. Unlike $Fut4^{-/-}Fut7^{-/-}$ mouse neutrophils, however, human knockdown leukocytes lacking FUT4 and FUT7 only exhibited partial reduction in rolling interaction on E-selectin. In this case, the third α1,3-fucosyltransferase FUT9 played a dominant role since leukocyte adhesion was reduced by 50% upon knocking-down FUT9 alone, >70% in dual knockdowns lacking FUT7 and FUT9 and 85% in triple knockdowns lacking FUT4, FUT7 and FUT9. Gene silencing results were in agreement with gain-of-function experiments where all three FUTs conferred E-selectin rolling in HEK293T cells. Overall, the study advances new tools to study human glycoT function and it suggests a species-specific role for FUT9 during the biosynthesis of human E-selectin ligands.

(105) A novel fucosylated TLR-4 mediates fucose-dependent interkingdom communication between mutualist microbiota and mammalian gut

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An essential component of innate immune response to microbial infection includes transcellular inflammatory signaling by cell surface toll-like-receptors (TLRs). In the intestinal mucosa, Mammalian toll-like receptors (TLRs) are activated by characteristic microbial molecules and precipitate a cascade of signaling pathways that generally result in an appropriate inflammatory response. The critical signaling events of the inflammatory response are the activation of nuclear factor kappa B (NF-kB) and interferon regulatory factor 3 (IRF3). However, during initial gut colonization by normal microbiota, a novel form of TLR4 that is fucosylated appears. This post-translational modification allows TLR4 to be activated by fucose-specific ligands, stimulating a non-inflammatory (NF-kB and IRF3 independent) signaling pathway that mediates communication between a component of the gut microbiota and the nucleus of the intestinal epithelium. The ERK and JNK signaling pathways are essential to this signaling. Activation of these pathways leads to specific transcription of the secretor (fucosyltransferase 2; *fut2*) gene resulting in induction of fut2 mRNA and FucT II activity. The resultant fucosylation of the mucosa creates a niche that facilitates colonization by a distinct microbial community. This is the first description of this important type of interkingdom communication between the mammalian gut microbiota and the intestinal epithelium and of its mechanism. This communication seems to be central to the succession of the normal mammalian microbiota, apparently by facilitating relationships with mutualistic pioneering or keystone species able to utilize the fucose of the mucosal glycocalyx. This may help explain recent reports of a significant association between secretor gene expression and intestinal inflammatory diseases such as necrotizing enterocolitis and Crohn's disease. Supported by HD013021, AI075563 and DK 070260.

(106) Expression of heparan sulfate 6-*O*-sulfotransferase-1 is stimulated by adrenaline via the Src.ERK signaling pathway in L-M cells

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Heparan sulfate (HS) is a randomly sulfated polysaccharide and presents ubiquitously on the cell surface and in the extracellular matrix. HS is known to participate in a variety of biological activities such as growth factor signaling, cell differentiation and tumor metastasis. Many of these functions are thought to be dependent on the sulfated structures of HS, which are modified by various enzymes including sulfotransferases. However, it has been unclear how cells regulate the structures of HS. To determine the mechanisms of structure alterations for HS, we studied a signal transduction of a sulfotransferase in mouse fibroblast cells (L-M cells). Here, we provide evidence that expression of 6-*O*-sulfotransferase-1 (6-OST-1), one of heparan sulfate sulfotransferases, is stimulated by adrenaline via Src.ERK signaling pathway in L-M cells. Adrenaline induces the secretion of nerve growth factor in L-M cells. In addition, we found that HS structures on the cell surface were altered when the cells were treated with adrenaline. Although the amount of HS on the cell surface was not changed by adrenaline treatment, the HS structure was greatly changed at 6 h after the adrenaline treatment. The ratio of 6-*O*-sulfated disaccharides was increased, whereas the non-sulfated disaccharides ratio was oppositely decreased. The mRNA

expression level of 6-OST-1, the major type of 6-*O*-sulfotransferases expressed in L-M cells, increased at 90 min after the adrenaline treatment. To gain insights into the signaling pathway of 6-OST-1, the effect of various adrenergic receptor antagonists and signal transduction inhibitors on the up-regulation of the mRNA expression of 6-OST-1 by adrenaline was investigated. As a result, a β3 adrenergic receptor antagonist, a Src inhibitor and an ERK1/2 inhibitor completely suppressed the up-regulation of 6-OST-1 mRNA expression. Furthermore, pretreatment with an ERK1/2 inhibitor before the adrenaline treatment inhibited the structural alteration of HS by adrenaline. These observations indicate that the signal transduction of 6-OST-1 is regulated by the Src.ERK signaling pathway through β3 adrenergic receptor in response to adrenaline in L-M cells. Our study provides new insights into the understanding of the signaling pathway regulating HS structures.

(107) The subendothelial matrix alters flow-induced nitric oxide production to regulate NF-kB activation

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Shear stress generated by distinct blood flow patterns modulates endothelial cell phenotype to spatially restrict atherosclerotic plaque development. Signaling through p21-activated kinase (PAK) mediates several of the deleterious effects of shear stress, including enhanced NF-k B activation and proinflammatory gene expression. While shear stress activates PAK in endothelial cells on a fibronectin matrix, basement membrane proteins limit shear-induced PAK activation and inflammation through a protein kinase A-dependent pathway; however the mechanisms underlying this regulation were unknown. We now show that basement membrane proteins limit membrane recruitment of PAK2, the dominant isoform in endothelial cells, by blocking its interaction with the adaptor protein Nck. This uncoupling response requires protein kinase A-dependent nitric oxide production and subsequent PAK2 phosphorylation on Ser20 in the Nck-binding domain. Importantly, shear stress does not stimulate nitric oxide production in endothelial cells on fibronectin, resulting in enhanced PAK activation, NF-kB phosphorylation and ICAM-1 expression. These data demonstrate that differential flow-induced nitric oxide production regulates matrix-specific PAK signaling and describe a novel mechanism of nitric oxide-dependent NF-k B inhibition.

(108) Characterization of the specificity of CTD110.6, an *O*-GlcNAc reactive antibody, under conditions of cellular starvation and stress

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Under normal cellular conditions, the modification of nuclear, cytoplasmic and mitochondrial proteins by *O*-linked β-*N*-acetyl-D-Glucosamine (*O*-GlcNAc) has been shown to dynamically modify and regulate over 1500 proteins. *O*-GlcNAc is analogous to protein phosphorylation and has been implicated in regulating signal transduction, protein-protein interactions, transcription, the cell cycle, nutritional signaling and the cellular stress response. Detecting changes in the levels of *O*-GlcNAc can be assayed using a wide variety of techniques. One commonly used antibody that recognizes *O*-GlcNAc, CTD110.6, has recently had its specificity called into question. It has been reported that under conditions of glucose starvation, *N*-glycan biosynthesis is attenuated resulting in an increase in N-linked chitobiose, which cross-reacts with CTD110.6. These data have suggested that stress-induced changes in *O*-GlcNAc may be due to changes in *N*-linked chitobiose rather than O-GlcNAcylation. To investigate this possibility, we have examined CTD110.6 reactivity in response to a variety of forms of cellular stress in OGT wild-type and null cells. Our findings confirm that the increased signal we observe using CTD110.6 during stress treatments are in fact due to increases in *O*-GlcNAc and not *N*-linked chitobiose. As cells become glucose starved during injuries such as ischemia-reperfusion injury, we have investigated changes in *O*-GlcNAc and chitobiose during times of glucose starvation. Our studies have confirmed that glucose starvation results in increases in *O*-GlcNAc, but that severe nutrient deprivation (glucose/FBS) results in changes in *N*-linked chitobiose, which react with CTD110.6. To delineate between *O*-GlcNAc and *N*-linked chitobiose, we have developed a western blot approach that can be used to confirm the specificity of a wide variety of *O*-GlcNAc-specific antibodies and lectins. This protocol uses a combination of PNGaseF treatments and on-blot mild β-elimination. Currently, we are determining which other *O*-GlcNAc-specific antibodies cross-react with the *N*-linked chitobiose modification and are focusing on identifying the factor(s) in FBS which suppresses the induction of chitobiose. This project is funded in part by a Program of Excellence in Glycosciences (P01HL107153).

(109) New roles of microneme GBPs in Toxoplasma gondii infection

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Toxoplasma gondii actively invades host cells in a process that critically depends on MICs, proteins released by micronemes. Microneme proteins 1 (*Tg* MIC1), 4 (*Tg*MIC4) and 6 (*Tg*MIC6) form a complex on the parasite surface, which promotes *T. gondii* adhesion to host cells. *Tg*MIC1 and *Tg*MIC4 are both glycan-binding proteins (GBPs), with specificity to sialic acid- and galactose-terminating glycans, respectively. These GBPs constitute the subcomplex Lac +, obtained through adsorption of soluble *Toxoplasma* antigens to immobilized lactose. When used to immunize C57BL/6 mice, Lac + conferred resistance against *T. gondii* infection. Protection was also provided by mouse vaccination with recombinant forms of microneme GBPs, as demonstrated by increased survival to infection and reduced parasitism, associated with Th1 immunity. Glycoarray analysis identified the better recognized glycans by recombinant *Tg*MIC1 (α2-3

sialyllactosamine) and *Tg*MIC4 (β1-4 or β1-3 galactosamine). Coherently, r*Tg*MIC1 binding to macrophage surface was inhibited 49% by α2-3 sialyllactosamine, whereas lacto- *N*-biose inhibited r*Tg*MIC4 binding by 48%. We also examined the activities exerted by microneme GBPs on innate immune cells. Both GBPs activated macrophages to produce inflammatory mediators through a TLR2- and a TLR4-dependent mechanism, since the response was significantly inhibited when cells from MyD88, TLR2 or TLR4-KO mice were assayed. Furthermore, r*Tg*MIC1- and r*Tg*MIC4-activated macrophages enhanced their migratory and phagocytic capabilities. By using HEK293 cells transfected with TLR-4, TLR-2/1 and TLR-2/6, we demonstrated that direct interactions of r*Tg*MIC1 and r*Tg*MIC4 with TLRs were responsible for cell activation, manifested by NF-kB activation and IL-8 production. Finally, HEK293 cells transfected with mutated TLR2 molecules for their *N*-glycosylation sites allowed to localize the *N*-glycan targeted by r*Tg*MIC1, but not by r*Tg*MIC4. Therefore, our study attributes new roles to microneme GBPs in host-parasite relationship exerted through their interaction with TLRs, which results in activation of innate immune cells. Financial support: FAPESP and NIH (grants 5G12RR008124-16A1, 5G12RR008124-16A1S1 and G12MD007592)

(110) HS3ST2 overexpression increases invasiveness of MDA-MB-231 breast cancercells via up-regulation of protease expression and MAPK signaling

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Heparan sulfate proteoglycans (HSPGs), which are ubiquitously present virtually in all cells, have been shown to be involved in the process of breast cancer metastasis partially due to their heterogeneous sulfation patterns, which allow for specific binding of a multitude of ligands relevant to tumor progression. HS3ST2, one of the enzymes involved in the 3-O-sulfation modification of the HSPGs, is known to be silenced by hypermethylation in breast cancer. The aim of this study was to elucidate the role of HS3ST2 in breast cancer cell behavior in vitro using an ectopic overexpression approach. HS3ST2 was stably transfected into the MDA-MB-231 breast cancer cell line, and phenotypical changes due to altered heparan sulfation were investigated in vitro using matrigel invasion chamber assays, cell proliferation assays, immunofluorescence microscopy, real-time PCR analysis, immunoblotting, gelatin zymography and pH video imaging techniques. Comaped with controls, HS3ST2-transfected MDA-MB-231 cells showed a highly significant increase in invasiveness and motility is accompanied by significantly increased expression of several matrix metalloproteinases (MMPs), including MMP9 and MMP13, as well as annexin 10, cadherin 11 and E-cadherin. Furthermore, E-cadherin showed a membranous redistribution upon HS3ST2 overexpression. Treatment of cells with TIMP-1, a protease inhibitor, hampered invasion, suggesting a role of MMPs in increased invasiveness. In addition, dysregulation of ion transporters and significantly increased cytosolic acidification were observed in HS3ST2 expressing MDA-MB-231 cells. HS3ST2 overexpression lead to increased basal and FGF-specific signaling through the p44/42 MAPK pathway, which depended on the presence of heparan sulfate. Increased MAPK activation was accompanied by a significantly increased expression of the transcription factor TCF4. MAPK inhibition with an MEK1/2 inhibitor also hampered invasion, providing a clue that increased invasiveness might be also due to increased MAPK signaling. Also, preliminary qRT PCR analysis revealed down-regulation of MMPs in MEK 1/2 inhibitor-treated cells.This study provides the first in vitro evidence of the involvement of HS3ST2 in breast cancer cell invasion. Increased activation of the p44/42 MAPK signaling pathway and of TCF4 in the presence of HS3ST2-specific sulfation patterns emerge as novel mechanistic aspects leading to increased expression of proinvasive gene products. These results suggest that increased invasion in HS3ST2 overexpressing MDA-MB-231 cells is due to increased expression of proteases and increased MAPK signaling.

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(111) Heparan sulfate-dependent oligomerization of RAGE is essential for signal transduction

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Vascular inflammation induced through the activation of receptor for advanced glycation endproducts (RAGE) accentuates the progression of atherosclerosis. RAGE oligomerization is prerequisite for signal transduction, but how oligomerization is regulated remains unknown. Recently, we reported that RAGE signaling depends on its interaction with heparan sulfate (Xu et al. 2011), and prior studies showed that signaling depends on receptor oligomerization. Here, we show that heparin-derived oligosaccharides with a minimal length of dodecasaccharide are capable of inducing a stable tetramer of the RAGE extracellular domain. Gel filtration chromatography showed that the complex consisted of four RAGE monomers and two dodecasaccharides. Site-directed mutagenesis mapped two heparan sulfate-binding sites onto the Ig-like V domain (K39, K43, K44, R104 and K107) and the C1 domain (R216 and R218). Furthermore, we constructed a structural model of the tetrameric RAGE VC1 domain based on small-angle X-ray scattering (SAXS) data collected in solution and the crystal structure of the monomeric VC1 domain. The model suggests that two

dodecasaccharides could bind at the core of the tetramer with each individual chain in contact with all four monomers. The SAXS-derived model also identified a hydrophobic oligomeric interface of RAGE tetramer, which was subsequently verified by mutagenesis. Moreover, these structural insights facilitated the identification of monoclonal antibodies that specifically target the heparan sulfate-binding sites and oligomerization interface, which can be tested to target RAGE–heparan sulfate interactions in atherosclerosis.

Reference

Xu, *et al* 2011. *J Biol Chem*. 286:41736.

(112) Aortic and lung disease in homozygous Fbn1 GT-8 mutant mice

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Fibrillin-1 is a ubiquitous extracellular matrix molecule involved in the control of growth factor signaling. Mutations in *FBN1* are primarily responsible for Marfan syndrome (MFS) and lead to similar clinical features presumably through common mechanisms. Abnormal activation of TGF-b signaling is one such mechanism. We hypothesize that aberrant growth factor signaling in MFS is due to cellular responses to structurally compromised microfibrils. To address this hypothesis, we are characterizing aortic and lung disease in homozygous mice of our GT-8 ("green truncated" from founder mouse 8) mouse line, which secretes and assembles a mutant fibrillin-1 molecule that is truncated and tagged with green fluorescent protein. These mice exhibit microfibril fragmentation in various tissues and demonstrate fragmentation of the aortic elastic lamellae (Charbonneau et al. 2010). Homozygous mice die between P9 and P18, with most deaths occurring around P14. Starting at P7, homozygous GT-8 mice develop enlarged distal airspaces reminiscent of emphysema that increase in severity until their death. qPCR analyses of both the aorta and lung show elevated levels of TGF-b responsive genes. Time course experiments were conducted to determine when TGF-b signaling reporters are increased and to investigate candidate upstream activating events. Morphological evidence of disease was correlated with these molecular data. Of the matrix metalloproteinases (MMPs) that cleave fibrillin-1, MMP-12 is known to be required for cigaret smoke-induced emphysema. To determine whether MMP-12 plays a role in our *Fbn1* genetic form of emphysema, we crossed the GT-8 allele onto an MMP-12 null background. Surprisingly, loss of MMP-12 impacted disease features in both the lung and the aorta of homozygous mice. However, while TGF-b signaling is abnormally activated in both cases, results show that disease progression in the lung and aorta exhibit important tissue-specific differences.

Reference

Charbonneau, *et al* 2010. *J Biol Chem*. 285:24943–24955.

(113) Fibrillins integrate BMP and Notch signaling during muscle development

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Fibrillins are large multi-functional matrix molecules that control growth factor signaling. Mutations in *FBN2* cause congenital contractural arachnodactyly characterized primarily by contractures of the large and small joints that resolve over time. In an *Fbn2* null mouse model, forelimb contractures were observed at birth, resolving after the first week of life (Arteaga-Solis et al. 2001). Additionally, these mice have reduced skeletal muscle mass due to a delay in differentiation mediated by the abnormal activation of BMP signaling (Sengle et al. manuscript in preparation). Because BMP signaling and Notch signaling work together to control skeletal muscle development, we examined Notch signaling components in *Fbn2* null muscle. *Fbn2* null muscle showed increased Jagged-2 and Notch-3 expression at P0, and western blots also showed Jagged-2 protein up-regulated in the null comaped with wild-type littermates. We also tested for direct interactions between Notch receptor or Notch ligand peptides and fibrillin-1 and fibrillin-2 recombinant peptides. Both fibrillins bind to Jagged-1, Jagged-2, Deltalike-1, Deltalike-4 and Notch-1, Notch-2 and Notch-3. Using various fibrillin wild-type and mutant recombinant peptides, a universal binding site in fibrillins was narrowed down to a region containing cbEGF domains 1 and 2. Interactions between Notch and Delta were previously shown to be mediated by cbEGF domains. However, since fibrillins are composed of 43 cbEGF domains, there is clear specificity in which fibrillin domains bind to Notch or Notch ligands. To test for a genetic interaction, *Fbn2* null mice were crossed with Jagged-2 mutant mice. Muscle mass was remarkably reduced in the *Fbn2 null; Jag2* mutants comaped with *Fbn2* null mice. In contrast, crossing Jagged-1 mutant mice with *Fbn2* null mice appeared to have no effect on the *Fbn2* null muscle phenotype. Taken together, these data suggest that fibrillins may physically integrate BMP and Notch signaling during muscle development.

Reference

Arteaga-Solis E, Gayraud B, Lee SY, Shum L, Sakai L, Ramirez F. 2001. Regulation of limb patterning by extracellular microfibrils. *J Cell Biol.* 154(2):275–281.

(114) Pathogenesis of Thick Skin in Weill-Marchesani Syndrome

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Fibrillin-1 mutations can cause Weill-Marchesani Syndrome (WMS), an acromelic dysplasia characterized by short stature, brachydactyly, hypermuscularity and thick skin. We replicated a human WMS mutation in mice and showed recapitulation of some features of human WMS, in particular thickened skin with ultrastructurally abnormal microfibrils (Sengle et al., 2012). We report here a new skin phenotype in WMΔ mutant mice. More hair follicles are present in the

early post-natal period, preceding abnormal collagen deposition. Increased b-catenin accumulation was also found during the early post-natal period. The mechanism leading to dermal fibrosis in WMS patients and WMΔ mice remains unknown. b-Catenin signaling has been implicated in scleroderma, and excessive collagen deposition indicates a role for TGFb. To further investigate the role of TGFb in WMS, we are performing genetic analyses of WMΔ mice crossed with TGFb1 haploinsufficient, TGFb2 haploinsufficient and TGFb3 haploinsufficient mice. In addition, we have examined skin fibroblast cultures from two WMS patients. Western blot analyses of WMS fibroblasts showed similar amounts of phosphoSMAD2/3 in both unstimulated and TFGb-stimulated cells, suggesting that TFGb signaling is constitutively active. This apparent "always on" activation and unresponsiveness to TGFb stimulation was confirmed by qPCR. Patient cells expressed up to 5-fold higher levels of TGFb responsive genes when comaped with control cells. Unlike control cells, patient cells had no change in expression of these genes following TGFb stimulation. WMΔ mice and WMS fibroblasts may be useful model systems for elucidating fibrillin matrix interactions with TGFb and b-catenin signaling pathways in the development of skin fibrosis.

Reference

Sengle G, Tsutsui K, Keene DR, Tufa SF, Carlson EJ, *et al* 2012. Microenvironmental regulation by fibrillin-1. *PLoS Genet*. 8:e1002425.

(115) Novel insights into fibrillin-1 and TGFβ contributions to aortic disease in Marfan syndrome

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Thoracic aortic aneurysm (TAA) in Marfan syndrome (MFS) is associated with dilatation of the aortic root and ascending aorta, medial layer degeneration and elevated TGFβ signaling secondary to structural or quantitative defects in fibrillin-1 (*FBN1*). An *Fbn1* conditional null allele ($Fbn1^{Lox}$) was recently employed to gain additional insights into TAA pathogenesis. In contrast to the normal phenotype of mice with *Fbn1* inactivation in endothelial cells (using *Cdh5-Cre* mice), mice without fibrillin-1 in the medial layer of the aortic root and entire aortic tree (using *SM22α-Cre* mice) or only the aortic arch (using *Wnt1-Cre* mice) died from ruptured dissecting TAA. Hence, fibrillin-1 deficiency in the medial layer of the ascending aorta is necessary and sufficient to cause TAA in MFS. As the TGFβ neutralizing antibody (TGFβNab) 1D11 has been shown to prevent aneurysm formation in mice with non-lethal TAA ($Fbn1^{C1039G/+}$ mice), TGFβ antagonism was also performed in mice with early onset of severely progressive (lethal) TAA ($Fbn1^{mgR/mgR}$ mice). Surprisingly, neonatal 1D11 treatment precipitated TAA dissection and rupture earlier than in untreated $Fbn1^{mgR/mgR}$ mice. By contrast, 1D11 administration after completion of post-natal vessel growth nearly doubled the average survival of $Fbn1^{mgR/mgR}$ mice. Hence, TGFβ signaling has an aortoprotective function during early TAA formation presumably by promoting ECM production and aortic wall maturation. Consistent with this hypothesis, *Fbn1* inactivation soon after birth (P1) or at P4 (using $R26CreER^{T}$ mice) resulted in death from ruptured dissecting TAA within the subsequent 30 and 60 days of life, respectively.

(116) ADAMTS9 regulates aortic vascular smooth muscle cell function

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The secreted metalloproteinase ADAMTS9 is the most conserved member of the ADAMTS protease family. Mice homozygous for Adamts9 deletion (Adamts9 − /−) die before gastrulation at embryonic day 7.5 (E7.5). Adamts9 + /− mice survive, but develop defects, in the aortic wall, myocardium and heart valves, along with spontaneous corneal neovascularization. ADAMTS9 is strongly expressed in aortic vascular smooth muscle cells (VSMCs). To investigate the functions of ADAMTS9 in aortic VSMC in vitro, we performed siRNA knock-down and analyzed the effects on VSMC proliferation, migration and differentiation. ADAMTS9 silencing reduced aortic VSMC proliferation measured via BrdU incorporation. VSMC migration, analyzed both by scratch wound healing assay and trans-well assay induced by platelet-derived growth factor BB (PDGF-BB) was also reduced by ADAMTS9 knockdown. ADAMTS9 knockdown reduced PDGF receptor α and b expression as well as levels of p-Erk1/2, the down stream mediator of PDGF receptor signaling. Furthermore, expression of α-actin and SM22α, the markers of VSMC differentiation, was also decreased by knockdown. In order to study the in vivo significance of ADAMTS9 in vascular physiological homeostasis, Adamts9 was specifically deleted in VSMC by crossing mice harboring a floxed Adamts9 allele with Tagln-Cre mice. The aorta was fixed by perfusion and analyzed histologically. Preliminary results indicated that, ADAMTS9 deletion led to a mild dilatation of the abdominal aorta in 1-year-old male mice. Ultrastructural analysis of the aortic wall suggested increased pericellular matrix deposition around VSMC. These results are preliminary and analysis of additional mice is ongoing. In summary, our preliminary data suggest that ADAMTS9 may regulate physiological functions of aortic VSMC via the PDGFR axis. ADAMTS9 deficiency may induce VSMC dysfunction and result in the dilatation of the abdominal aorta.

(117) Serum *N*-glycan profiling has potential to predict cardiovascular disease (CVD) event and prognosis in hemodialysis (HD) patients

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Hemodialysis (HD) patients have been considered extremely high risk for cardiovascular disease (CVD) and mortality.There are well-known risk factors (age, diabetes, dialysis duration, intima

media thickness etc.) for CVD and survival in HD patients, but no attempt has been made to evaluate serum *N*-glycans for prognostic potential. In this study, we evaluated serum *N*-glycan profiling for CVD onset and prognostic potential in HD patients, utilizing a glycoblotting method that allows high-throughput, comprehensive and quantitative glycan analysis. We performed a serum *N*-glycan analysis of 100 HD patients at June 2008 using the glycoblotting method that allows high-throughput, comprehensive and quantitative glycan analysis and followed up for 3 years. Whole human serum was treated to release reduced *N*-glycans. The total *N*-glycans released into the digested mixture was directly subjected to glycoblotting using BlotGlyco beads. The recovered oligosaccharides were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry utilizing an internal standard. Age, gender, HD duration, existence of diabetes mellitus, aortic calcification index, maximum intima media thickness (max-IMT) and the intensity of the *N*-glycans were analyzed by the Cox regression multivariate analysis to evaluate an independent predictor for CVD event and prognosis. A receiver operating characteristic curve was used to determine the cutoff value of *N*-glycans intensity for CVD event and prognosis. Selected *N*-glycans were evaluated by the log-rank test with the Kaplan–Meier method to identify the potential to be a predictive indicator for CVD event and prognosis. Each patient was categorized according to the number of risk factors to evaluate the predictive potential of risk criteria for prognosis. In total, 56 kinds of *N*-glycans were identified from HD patients. Peak 20 (P20) has potential to be a CVD event predictor, and max-IMT, P20 and P49 have potential to be a predictive indicator for prognosis in HD patients. Risk classification according to the number of independent risk factors showed a significant poor overall survival in the high-risk group. Serum *N*-glycan analysis has potential to predict CVD event and prognosis. The glycoblotting method provides a novel knowledge on CVD event and prognosis in HD patients.

(118) The tumor growth inhibitory effect of ADAMTS1 is accompanied by the inhibition of tumor angiogenesis

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Angiogenesis plays an important role in tumor progression. Several reports have demonstrated that ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin motifs 1) inhibited angiogenesis via multiple mechanisms. The aim of this study was to investigate the effect of ADAMTS1 on endothelial cells in vitro and on tumor growth with regard to angiogenesis in vivo. We examined the effects of the transfection of ADAMTS1 using two constructs, full-length ADAMTS1 (full ADAMTS1) and catalytic domain-deleted ADAMTS1 (delta ADAMTS1). Transfection of both the full ADAMTS1 and delta ADAMTS1 gene constructs demonstrated the secretion of tagged ADAMTS1 protein into the conditioned medium, so we examined the effects of ADAMTS1-containing conditioned medium on endothelial cells. Both types of conditioned media inhibited endothelial tube formation, and this effect was completely abolished after immunoprecipitation of the secreted protein from the medium. Both types of conditioned media also inhibited endothelial cell migration and proliferation. We then examined the impact of ADAMTS1 on endothelial cell apoptosis. Both conditioned media increased the number of Annexin V-positive endothelial cells and caspase-3 activity and this effect was attenuated when z-vad was added. These results indicated that ADAMTS1 induced endothelial cell apoptosis. We next examined the effects of ADAMTS1gene transfer into tumor-bearing mice. Both full ADAMTS1 and delta ADAMTS1 significantly inhibited the subcutaneous tumor growth. Collectively, our results demonstrated that ADAMTS1 gene transfer inhibited angiogenesis in vitro and in vivo, likely as a result of the induction of endothelial cell apoptosis by ADAMTS1 that occurs independent of the protease activity.

(119) A correlative method for imaging identical regions of soft tissue by micro-CT, light microscopy and electron microscopy: evaluating adipose tissue in a model system

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Micro computed X-ray tomography (micro-CT) is a versatile tool conventionally used to generate high-resolution 3D images for the non-destructive evaluation of mineralized tissues. We present a method of correlative microscopy incorporating micro-CT, the light microscope and transmission electron microscope to examine and evaluate soft tissues. This method takes advantage of the contrast imparted to soft tissues by Osmium fixation. Osmium is a traditional fixation component for electron microscopy and due to its strong X-ray attenuation, mediates the low-level visualization of many soft tissues in the micro-CT. Samples initially prepared for transmission electron microscopy can be imaged in the micro-CT. Samples can then be oriented for microtomy so that sections may be imaged in the light microscope and aligned with those composed using micro-CT with an accuracy of a few microns. Structural components may be further verified in the transmission electron microscope by observing the next serial ultrathin section which allows precise correlation of all three imaging techniques. Using this correlative method, we examine a model system to compare and quantitate white adipose tissue within mouse neonate forelimbs from a wildtype mouse and a littermate harboring a lipid-altering mutation. We demonstrate that discrete lipid droplets as small as 15 μm and droplets separated by as little as 35 μm can be measured in the micro-CT which validates this to be the highest resolution, nondestructive technique for the evaluation of fat content.

(120) Antagonistic regulation of transforming growth factor-β by fibulin-4a andfibulin-4b is required for cardiovascular and musculoskeletal development in zebrafish

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Human mutations in the *fibulin-4* (*FBLN4, EFEMP2*) gene cause a recessive disease with arterial tortuosity, aneurysms, developmental emphysema, bone fragility and cutis laxa. Our goal was to elucidate the molecular mechanisms of this disease by using zebrafish as a model organism. The zebrafish genome has two paralogs *FBLN4*, *fbln4a* and *fbln4b*. In situ hybridization and immunostaining showed complementary and often adjacent expression of the two paralogs during segmentation and neural crest migration. For example, whereas *fbln4a* was expressed in the entire width of somites, *fbln4b* was restricted to the medial surface of somites. Knockdown of *fbln4a* using antisense morpholino oligonucleotides resulted in severe cardiovascular phenotype at 2 days post-fertilization including blood pooling at the heart and a decrease in both circulation and heart rate. *Fbln4b* knockdown also showed a severe phenotype at 2 days post-fertilization including cardiac edema, decreased heart rate, muscle dysfunction leading to reduced tail twitching and circulation failure with pooling of the blood in the caudal region. *In situ* hybridization for cardiac marker *cmlc2* and confocal microscopy of transgenic embryos expressing green fluorescent protein in the endothelium illustrated both cardiac and vascular defects. To test if transforming growth factor-β (tgfb) signaling was altered in either *fbln4a* or *fbln4b* deficient embryos, we treated them with a small molecule tgfb receptor 1 inhibitor, LY-364947. The inhibitor treatment rescued vascular abnormalities of *fbln4b* morphants but made the phenotype worse in *fbln4a* morphants suggesting antagonistic roles of the two genes. The specific tgfb isoforms responsible for altered sig naling in *fbln4b* morphants was identified by double morpholino silencing of *fbln4b* and *tgfb1b*, *tgfb2* or *tgfb3*. *Tgfb2* and *tgfb3* but not *tgfb1b* knockdown rescued both the cardiac and vascular defects of *fbln4b* morphants. Based on their adjacent localization and antagonistic function, we propose that the *fbln4* paralogs in zebrafish define distinct and sharply demarcated domains of tgfb activity ensuring highly localized differentiation responses by mesodermal and neural crest derivatives.

(121) Cardiac neural crest-specific vascular smooth muscle cell dysregulation results inregional proteoglycan misexpression and biomechanical dysfunction in a mouse model of aortopathy

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Aorto[p]athy is a subclinical disease associated with sudden cardiac death that is characterized by vascular smooth muscle cell (VSMC) and elastic fiber abnormalities. Elastin insufficient (*Eln +/*) mice demonstrate latent aortic root dilation, increased VSMCs and elastic fiber fragmentation. We hypothesized that aortopathy originates in the cardiac neural crest (CNC) regions of the aorta. Aorta tissues from adult and aged wild-type (WT) and *Eln +/−* mice were examined by anatomic (root vs ascending) and developmental (CNC vs non-CNC) regions. Tissue was analyzed for proteoglycans (aggrecan, biglycan), ECM remodeling enzymes (MMP-2, Cathepsin L), CNC-derived cells (Sonic Hedgehog), VSMCs (α-SMA, SM22), elastic fiber architecture (Hart's stain) and regional morphometrics. Spatial distribution of VSMC and CNC markers was determined using a novel 3D reconstruction approach. Aortic distensibility and circumferential cyclic strain were quantified using magnetic resonance imaging. Regional aorta tissue tensile stiffness was determined using micropipette aspiration and a half space model adjusted for tissue thickness. The CNC side of WT ascending aorta was significantly thicker and stiffer than non-CNC ($P < 0.05$). Similar differences were observed in *Eln +/−* mice, but were more pronounced at the aged stage ($P < 0.01$). Aggrecan was localized to CNC aorta in *Eln +/−*, but absent in WT. Further, biglycan, MMP-2 and Cathepsin-L were increased in the aortic root only. The stiffness of ascending aorta CNC and non-CNC regions was significantly higher than the aortic root in WT and *Eln +/−* mice ($P < 0.0001$) and increased with age ($p < 0.05$). While tissue stiffness values were not different among specific WT aortic root sinuses, the *Eln +/−* - non-coronary sinus (non-CNC) paradoxically demonstrated increased stiffness when compared with CNC sinuses ($P < 0.0001$). These findings demonstrate that CNC-derived aorta tissue is spatiotemporally associated with VSMC abnormalities, maladaptive matrix remodeling and biomechanical dysfunction in *Eln +/−* mice, suggesting a primary role in the development of aortopathy.

(122) Molecular mechanisms of anticoagulant activity of annexins A4 and A5 : Thrombin sensitivity and cell surface translocation

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Annexins are a family of Ca^{2+}-binding proteins, for which 12 annexin genes have been identified in the human genome. Annexin A4 (anxA4) and annexin A5 (anxA5) were originally purified from the placenta as anticoagulant proteins and strongly inhibited in vitro blood coagulation reaction, suggesting that these proteins have a role in regulating blood coagulation in vivo. AnxA4 and AnxA5 exhibit the highest amino acid sequence homology among annexin family proteins and structural features common to the two annexins may be required for expression of the anticoagulant activity-dependent phospholipid binding is a typical activity of annexins and several annexins bind to heparan sulfate/heparin, a major component of cell surface proteoglycans. It was speculated that heparan sulfate/heparin is a binding partner for annexins exhibiting extracellular activities including the regulation of blood coagulation. We investigated the comparative anticoagulant activities of anxA4 and anxA5 using the in vitro plasma coagulation assay. The results showed that these proteins inhibit coagulation to a similar extent. Cell surface translocation of anxA4 and anxA5 was confirmed by immunofluorescence of cells transfected with anxA4 and anxA5 constructs, suggesting that these proteins suppress procoagulant conditions on the cell surface. When thrombin, a serine protease responsible for the fibrin production in the coagulation reaction, was allowed to react with intact anxA4 and anxA5, anxA4 was slightly cleaved but anxA5 was resistant. On the other hand, thrombin completely degraded

heat-denaturated anxA4 and anxA5. The results raise a possibility that anxA4 and anxA5 inhibit thrombin on cell surfaces.

(123) Aortic Disease in the Heterozygous GT-8 Mouse Model of Marfan Syndrome

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Heterozygous GT-8 mice display progressive fragmentation of elastic lamellae in the aortic root (1). By 8 months of age, aortic elastic lamellae are severely fragmented. To determine whether doxycycline or losartan can rescue aortic dilatation and fragmentation of elastic lamellae, treatments were initiated when mice were 2 months old using established protocols (2,3). Mice were sacrificed and examined at 8 months of age. There were 6 groups of mice: untreated wildtype and heterozygous GT-8 littermates; wildtype and heterozygous GT-8 littermates treated with doxycycline; and, wildtype and heterozygous GT-8 littermates treated with losartan. Aortic roots from all groups were examined for fragmentation of elastic lamellae. Elastic lamellae in aortic roots from both male and female GT-8/+ mice were significantly more fragmented than wildtype littermates. However, when large breaks extending through multiple layers of elastic lamellae were counted, male GT-8/+ mice were more severely affected than female GT-8/+ mice, which is consistent with the incidence of aortic dissection (74% male) in humans with Marfan syndrome (4) and suggests that GT-8/+ mice may be a good model for the pathogenesis of severe aortic disease in MFS. Treated mice showed no improvement in fragmentation, but a trend toward a reduction in the numbers of large breaks was observed, though was not statistically significant.

Treatments appeared to ameliorate aortic root wall thickness and area, but these trends were also not statistically significant. Male GT-8/+ mice are more severely affected than unbred female GT-8/+ mice. Female GT-8/+ mice that have been bred show fragmentation equivalent to their male counterparts. Currently available treatment protocols do not prevent aortic disease in these mice and treatments that inhibit fragmentation of the elastic lamellae should be considered. 1. Charbonneau et al., J. Biol. Chem., 285: 24943-55, 2010. 2. Habashi et al., Science, 312:117-21, 2006. 3. Xiong et al., J. Vasc. Surg., 47:166-72, 2008. 4. LeMaire and Russell, Nature Rev. Cardiol., 2:103-13, 2011.

(124) Zipper-like folding of the C-terminal quarter fragment of type III collagen model polypeptides with Ehlers-Danlos Syndrome mutations

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Vascular Ehlers-Danlos syndrome (EDS) type IV is the most severe form of EDS due to the lethal rapture of arteries or aorta. The disease is caused by dominant-negative mutations in the procollagen type III. In many cases, one of the Gly residues is mutated to another amino acid. From the analysis of cultured skin fibroblast from EDS type IV patient, it is suggested that the type III procollagen is overmodified. A slow folding of the collagen helix will result in overmodifications. However, little is known about the rate of folding of EDS type III collagen. The triple helix is folded in a zipper-like fashion from the C to the N terminus. In order to understand the molecular mechanism of the effect of mutations, a system was developed for bacterial production of homotrimeric model polypeptides. The C-terminal end of the natural human type III collagen 252 residues were attached to $(GlyProPro)_7$ with a part of type XIX collagen NC2 domain, which forms a thermally stable trimer. The natural collagen domain forms a triple helical structure without 4-hydroxylation of proline. At 35°C, the natural collagenous part is denatured, but the C-terminal GPP7-NC2 is stable. The initial refolding phase of the natural type III collagen part showed the linear phase by CD as described by Bächinger et al. Eur J. Biochem. (1980) 106, 619. We used this system for the two known EDS mutations (Gly to Val) in the middle and at the C terminus. WT, G910A, G910V, G1018A and G1018V proteins were made. All the mutated proteins form the triple helical structure at 4°C. From the analysis of refolding experiments, we found that the effect of Gly to Val mutation is much more severe compared with Gly to Ala. This is the first report on the delayed folding of collagen with mutated Gly in EDS.

(125) Latent TGFβ-binding protein 4 converts microfibrils to elastic fibers by recruiting fibulin-5

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Elastic fiber assembly requires deposition of elastin monomers onto microfibrils, the mechanism of which is largely unknown. Here, we show that latent TGFβ binding protein 4 (LTBP-4) converts microfibrils to elastic fibers by recruiting fibulin-5, a tropoelastin-binding protein necessary for elastogenesis. Decreased expression of LTBP-4 in human skin fibroblast cells by siRNA treatment abolished the linear deposition of fibulin-5 and tropoelastin on microfibrils. Notably, addition of recombinant LTBP-4 to cell culture medium not only rescued the deposition of fibulin-5 and tropoelastin, but also induced excessive elastogenesis without changing the expression of elastic fiber components. This elastogenic property of LTBP-4 is independent of bound TGFβ, since TGFβ-free recombinant LTBP-4 was as potent an elastogenic inducer as TGFβ-bound recombinant LTBP-4. Without LTBP-4, fibulin-5 and tropoelastin deposition was discontinuous and punctate in vitro and in vivo. These data suggest a new function for LTBP-4 as an essential scaffold for fibulin-5 during elastic fibrogenesis and as a potential therapeutic target for elastic fiber regeneration.

(126) The role of β1 integrin in mediating vascular extracellular matrix integrity

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β1 integrin is ubiquitously expressed in the vasculature, and together with several α subunits, binds numerous proteins in the

extracellular matrix (ECM). β1 integrin has also been implicated in the organization of fibrillar fibronectin and collagen I, associating with proteins known to be involved in ECM cross-linking, namely tissue transglutaminase. To evaluate its specific biological function in vascular development, we used the CRE-lox system to delete β1 integrin in vascular smooth muscle cells (vSMCs). Deletion was embryonic lethal by E18.5 due to cardiovascular defects. Predominating was site-specific aneurysm formation at the bifurcations present at the brachiocephalic and common carotid arteries. Histological examination of the aneurysms showed discrete aggregates of fully differentiated vSMCs that did not organize into concentric layers. Assessment of elastin, collagen IV, fibronectin and laminin by immunofluorescence showed lack of lamellar organization exclusively in aneurysm sites, while the descending aorta (DA) in knockout animals resembled that of controls. However, further evaluation of the 3D architecture of the DA at E18.5 by scanning electron microscopy revealed a significant increase in lumen size in knockouts, a decrease in the thickness of the tunica media and loosely compacted ECM. In regions across the aneurysm, vSMCs are dysmorphic with aberrant cell–cell and cell–matrix connections. We hypothesize that a defect in ECM cross-linking renders the vascular wall unstable and susceptible to aneurysm formation. In vitro analysis of ECM cross-linking, using WT and β1 integrin null vSMCs show that in its absence, fibronectin assembly into fibrils is greatly diminished despite no differences in protein levels. These results suggest that in the absence of β1 integrin, ECM components are able to be secreted, but are unable to be fully organized. This provides insight into the influence of β1 integrin in forming an ECM network that can contribute to the overall mechanical integrity of developing vasculature.

(127) Core 1 O-glycosylation is required for the stability of podoplanin on lymphaticendothelial cells

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Our published data show mice lacking endothelial T-synthase (EHC T-syn$^{-/-}$), a key glycosyltransferase for core-1 derived *O*-glycosylation, exhibited disorganized and blood-filled lymphatic vessels. We found that the expression of podoplanin (PDPN), a transmembrane mucin-type *O*-glycoprotein, was significantly reduced in EHC T-syn$^{-/-}$lymphatic endothelial cells (LECs), and that PDPN-deficient mice developed mixed blood/ lymphatic vessels resembling the EHC T-syn$^{-/-}$defects. These results indicate that PDPN controls blood and lymphatic vessel separation during development and that *O*glycosylation regulates PDPN level in LECs. However, how core 1 *O*-glycosylation contribute to PDPN expression in LECs is unclear.

To address this question, we analyzed PDPN on LECs freshly isolated from wild type (WT) or EHC T-syn$^{-/-}$mice by flow cytometry. T-syn$^{-/-}$LECs displayed markedly reduced surface PDPN level compared with that of WT LECs. However, after 5 7 days of *in vitro* culture, both WT and T-syn$^{-/-}$LECs exhibited a similar surface PDPN level. This experiment suggests that *O*-glycan-deficient PDPN is susceptible to proteolytic shedding *in vivo*. Consistent with this hypothesis, serine protease trypsin, elastase, or cysteine protease calpain-2 cleaved *O*-glycan-deficient PDPN, but not WT PDPN expressed in LECs. Furthermore, lymph isolated from WT mice cleaved *O*-glycan-deficient PDPN, but not WT PDPN. Protease inhibitors (a combination of aminopeptidases inhibitor, serine, cysteine, aspartic acid, and metalloproteases inhibitors, Pierce Halt^{TM}), or EDTA rescued the reduced level of *O*-glycandeficient PDPN caused by lymph treatment, while benzamidine or PMSF rescued partially. These data suggest that protease(s) in the lymph, most likely metalloproteases, are one of the candidate enzymes that cleave *O*-glycan-deficient PDPN. Although glycosylation has been assumed to protect proteins from proteolytic degradation, this is the first example to our knowledge that mucin-type *O*-glycosylation is required for the stability of an *O*-glycoprotein *in vivo*.

(128) Fibronectin and heparan sulfate in microfibril assembly and homeostasis

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Fibrillins constitute the major backbone of multi-functional microfibrils in elastic and non-elastic extracellular matrices. Proper expression, assembly and homeostasis mechanisms are central to the formation and function of these microfibrils. These properties are often compromised in pathological situations such as Marfan syndrome and other fibrillinopathies caused by mutations in fibrillins. In the present study, we report on the role of fibronectin and heparan sulfate proteoglycan in the assembly and homeostasis of fibrillins in vitro and in vivo. We demonstrated through peptide inhibition studies in human dermal fibroblasts, the requirement of a preassembled fibronectin network for fibrillin-1 network assembly and homeostasis in early stages. We observed by immunoprecipitation that fibronectin is present in extracted mature microfibrils indicating a non-transient mechanism for the involvement of fibronectin in microfibril assembly. To study the role of fibronectin on the formation, homeostasis and function of microfibrils in vivo, we generated a conditional and tamoxifen-inducible fibronectin knockout mouse model in smooth muscle. Tamoxifen treatment of 5-day-old homozygous mice resulted in much smaller body sizes than their wild-type littermates. Histological analysis of blood vessels (aorta) shows disrupted architecture of the vessel wall. Homozygous tamoxifen-injected mice were also characterized by enlarged and reduced alveolar spaces in the lung. These findings suggest that fibronectin is a critical matrix protein in the post-natal development and homeostasis of blood vessels and lungs. In this study, we further characterize the role of heparin/heparan sulfate in microfibril assembly and its interaction with fibrillins. We demonstrate that fibrillin-2 and -3 interact with heparin/heparan sulfate in solid phase binding assay and that fibrillin multimerization increases the avidity for heparin/heparan sulfate. We also show

that heparin/heparan sulfate acts as a regulator of fibrillin homo- and heterotypic interactions, which are critical for microfibril assembly. Our results determine the sequence of events leading to microfibril assembly, consolidated in a new model.

(129) Cartilage Inhibits Angiogenesis via Type IIB Procollagen N-Propeptide

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The goal of this study was to determine the role that PIIBNP plays in cartilage in cartilage avascularity. We have previously shown that recombinant PIIBNP induces death of tumor cells (Wang Z et al. 2010. J Biol Chem. 285:20806) and osteoclasts (Hayashi et al., 2011. Bone 49:644) via binding to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. To begin to determine whether PIIBNP plays a role in angiogenesis, we investigated its effect on endothelial cells. Recombinant PIIBNP was produced by GST-PIIBNP fusion proteins in bacteria. Cartilage and chondrocytes were isolated from newborn mouse pups and tested in the aortic ring angiogenesis assay. Human umbilical vein endothelial cells (HUVEC) tube formation and mouse corneal angiogenesis assays were performed. PIIBNP was injected into mouse pups at days 2, 4, 6, 8 and 10 post-partum. Results from cartilage-aorta coculture experiments showed that microvessel outgrowth from aorta was seen in the presence of bone, but not in the presence of cartilage. Native PIIBNP was identified in cartilage and chondrocyte conditioned medium (MA Weis and D Eyre, Seattle, WA). Chondrocyte conditioned medium inhibited microvessel outgrowth in mouse aortic angiogenesis assays. The MMT inhibitor GM6001 inhibited the production of PIIBNP in cartilage and chondrocytes and thus anti-angiogenic activity was ablated. Injection of PIIBNP into mouse pups significantly reduced blood vessel formation. These studies using recombinant and native PIIBNP showed that PIIBNP inhibits angiogenesis both in vitro and in vivo. Direct injection of PIIBNP inhibits vessel formation. These data strongly suggest that the PIIBNP liberated from each type II procollagen molecule during cartilage formation likely inhibits angiogenesis.

(130) Identification of a high-mannose ICAM-1 glycoform in activated endothelial cells

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A hallmark of inflammation is immune cell trafficking into the inflamed tissue, a process facilitated by carbohydrate structures on adhesion molecules of vascular endothelial cells. Recently we showed that pro-inflammatory stimuli increases hypoglycosylated *N*-glycan content on the endothelial cell surface and that these residues mediate monocyte adhesion. Previous reports have shown that the glycosylation status of intracellular adhesion molecule-1 (ICAM-1) controls interactions with the leukocytes receptors (1991, Cell. 65:961–971) . In these studies, ICAM-1 produced in the presence of the class 1 α-mannosidase inhibitor 1-deoxymannojirimycin exhibited increased binding to integrin Mac1 (CD11b/CD18). In the current studies, we sought to determine if ICAM-1 is hypoglycosylated in activated endothelial cells. ConA affinity pull-downs of TNF-α stimulated endothelial cells were performed and selectively precipitated and enriched a lower molecular weight ICAM-1 glycoform (75–80 kDa) comaped with the major ICAM-1 glycoform (95 kDa). This lower molecular weight ICAM-1 migrates at the same size as ICAM-1 produced in the presence of the class 1 α-mannosidase inhibitor kifunensine, indicating a high-mannose ICAM-1 glycoform (HM-ICAM-1). The presence of high-mannose structures was confirmed by Endo H digestion which selectively degraded HM-ICAM1, but not the major ICAM-1 glycoform of TNF-α-activated endothelial cells. Critically, subcellular fractionation studies revealed that HM-ICAM-1 was present on the plasma membrane and immunofluoresece staining of kifunensine pretreated, ans TNF-α-stimulated cells confirmed that HM-ICAM-1 can traffic to the cell surface. Furthermore, HM-ICAM-1 is fully functional as it supports THP-1 monocyte adhesion and facilitates ICAM-1-dependent VE-cadherin phosphorylation. To conclude, a functional HM-ICAM-1 glycoform is present and functional on activated endothelial cells and represent a novel target in anti-inflammatory therapies.

Reference

Diamond MS, Staunton DE, Marlin SD, Springer TA. 1991. Binding of the integrin Mac-1 (CD11b/CD18) to the third immunoglobulin-like domain of ICAM-1 (CD54) and its regulation by glycosylation. *Cell*. 65 (6):961–971.

(131) Role of endothelial *N*-glycan mannose residues in monocyte recruitment during atherogenesis

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Up-regulated expression of endothelial adhesion molecules and subsequent binding to cognate monocyte receptors are established paradigms in atherosclerosis. However, these proteins are the scaffolds, with their post-translational modification with sugars providing the actual ligands. We recently showed that tumor necrosis factor-α increased hypoglycosylated (mannose-rich) *N*-glycans on the endothelial surface. In the present study, our aim was to determine whether (i) hypoglycosylated *N*-glycans are up-regulated by proatherogenic stimuli (oscillatory flow) in vitro and in vivo and (ii) mannose residues on hypoglycosylated endothelial *N*-glycans mediate monocyte rolling and adhesion. Staining with the mannose-specific lectins, concanavalin A and *Lens culinaris* agglutinin was increased in human aortic endothelial cells exposed to oscillatory shear or TNF-α and at sites of plaque development and progression in both mice and human vessels. Increasing surface *N*-linked mannose by inhibiting *N*-glycan processing potentiated monocyte adhesion under flow during tumor necrosis factor-α stimulation, but not under static conditions. Enzymatic removal of high-mannose *N*-glycans with Endo H, or masking mannose residues with lectins (concanavalin A, *L. culinaris* agglutinin and *Galanthus nivalis* lectin) significantly decreased monocyte adhesion under flow. These effects occurred without altering

induced expression of adhesion molecule proteins. To conclude, hypoglycosylated (high-mannose) *N*-glycans are present on the endothelial cell surface at sites of early human atherosclerotic lesion development and are novel effectors of monocyte adhesion during atherogenesis.

(132) Role of carbohydrate-binding domains of human annexin A5 in the regulation of blood coagulability

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Annexin A5 (AnxA5) has been known as a potent anticoagulant protein in in vivo and it was reported that ANXA5 gene variants could influence the risk for recurrent pregnancy loss caused by hypercoagulation disorders (2007, Hum Mol Genet. 16:573–578). A mechanism for the expression of the anticoagulant activity of AnxA5 has been proposed; AnxA5 as trimers binds to phosphatidylserine (PS) externalized on the surfaces of the outer leaflet of the plasma membrane, over which it forms lattice structure, blocking the PS from availability for PS-dependent coagulation enzyme reactions. On the other hand, AnxA5 has been shown to bind to glycosaminoglycans, such as heparin/heparan sulfate (1998, J Biol Chem. 273:9935–9941) and two carbohydrate recognition domains (CRDs) have been determined by X-ray crystallography (2001, Structure. 9:57–64). However, the contribution of the carbohydrate binding property to the anticoagulant activity of AnxA5 remains to be clarified. In this study, we prepared recombinant human AnxA5 and its mutants (mutCRD1a, mutCRD1b, mutCRD2a and mutCRD2b) by substituting amino acid residues localized in the CRDs (CRD1 and CRD2) and compared their PS- or heparin-binding and anticoagulant activities. No changes was observed in the PS-binding activity among wild-type and mutated AnxA5 proteins in a solid-phase-binding assay. Meanwhile, the heparin binding and anticoagulant activities of mutCRD1a, mutCRD1b and mutCRD2b were remarkably reduced. A synthetic peptide, 12pep-CRD1, composed of 12-amino acid residues corresponding to CRD1 exhibited anticoagulant activity; furthermore, the anticoagulant activity of mutCRD1a was recovered by the addition of 12pep-CRD1. These results indicate that AnxA5 regulates the blood coagulability via its CRDs. Possible cell surface carbohydrate ligands of AnxA5 will be discussed.

(133) LTBP4 regulates both the magnitude and the direction of TGFβ response

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Mutations in the latent transforming growth factor-β binding protein 4 (LTBP4) cause autosomal recessive cutis laxa type 1c associated with severe pulmonary, gastrointestinal and urinary abnormalities. Previous studies showed elevated extracellular transforming growth factor-β (TGFβ) activity in cultured cells with LTBP4 mutations. Our goal was to investigate the mechanisms of TGFβ dysregulation in response to LTBP4 deficiency. We studied dermal fibroblasts from four patients with confirmed LTBP4 mutations and age-matched controls. Immunofluorescent staining and immunoblotting confirmed the loss of LTBP4 expression in mutant cells. Luciferase reporter assays and immunoblotting showed elevated extracellular TGFβ activity but reduced SMAD2 phosphorylation in LTBP4 mutant fibroblasts. TGFβ1 supplementation caused elevated phosphorylation of both SMAD2 and ERK in controls, but surprisingly suppressed phosphorylation of both these molecules in mutant cells. The expression of inhibitory SMAD6 and SMAD7 did not change and therefore failed to explain this aberrant TGFβ1 response. However, administration of a chemical inhibitor of TGFβ receptor 1 (TGFBR1) kinase, LY-364947, eliminated the negative TGFβ1 responses by LTBP4deficient cells. We conclude that LTBP4 regulates both the direction and magnitude of phosphorylation responses to TGFβ1 in a TGFBR1dependent manner. Additional, *in vivo* studies will be needed to determine whether altered TGFβ response in LTBP4-deficient cells is a developmental adaptation to correct for a persistently enhanced extracellular TGFβ activity or a true disease-causing mechanism.

(134) Functional effect of dietary fiber from *Costaria costata* residue on reducing serum lipids

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The soluble dietary fiber (SDF) was extracted from *Costaria costata* residue using sodium chloride activation method and insoluble dietary (IDF) fiber was then obtained after extraction. The dietary fiber (DF) which included SDF, IDF, MDF of soluble and insoluble in the ratio of 1:3 and the original *Costaria costata* residue, were fed to the mice for the studies of the functional effect of DF from *Costaria costata* residue. The serum total cholesterol (TC), triglyceride(TG), high-density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), fecal cholesterol and liver fat (LF)of mice were determined respectively, and the atherosclerosis index(AI), liver index(LI) and spleen index were calculated separately. The results showed that the test diets with high lipid can form the high-fat model mice.The serum TC and TG levels were reduced significantly for the mice feed by low, medium and high doses of IDF and also MDF with a high dose. The MDF with a medium dose was also effective for a reducing serum TC level. The serum LDL-C levels were reduced significantly for all the mice feed by SDF, IDF, MDF and *Costaria costata* residue with all three doses while the HDL-C levels were improved by all of those. All the sample groups fed by DF showed that the AI and LF were decreased compared with those for high-fat model mice and the TC in excretion was accelerated. The LI appeared reduction for the sample feed with IDF of medium and high doses while spleen index was significantly improved for the samples feed with SDF, MDF with all three

doses and *Costaria costata* residue with a high dose. All those indicated that DF from *Costaria costata* residue can be applied for preventing atherosclerosis and coronary heart disease.*Keywords:* *Costaria costata* / dietary fiber / animal experiment / reducing serum lipids

(135) Identification of key modulators involved in the initiation of aortic aneurysm in fibulin-4-deficient mice

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Fibulin-4 (*Fbln4*) is a secreted extracellular matrix protein expressed in blood vessels and associated with microfibrils surrounding elastic fibers. *Fbln4*-null mice exhibit perinatal lethality with rupture of aortic aneurysm and a marked disruption of elastic fibers. The aortic aneurysm phenotype was recapitulated by smooth muscle cell (SMC)-specific deletion of *Fbln4* in vivo ($Fbln4^{SMKO}$), in which severe aneurysm developed exclusively in the ascending aorta at post-natal day 90 (P90). To identify key modulators involved in the initiation of aortic aneurysm in $Fbln4^{SMKO}$ mice, we performed proteomics analysis of the $Fbln4^{SMKO}$ ascending aorta and compared with that of wild-type. The early stage of aneurysm was evaluated in $Fbln4^{SMKO}$ mice by histology. The protein expression profiles were obtained from wild-type and $Fbln4^{SMKO}$ ascending aortae at each stage of aneurysm development (P1, P7, P14, P30 and P90) by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). Several differentially expressed proteins were identified by mass spectrometry and validated using qPCR and western blot analysis. Macroscopic observations revealed that dilatation of the ascending aorta was not observed at P7 but became detectable at P14. Histologically, focal lesion of SMC proliferation was already noticeable in the subendothelial region at P7 and became evident at P14 with a thickened aortic media. 2D-DIGE analysis showed that protein profiles between P7 and P14 were dramatically changed and molecules involved in actin dynamics, including cofilin, tropomyosin, ezrin/radixin/moesin (ERM) protein, destrin and S100a13 were differentially expressed between wild-type and in $Fbln4^{SMKO}$ aortae. Our study indicated that the protein expression significantly changed after P7, coinciding with the initiation of proliferative changes in the $Fbln4^{SMKO}$ aorta. *Fbln4* deficiency contributed to aneurysm formation by facilitating the alteration in actin dynamics.

(136) Deciphering α-dystroglycan glycosylation in cancer. More than LARGE

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Alpha-dystroglycan mediated cell anchorage to the extracellular matrix has been shown to be compromised in epithelial-derived cancers, such as breast, prostate, ovarian and lung cancers. The α-dystroglycan receptor function is mediated by a laminin binding sugar moiety which is *O*-mannosyl linked. Post-translational modification of α-dystroglycan is carried out by a battery of glycosyltransferases that include, POMT1, POMT2, POMGnT1, Fukutin, FKRP, iGnT and LARGE1. LARGE1 plays a central role in the synthesis of the laminin binding glycan. It is a bifunctional glycosyltransferase with both xylosyltransferase (Xyl-T) and glucuronyltransferase (GlcA-T) activities synthesizing a novel heteropolysaccharide structure on α-dystroglycan which is essential for dystroglycan receptor function. We recentl y demonstrated that dystroglycan functional glycosylation and binding to its main ligand laminin, is lost in epithelial-derived cancer cell lines as a result of the silencing of LARGE1. To investigate if other genes may underlie α-dystroglycan hypoglycosylation in cancer we analyzed the α-dystroglycan glycosylation status and the expression levels of all known dystroglycan modifying glycosyltransferases in a large collection of commercially available cancer cell lines. We discovered a number of cancer cell lines that express low levels of LARGE1 however they showed normal αdystroglycan glycosylation and receptor function. Analyzing the glycosyltransferase expression profile of these cells we detected high-expression levels of LARGE2, a LARGE1 paralog. These data suggest that LARGE2 can compensate for the loss of LARGE1 expression. Additionally, we identified cell lines with normal expression of LARGE1 and LARGE2, however they were characterized by a lack of α-dystroglycan functional glycosylation. We provide genetic and biochemical evidence to define the genetic mechanisms involved in α-dystroglycan hypoglycosylation during cancer progression.

(137) Investigations on new strategies for treating PMM2-CDG

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Congenital disorder of glycosylation type Ia (CDG-Ia, PMM2-CDG) leads to a severely reduced activity of the enzyme phosphomannomutase 2 (PMM2), which converts mannose-6-phosphate (Man-6-P) to Man-1-P in the cytosol. Patients develop

hypoglycosylation of proteins that result in multisystemic disorders with a mortality rate of 20% in the early onset of life. To investigate the pathophysiology of this disorder with the intention of developing effective therapeutic approaches, we generated a model for PMM2-CDG by introducing two separate mutations into the mouse Pmm2 protein, R137H and F118L. Pmm2 compound heterozygous embryos show a residual Pmm2 activity of 11% comaped with wild-type littermates and develop a hypoglycosylation phenotype that results in embryonic death by 10.5 dpc. Supplying mannose to pregnant dams normalizes the hypoglycosylation and rescues the embryonic lethality, demonstrating a prenatal benefit of mannose in our Pmm2 mouse model. However, postnatal mannose treatment does not help PMM2-CDG patients. One explanation might be that phosphomannose isomerase (PMI) competes with PMM2 for Man-6-P and diverts its metabolism into glycolysis. Inhibition of PMI, therefore, should restrict this route of removal and allow increased availability to attenuated PMM2. Using RNAi and genetic strategies (Pmi-Y255C mutation) to attenuate Pmi in, R137H/F118L, Pmm2 mouse embryonic fibroblasts, we demonstrate that reduced Pmi activity in Pmm2 hypomorphic samples restores efficient mannose flux into glycosylation. Furthermore, using ^{13}C-mannose and glucose heavy label tracers, we demonstrate that increasing the concentration of mannose in media is not substantially removed by attenuated Pmi but is instead preferentially diverted into glycosylation. Together, these data support the idea that inhibition of PMI, in combination with mannose therapy, is beneficial in correcting hypoglycosylation and warrants further studies as a potential post-natal therapy for PMM2-CDG.

(138) Heparin blocks autophagy in hyperglycemic dividing mesangial cells and initiates synthesis of a monocyte–adhesive hyaluronan matrix after completing cell division

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Our previous studies have shown that mesangial cells stimulated to divide in hyperglycemic medium activate hyaluronan synthases that then synthesize hyaluronan in intracellular compartments. This initiates an ER stress-autophagy response and up-regulates cyclin D3 that then mediates formation of a monocyte–adhesive hyaluronan matrix after completing cell division (Ren et al. 2009; Wang et al. 2011). This process occurs in vivo in streptazotocin-treated diabetic rat glomeruli with increasing autophagic mesangial cells and the formation of an extensive hyaluronan matrix with embedded macrophages. This leads to extensive nephropathy and proteinurea by 6 weeks. Previous studies showed that daily IP injection of a small amount of low-MW heparin in diabetic rats prevented the nephropathy and proteinurea over an 8-week period (Gambaro et al. 1992, 1994). Our new studies show that mesangial cells stimulated to divide in the hyperglycemic medium in the presence of 0.2 μM heparin do not activate hyaluronan synthesis in intracellular compartments nor induce the autophagy and cyclin D3 responses. Nevertheless, at the end of cell division, the mesangial cells synthesize a much larger monocyte–adhesive matrix. In vivo in the heparin-treated diabetic rat, the hyaluronan content in glomeruli increases greatly in weeks 1 and 2 and then declines to near normal by 6 weeks, at which time there are large numbers of macrophages present, but no evidence for autophagic mesangial cells. These results suggest that the dialog between the mesangial cells and influxed macrophages in the diabetic glomeruli is pro-inflammatory with accumulation of a fibrotic hyaluronan matrix that compromises kidney function. In contrast, in the heparin-treated diabetic rat the mesangial cells maintain their normal function after completing cell division, but still activate synthesis of the extensive hyaluronan matrix as an effective way to deal with the continued high-glucose stress. In this case, the influxed macrophages remove the matrix and do not initiate fibrotic responses and by 6 weeks there is a steady state of hyaluronan synthesis by the mesangial cells and its removal by the macrophages.

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(139) Controlling the source of mannose in *N*-glycans

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Exogenous mannose can directly contribute to *N*-glycan synthesis through Man-6-P. More commonly, glucose provides Man-6-P through phosphomannose isomerase (MPI, Fru-6-Pß > Man-6-P). A very modest increase in exogenous mannose rescues patients with mutations in *MPI* and mice deficient in Pmm2 (Man-6-PàMan-1-P), but the quantitative contributions of each pathway are unknown. We developed a highly sensitive and inexpensive stable isotope labeling method using (1,2-C) glucose and (4-C) mannose to measure their contributions to *N*-glycans under physiological conditions (5 mM glucose and 50 μM mannose). *N*-Glycans were released and hydrolyzed and mannose were derivatized and analyzed by GC-MS to reveal its origin. Analysis of various cell lines showed that mannose directly contributes 10–45% to newly synthesized *N*-glycans, showing intracellular preference of up to 100-fold for exogenous mannose based on relative exogenous hexose concentration. Mannose directly contributed 30% to *N*-glycans in normal human fibroblasts, and this increased to 80% in MPI-deficient CDG-Ib fibroblasts. Increasing exogenous mannose 20-fold above physiological levels eliminated glucose contribution altogether without increasing intracellular Man-6-P. Thus, both MPI activity and exogenous mannose strongly influence the metabolic flux of mannose into the glycosylation pathway. However, differences in mannose contribution between cell lines

were not exclusively determined by the ratio of Man-6-P degradation (MPI) to biosynthetic (PMM2) activities. Some lines with similar MPI:PMM2 ratios use exogenous mannose with substantially different efficiencies. We hypothesize that the differential expression of mannosecompetent hexose transporters or variable intracellular distribution of MPI and PMM2 also determines mannose delivery to *N*-glycans. Gluconeogenesis and pre-formed glycogen did not provide any mannose for N-glycosylation in fibroblasts. In summary, glucose is a preferred source of mannose for N-glycosylation, but exogenous mannose is much more efficiently used and substantially increases flux into the N-glycosylation pathway. While the MPI:PMM2 ratio is a key intracellular determinant, additional undefined factors also contribute. This work was supported by R21HD062914, R01DK55615 and The Rocket Fund.

(140) Characterization of the mucin-type *O*-glycans in IgA1 from patients with IgA nephropathy and healthy individuals

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Mucin-type O-glycosylation is a common post-translational modification, but the functional roles of *O*-glycans are poorly understood. A crucial step of *O*-glycan synthesis requires the co-expression of the core 1 galactosyltransferase 1 (C1GalT1 or T-synthase) and its molecular-specific chaperone Cosmc (core 1 Gal-T-specific molecular chaperone, Cosmc). Defects of T-synthase and/or Cosmc invariably lead to the expression of the Tn antigen (GalNAca 1-Ser/Thr), or its sialylated version, Neu5Aca2,6GalNAca1-Ser/Thr, Sialyl Tn (STn) antigen. Such epitopes are associated with pathology, such as Tn syndrome, neoplasic transformations and IgA nephropathy (IgAN). IgAN is the most common glomerulo-nephritis worldwide and is histologically characterized by deposits of IgA1. In contrast to IgA2, the IgA1 hinge region is uniquely *O*-glycosylated and galactose deficiency of these glycan structures is a hallmark of the IgAN. However, the molecular mechanism for decreased galactose in *O*-glycans in IgA1 of IgAN patients is unclear. We compared IgA1 in serum samples from multiple IgAN patients and control individuals. It is noteworthy that the total levels of IgA1 in IgAN patients were 2-fold elevated comaped with control donors. However, we found that IgA1 from both sets of samples express Tn antigen. Unexpectedly, the pool of IgA1 of almost all samples contains two distinct glycoforms: fully galactosylated (sialyl core 1 structure) and fully non-galactosylated (Tn and/or STn). Importantly, the IgA1 fraction with the Tn antigen from IgAN patients and control individuals was enzymatically restored to core 1 structure in vitro by the incubation of denatured IgA1 with recombinant T-synthase and the UDP-Gal donor, thus confirming the presence of authentic Tn antigen. This also raises the raising the question of the in vivo deficiency of T-synthase in "normal conditions" in healthy individuals in a subpopulation of B cells, as well as the role of Tn/STn on IgA1 in the pathogenesis of IgAN.

(141) Advanced age glycosaminoglycans of β-amyloid astrocytes

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Neurofibrillary tangles, characteristic of Alzheimer's disease (AD) and normal controls were obtained using the tissue culture. Patients and controls were males of an advanced age. Cultures were grown for 16 days; removed cells were washed and used to isolate glycosaminoglycans (GAGs). Briefly, the sample was degraded with repeated pronase and benzonase digestions before the removal of GAGs through β-elimination procedures. GAG composition analysis was accomplished using the individual digestions of the isolate GAGs with the enzymes chondroitinase ABC, AC, hyaluronidase and heparinase I, II and III. The produced disaccharides were separated using an Agilent Waters Spherisorb Analytical column HPLC (SAX-HPLC), employing a sodium phosphate gradient (2.5 mM to 1.2 M, pH 3.5) and post-column detection using 2-cyanoacetate as a fluorescent label. The results display CS oligosaccharides as the most abundant GAG of β-amyloid tangles of Alzheimer's disease, with similar results in the controls. Although heparan sulfate proteoglycan has specific association with glial plasma membranes, it was missing from these results. The discrepancy should stem from the fact that only prenatal and neonatal rats have been the source of most Alzheimer's disease GAG.

(142) Antigen-specific IgG glycosylation is strongly influenced by infection status while bulk IgGs remain constant

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Regulation of IgG glycosylation is poorly understood, but may be of crucial importance in understanding immunological control of infection during natural infection and protective vaccination. Studies of human IgG glycosylation have mainly focused on bulk antibodies in which minor skewing of glycan structures have been shown in autoimmune disease and aging, but very few differences have been observed in natural infection. Patients from a variety of infected cohorts were studied including HIV positive, influenza infected, Hepatitis C infected and longitudinal samples of seasonal influenza vaccine recipients. Bulk antibodies were isolated using Melon Gel IgG Purification kits and specific antibodies were isolated using antigens (HIV envelope, flu HA, flu vaccine antigen and HCV envelope) immobilized in a sepharose resin. Glycans were removed from bulk and specific IgGs using PNGase and the glycans were labeled with APTS and analyzed by capillary electrophoresis on an ABI 3130Xl sequencer. We found that though bulk IgG glycosylation was not strongly affected by infection or vaccination, the antibodies raised directly to the antigen in question were distinct from the bulk population and distinct from those antibodies from other types of infection. Antigen-specific antibodies contained more inflammatory species, including a reduction

in fucose, sialic acid and galactose to the *N*-glycan structures while the bulk population retained a normal distribution of non-inflammatory structures. This is the first study, to our knowledge, that has investigated the IgG glycosylations of such a large and varied cohort of infection and vaccination. This study provides evidence for a distinct and regulated glycosylation response during natural infection or vaccination, which suggests that these particular responses can be tuned in the context of vaccination to better tailor the best protective response.

(143) Type 1 diabetes: Involvement of the extracellular matrix in immune-mediated pancreatic islet destruction

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Type 1 diabetes (T1D) affects millions of individuals worldwide with prevalence and incidence rising annually. Although the mechanism triggering T1D is still not clear, the entire process must rely on the migration of inflammatory cells from the blood stream into the pancreatic islets via interaction with the extracellular matrix (ECM) that lies between islet capillaries and endocrine cells. The major aim of this study was to define the characteristic constituents of non-inflammatory ECM in the pancreatic islets and the nature of changes in this specialized ECM relative to the onset and progression of autoimmune islet destruction in a T1D mouse model. For this purpose, the DORmO (DO11.10xRIPmOVA) double-transgenic mouse model of T1D was used. This model closely parallels the autoimmunity and inflammatory β-cell destruction found in humans. The mice were evaluated at 7, 10, 15 and 36 weeks of age using immuno- and affinity-histochemistry. We demonstrate that the increase in blood glucose starting at 10 weeks of age is accompanied by a significant increase in hyaluronan (HA) and a dramatic decrease in inter-α-trypsin inhibitor (IαI) accumulation in the pancreatic islets. The intensity, distribution and amount of HA staining in the pancreatic islets increased even before the animals became measurably diabetic. The HA distribution inside the islet was mainly around the invading cells and the microvasculature. Interestingly, as soon as the animals became diabetic, immunostaining for IαI, which is known to form complexes with HA, exhibited opposite staining patterns to those of HA. The present study focuses on the role of HA-enriched ECM molecules in creating a permissive environment for autoimmune attacks. Based on the findings in other inflammatory diseases, it is likely that the HA-enriched ECM mediates the invasion and destruction of islet tissue by T cells and is therefore crucial to designing effective new therapies to treat T1D.

(144) New Approach to Detect Changes in Sialylation of the Matrix Glycoprotein Vitronectin, which Regulates Matrix Restructuring During Liver Regeneration

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During tissue remodeling, the extracellular matrix molecules that are glycosylated are different from those of normal tissue owing to changes in the glycan modification. Vitronectin (VN) is a multifunctional glycoprotein present in plasma and in the restructuring matrix. Linking cellular adhesion and tissue lysis, VN plays an essensial role during tissue remodeling. We discovered that alteration of glycans modulate the biological activities of VN during liver regeneration. Plasma VN was purified from partially hepatectomized (PH) and sham-operated (SH) rats at 24 hours after operation and non-operated (NO) rats. We found that the adhesion and spreading of rat hepatic stellate cells (HSCs), fibrotic cells induced during hepatic inflammation, on PH-VN was decreased to 1/2 of that on NO- or SH-VN. That on desialylated NO-VN decreased to 1/2 of that of control VN, indicating the importance of sialylation of VN for the activation of HSCs (Sano et al. 2010). The presence of highly sialylated *O*-glycans and almost fully sialylated *N*-glycans, both of which dramatically decreased after PH indicated the importance of sialylation in controlling the biological activities of VN. To detect the changes in sialylation consecutively throughout the process, we developed a method using chromatofocusing combined with immunodetection that enables rapid separation and detection of the matrix glycoproteins according to their pI under physiological condition (Ogawa et al. 2012). The elution pattern of chromatofocusing was reproducible, and pI of PH-VN were found to increase to more than pH 5 until 72 h from pH 4.5 of NO-VN. Because recovered samples are available for activity measurement, it is useful to demonstrate when alterations of sialylation occurs and how biological activities of the glycoproteins change. Understanding the glycan modulation of VN may contribute to the development of a strategy to regulate matrix regeneration and fibrosis.

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(145) Sites of Glycosylation in Fibrillar Collagens and Structure of Type II Collagen Revealed via X-ray Diffraction

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Diabetes causes changes to the structure of extracellular matrix elements like collagen. The effects of diabetes on collagen can lead to complications like slow tissue repair, glaucoma, faster aging, altered periodontal cell behavior and neuropathy. In studying the molecular structure of fibrillar collagens in native, hyperglycemic and hypoglycemic conditions, we have tried to relate the effect of the environment to the structural changes detected collagen. Preliminary experiments suggest that at hyper- and hypoglycemic conditions of glucose, the susceptibility of collagen fibrils to digestive enzymes was higher when comaped with digestion at physiological concentration of sugar. We plan to present structural data that explains the observed phenomenon of at first, increased and then decreased resistance of cross-linked collagen fibrils to physiological enzymes and speculate on the effects seen in diabetes. In the course of this study, it was necessary to conduct a three dimensional characterization of type II collagen, analogous to work we have previously performed for type I collagen. The

major collagen found within Lamprey notochord sheath, perinotochord and dermis has been found to be indistinguishable from human type II collagen in terms of its solubility, chain composition, amino acid composition and susceptibility to vertebrate collagenase. While the composition of the type II collagen fibrils within Lamprey notochord in particular, as assessed by X-ray, diffraction and electron microscopy studies seem to be similar to human cartilage, there are important and useful differences. The collagen fibrils are relatively well aligned and allow the collection of well defined X-ray diffraction patterns and the more simple tissue organization allows more detailed electron microscopy visualization of the fibrils than that seen in previous studies. From these data we are able to ascertain the molecular and supramolecular structures of chordate fibrillar type II collagen at 1 nm resolution, which we present for the first time here.

(146) Identification of a novel protein–protein interaction involved in α-dystroglycan glycosylation

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α-Dystroglycan is an extracellular component of the dystrophin glycoprotein complex found in the sarcolemmma membrane of muscle fibers.α-Dystroglycan functions by interacting with several ligands present in the extracellular matrix including the agrin, perlecan, neurexin and most importantly laminin. These interactions are critically dependent on the glycosylation status of the α-dystroglycan protein. Pertubations in the glycosylation of α-dystroglycan disrupts the vital interaction with its ligands and impairs its attachment to the extracellular matrix subsequently leading to various forms of congenital muscular dystrophies. We have identified a novel protein–protein interaction that regulates α-dystroglycan glycosylation. Our results raise the possibility that this previously unrecognized interaction could be implicated in muscular dystrophy.

(147) Expression of the sialyltransferase ST6Gal-I contributes to cisplatin resistance intumor cells

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Although aberrant glycosylation has long been known to be a characteristic of tumor cells, specific changes in tumor glycosyltransferase expression and glycan structures remain largely unexplored. We present a novel role for the sialyltransferase ST6Gal-I, which is up-regulated in numerous cancers, in conferring resistance to apoptosis-inducing chemotherapy agents. ST6Gal-I adds the negatively-charged sialic acid sugar in a α2-6 linkage to the termini of *N*-linked glycans on selected receptors. Previously, our group has reported that α2-6 sialylation on the β1 integrin by ST6Gal-I abolishes galectin binding and protects from galectin-mediated apoptosis. Additionally, we have determined that ST6Gal-I sialylates the Fas and TNF-R1 death receptors and prevents apoptotic induction through these pathways. In light of reports that cisplatin activity stems partially from the activation of the Fas pathway, we explored the possibility that ST6Gal-I expression may promote tumor cell survival through cisplatin treatment and contribute to the emergence of drug resistance in tumors. To test this hypothesis, we utilized ovarian cancer cell lines with engineered ST6Gal-I expression and noted differential responses to cisplatin, as measured by caspase activation and cell viability assays. We observe that forced expression of ST6Gal-I in cells with low endogenous ST6Gal-I expression (OV-4) protects against cisplatin-mediated apoptosis, whereas shRNA-mediated knockdown of ST6Gal-I in a highly ST6Gal-I expressing cell line (Pa-1) sensitizes cells to cisplatin-mediated cell death. In a separate experiment, we subjected heterogeneously ST6Gal-I expressing, shRNA knockdown cells to low-dose cisplatin treatment for 3 weeks and found the surviving cell population had elevated ST6Gal-I expression, suggesting selection for cells with higher ST6Gal-I expression in the presence of cisplatin. Furthermore, ST6Gal-I up-regulation was observed in a second ovarian cell line (A2780) harvested for resistance to cisplatin. We propose that α2-6 sialylation of selected receptors by ST6GalI contributes to chemotherapy resistance by preventing apoptotic signaling through multiple pathways.

(148) Agonists of heparan sulfate synthesis for the treatment of multiple hereditary exostoses

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Multiple Hereditary Exostoses (MHE) is an autosomal dominant disorder characterized by formation of ectopic cartilage-capped growth plate-like exostoses (osteochondromas) next to long bones and other skeletal elements. MHE results from mutations in the genes *EXT1* or *EXT2*, which diminishes the capacity of cells in the growth plate and the surrounding perichondrium to make heparan sulfate. The mechanism by which a change in heparan sulfate content causes ectopic osteochondromas is unknown, but evidence suggests that the decrease in heparan sulfate affects one or more signaling pathways through which growth factors regulate the organization of chondrocytes in the growth plate. Regardless of the mechanism, restoring heparan sulfate levels might diminish the frequency of exostoses. All cells make heparan sulfate through a common mechanism. In this study we have used mutant Chinese hamster ovary (CHO) cell lines that express about 10-30% of the normal level of heparan sulfate due to mutations in *Ext1* and employed a primary cell-based screen to find potential drug candidates that augment heparan sulfate expression, using FGF2 binding as a surrogate marker. Our preliminary screening results have identified several small druggable molecules that increase FGF2 binding in wild-type and mutant Chinese hamster ovary cells. The increase in FGF2 binding is abrogated when cells are treated with enzymes that cleave heparan sulfate chains. Secondary and tertiary screens are underway to establish the utility of these agents to enhance heparan sulfate synthesis in chondrocytes, which give rise to exostoses. Such compounds might reduce exostoses in Ext-deficient mice, which would serve as a proof-of-principle for pharmacological manipulation of exostosis formation in MHE patients.

(149) Mannose-fed mice are resistant to weight gain on high fat diet

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High fat diet (HFD) causes metabolic syndrome. We studied the effects of mannose supplementation on HFD-fed C57/ BL6 mice. Mice were weaned on HFD and drinking water containing either 2% mannose (HFD +2%M) or no mannose (HFD). At 16 weeks of age, mice on normal chow weighed 29.9 ± 2.66g whereas HFD mice were heavier (40.0 ± 3.4g). Surprisingly, HFD +2%M mice weighed much less (29.4 ± 3.05g) than HFD mice. 2% galactose did not affect weight gain indicating a mannosespecific effect. HFD +2%M mice showed decreased fat mass (22.6 ± 4.5%) than HFD mice (31.2 ± 3.5%). In a treadmill exercise, the speed and time at exhaustion increased for HFD +2%M mice (s = 25 ± 0.8 m/ min, t = 13.0 ± 0.3 m) relative to HFD mice (s = 22.8 ± 0.8 m/ min, t = 10.8 ± 0.6 m) showing increased fitness with mannose. Histological analysis showed less fat in the livers from HFD +2% M mice than HFD mice. There was no difference in water intake, energy expenditure or activity. Calorie intake was actually higher for HFD +2%M than HFD group suggesting that the differences is neither due to increased activity and energy expenditure nor reduced food consumption. Impaired fat absorption was ruled out because the energy content of the feces with (3546 ± 57 Cal/ g) or without mannose (3429 ± 111 Cal/ g) was equivalent. HFD +2%M mice were slightly glucose intolerant yet slightly insulin sensitive. Adiponectin has insulin sensitizing effects and its levels are inversely proportional to body fat mass. HFD +2%M mice had increased plasma adiponectin levels than HFD mice. Weight reduction was observed only when mice received mannose at weaning but not when provided 6 or 13 weeks post weaning. One possibility could be a mannose effect on the gut microbiome, which is more susceptible to alterations as it is established early in life. Our results show some adverse effects of HFD are prevented when mice are weaned on 2% mannose in their drinking water. [Supported by The Rocket Fund and RO1-DK55615].

(150) Increase in *O*-glycosylated oncofetal fibronectin in high-glucose-induced epithelial-mesenchymal transition of cultured human epithelial cells

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Effects over cell migration and invasion has been largely studied in a process known as epithelial-mesenchymal transition (EMT), which is a hypothesized program of cell development characterized by loss of cell adhesion, repression of E-cadherin expression and increased cell mobility. EMT may be essential for numerous developmental processes including mesoderm and neural tube formation, as well as cancer progression. Here, we used hyperglycemic condition associated with the overexpression of glutamine fructose-6-phosphate amidotransferase (GFAT), an enzyme involved in the first and rate-limiting step of the formation of hexosamine products. Here, we show that high-glucose-induced EMT in A549 cells, as demonstrated by: TGF-β1 secretion, changes from epithelial to fibroblast-like cells, increased cellular motility and emergence of mesenchymal markers. Interestingly, the hyperglycemic condition increased onfFN expression, and this effect was enhanced in GFAT-overexpressing cells. The hyperglycemic condition associated with the overexpression of GFAT was able to up regulate the expression of ppGalNA-T6 gene, and mRNA levels of fibronectin IIICS, which contain the hexapeptide (VTHPGY) required for onfFN biosynthesis. Taken together, these results show for the first time that hexosamine pathway modulates the onfFN expression and may play and important role in EMT induction.

(151) Increased expression of sortilin, an alternate sorting receptor for lysosomal hydrolases, reduces the bioavailability of latent TGF-β in mucolipidosis II cells

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Dysregulation of transforming growth factor-β1 (TGF-β1) signaling underlies many skeletal and connective tissue disorders. Although similar bone and cartilage phenotypes are common in many lysosomal storage disorders, including mucolipidosis II (ML-II), little is known about the involvement of the TGF-β1 pathway in their pathogenesis and the mechanisms whereby impaired lysosomal function impacts growth factor signaling. ML-II arises due to defects in the biosynthesis of mannose 6-phosphate (M6P) residues, the carbohydrate tags that mediate lysosomal targeting of acid hydrolases. To address whether TGF-β1 signaling was altered in ML-II, we analyzed TGF-β1 activity in cultured human MLII fibroblasts and found decreased Smad phosphorylation, accompanied by impaired TGF-β1-dependent wound closure. Biochemical analyses revealed a striking increase in intracellular latent TGF-β1 within ML-II fibroblasts caused by reduced secretion of a newly synthesized protein. We further found that latent TGF-β1, largely insoluble but still capable of activation, accumulated in a dense compartment within ML-II fibroblasts corresponding to lysosomes. We show here that increased expression of sortilin, a sorting receptor known to mediate lysosomal delivery of some hydrolases, also targets to latent TGF-β to lysosomes. The levels of sortilin protein were greatly elevated in human ML-II fibroblasts but not in milder ML-III cases, indicating a relationship between its expression and the extent of M6P-dependent mistargeting. Subsequent experiments demonstrated that sortilin-1 and TGF-β1 from ML-II fibroblasts co-immunoprecipitate, in line with recent studies showing that sortilin binds to other TGF-β1 superfamily members. Lastly, co-expression of sortilin and latent

TGF-β1 in HeLa cells resulted in a substantial reduction in TGF-β1 secretion and decreased intracellular levels. Collectively, these findings demonstrate that increased sortilin expression, as a consequence of impaired M6P targeting, results in reduced TGF-β1 bioavailability in ML-II cells. We believe this unexpected and novel pathogenic mechanism contributes to the clinical manifestations associated with this disease.

(152) Heparan sulfate subdomains that are degraded by Sulf accumulate in cerebral amyloid ß plaques of Alzheimer's disease: Evidence from mouse models and patients

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Extracellular cerebral accumulation of amyloid β peptide (Aß) is a histopathological hallmark of Alzheimer's disease (AD). Heparan sulfate (HS) is a glycosaminoglycan abundant in the extracellular space. The state of sulfation within the HS chain influences its ability to interact with a variety of proteins. Highly sulfated domains within HS are crucial for Aß aggregation in vitro. Here, we investigated expression of the sulfated domains and HS disaccharide composition in the brains of transgenic AD mouse models and patients with AD. RB4CD12, a phage display antibody, recognizes highly sulfated domains of HS. The RB4CD12 epitope exists in large quantities in the basement membrane of brain vessels under physiological conditions. In the cortex and hippocampus of the mice and patients with AD, RB4CD12 strongly stained both diffuse and neuritic amyloid plaques. Interestingly, RB4CD12 also stained intracellular granules of certain hippocampal neurons in AD brains. Disaccharide compositions in vessel-enriched and non-vasculature fractions of the mice and AD patients were found to be comparable to those of non-transgenic and non-demented controls, respectively. The RB4CD12 epitope in amyloid plaques was substantially degraded ex vivo by Sulf-1 and Sulf-2, extracellular HS endosulfatases. These results indicate that formation of highly sulfated HS domains may be up-regulated in conjunction with AD pathogenesis and that these domains can be enzymatically remodeled in AD brains.

(153) Strategies for analysis of sialylated glycocondgugates in cells by various analytical techniques

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The Analytical Services Laboratory of the Complex Carbohydrate Research Center offers services for structural characterization of glycoproteins from universities, research institutions and industries worldwide. Here, we will show a recent project in our laboratory that employed various techniques for structural elucidation of glycoproteins derived from cells. In collaboration with Dr. Joseph Lau's laboratory, we developed strategies to facilitate the determination of relative contribution of α2,3 vs α2,6 linked sialic acids from total pools of cellular sialyl-glycans. We investigated the glycan profiles of total bone marrow nucleated cells from mice with an inactivated ST6Gal-1 gene (ST6Gal-1-null) when comaped with wild-type mice. Sialyl-glycans are typically minor components of *N*-glycan samples. In order to overcome this obstacle, we used weak ion exchange chromatography to enrich the sialyl-glycan fractions. Exoglycosidase digests coupled with MALDI analyses were used to detect the presence of sialic acids and distinguish between 2,3- and 2,6-linked residues. Sequential LTQ-MS^n lithium fragmentation analyses were then used to corroborate the findings of the exoglycosidase/MALDI experiment. These two techniques used in combination provide a powerful approach for the definitive characterization of sialylated glycan epitopes. This project, will contribute to defining the structural importance of terminal sialic acids in the functioning of cell surface receptors involved in the immune response and other processes.

(154) Secondary changes in lysosomal storage diseases: Cytoskeleton, pH and glycosidase forms

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Lysosomal storage diseases (LSDs) are associated with hereditary deficiencies of certain hydrolases or factors affecting lysosomal function. Little is known about some biochemical and intracellular parameters which are also change in LSDs and developed as secondary markers of pathological conditions. We investigated lysosomal and cytoplasmic pH, cytoskeleton system and isoform spectrum of certain lysosomal glycosidases in human fibroblasts with LSDs and at intracellular storage of non-hydrolyzable substances. Intralysosomal pH was determined spectrophotometrically using dye and fluorescein; lysosomal size was estimated visually by comparison with the size of the measuring diaphragm. Rearrangement of vimentin-type intermediate filaments (IFs) was analyzed by an immunofluorescence microscopy. Sucrose was used as a non-hydrolyzable sugar in experiments modeling lysosomal storage. Cytoplasmic pH of the pathological cells (mannosidoses and Fabry's disease) and sucrose loading cells did not differ from control values, but intralysosomal pH was significantly higher in the all pathological and sucrose-loading cells. A decrease in pH was accompanied with an increase in the size of the organelles, owing to the accumulation of non-hydrolyzable compounds (NCs). In fibroblasts from a patient with Krabbe's disease, which do not store NCs, there was no increase either in intralysosomal pH or in lysosome size. The immunofluorescence microscopy shows that unlike cytoplasmic microtubules, vimentin-type IFs are collected into ring-shaped structures in affected fibroblasts with LSDs or NCs. Transition from discoid to an extended cellular form is accompanied by the centrifugal dislocation of ring-shaped IF structures toward the cell's active edge with the gradual restoration of the radial fibrillar vimentin network. Spreading of affected cells occurred more slowly than that of control fibroblasts. The

effect of exogenous and endogenous product storage in lysosomes on the activity and multiple forms of α-L-fucosidase from human skin fibroblasts was also investigated. The sucrose load causes certain changes in a secretion level of α-L-fucosidase and multiple forms' spectra of the intracellular and secreted enzymes. Some changes of secreted α-Lfucosidase isoforms' spectra were found in Fabry's fibroblasts, characterized by the intralysosomal storage of di- and trihexosylceramides. It is proposed that the intralysosomal accumulation of NCs influences the α-L-fucosidase post-translational processing.

(155) Detection of prostate cancer-associated *N*-glycan on free PSA by Luminex bead-based immunoassay: A novel prostate cancer screening method with higher specificity than the conventional PSA test

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Serum prostate-specific antigen (PSA) assay is widely used for detection of prostate cancer. Since PSA is also synthesized from normal prostate, false-positive diagnosis cannot be avoided by the conventional serum PSA test. Recently, US Preventive Services Task Force announced that PSA-based screening leads to a small or no reduction in prostate cancer mortality after 10 years, but is associated with harms related to false-positive test results, subsequent evaluation and therapy, including over diagnosis and overtreatment. Thereby, the development of novel Pca screening method with improved specificity is of vital importance. In this study, we develop an assay system for the detection of Pca-associated Siaα2,3Gal-linkage as an additional terminal *N*-glycan on free PSA (fPSA) in serum with rapid bead-based immunoassay. We randomly selected sera from 40 patients with clinical T1/T2 prostate cancer and 40 patients with benign prostatic hyperplasia (BPH) from our serum bank. All patients received prostate biopsy due to elevated PSA concentration in our regional Pca screening program and their diagnoses were histologically proven. We used the Luminex xMAP magnetic bead array technology to measure serum Siaα2,3Gal-fPSA (S23fPSA), employing mouse monoclonal anti-human fPSA antibody (2E2) for coating beads and Siaα2,3Gal-specific *Maackia amurensis* lectin for detection. Serum S23fPSA amount was represented as a mean fluorescence intensity (MFI) value. MFI values of 40 Pca patients were determined (median: 1117) and 40 BPH patients were determined (median: 858). As a result, serum S23fPSA is significantly increased in Pca patients comaped with BPH patients. MFI values of both patients were analyzed receiver operating characteristic (ROC) curve. The optimal cutoff point was determined to 1023 for differential diagnosis of Pca from the ROC curve. The area under the curve (AUC) was determined 0.929 much higher than that of the conventional PSA test. About 82.5% of BPH patients (33/40) among S23fPSA negative (<1023) patients permitted the diagnosis of BPH without the prostate biopsy. Although the present study is small and preliminary, these results suggest that the detection of α2,3-linked sialic acids on fPSA in serum with Luminex bead-based immunoassay can discriminate Pca from BPH whose sera were positive for the PSA test.

(156) Regression of pathological changes of the dermal-epidermal junction in skin constructs after silencing the expression of the p.R2622Q and p.G2623C collagen VII mutants

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The structural integrity of skin depends on a complex system of extracellular matrix molecules that form a biological scaffold supporting epidermal and dermal components. One of the elements of this system is the dermal basement membrane whose functions include bonding the epidermis and the dermis. Mutations in collagen VII, a key structural component of the dermal membrane zone, are associated with dystrophic epidermolysis bullosa. It has been suggested that silencing the mutated *COL7A1* allele may restore the dermal basement membrane zone formed in the presence of collagen VII mutants, but no detailed tests were carried out to test this approach. To address this problem, we employed a model that utilized skin-like constructs in which engineered collagen VII mutant chains harboring the R2622Q or G2623C substitution were expressed conditionally, but the wild-type chains were expressed unconditionally. We demonstrated that switching off the production of the mutant collagen VII chains in skin constructs restores the organization of collagen VII and laminin 332 deposits in the dermal-epidermal junction to the level of control. We also demonstrated that remodeling of collagen IV deposits was not fully effective after silencing the expression of collagen VII mutants. Our study indicates that, while silencing mutant alleles of *COL7A1* may repair critical elements of the pathologically altered dermal basement membrane, it may not be sufficient to fully regress all structural changes imposed on the architecture initially formed in the presence of the mutant collagen VII chains. Supported by NIH R01AR054876.

(157) Allelic Differences Contribute to Variation in Ocular Dysgenesis, Myopathy and Neuronal Lamination Defects Caused by Col4a1 and Col4a2 Mutations

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The type IV collagens are one of the most abundant types of proteins in basement membranes. Mutations in type IV collagen α 1 (*COL4A1*) and α 2 (*COL4A2*) cause highly penetrant multi–system

disorders that affect different subset of organs with variable severity. Recently we reported that *Col4a1* mutant mice have ocular dysgenesis, myopathy and cortical neuronal lamination defects that model Muscle–Eye–Brain disease (MEB) and Walker–Warburg–Syndrome (WWS). We identified *COL4A1* variants in two MEB/WWS patients and demonstrated that the variants influenced biosynthesis of collagen IV α1α1α2 heterotrimers. Other patients with *COL4A1* or *COL4A2* mutations have very different clinical presentations. Here, we test the hypothesis that allelic heterogeneity contributes to variable expressivity of *COL4A1* and *COL4A2* using an allelic series of mice with an otherwise uniform genetic background. Typically, mutations in *COL4A1* or *COL4A2* cause intracellular retention of α1α1α2 heterotrimers at the expense of their secretion into basement membranes. Using primary cells from mice with different mutations we show that there are allele–specific effects on the levels of intracellular and extracellular COL4A1 and COL4A2 at the molecular level. Similarly, different mutations have very different effects on the severity of ocular anterior segment dysgenesis, optic nerve hypoplasia, myopathy and cerebral cortical defects.

These results conclusively demonstrate the effect of allelic heterogeneity in *COL4A1.* and *COL4A2.*induced disease. Such allelic heterogeneity may reveal mechanistic heterogeneity that can be exploited for therapeutic intervention and may lead to more accurate prognosis for patients with mutations in these two genes.

(158) Laminin-332 stabilizes lamellipodium dynamics through β1 integrin signaling in migrating Madin–Darby canine kidney epithelial cells

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Laminins (LM) are αβγ heterotrimeric proteins of the basement membrane that interact with each other forming a complex network which is believed to provide spatial cues for correct apical-basal polarization. We have previously shown that experimentally wounded Madin–Darby Canine Kidney (MDCK) epithelial cell cultures constitutively express LM-511, while LM-332 (a truncated form unable to polymerize) is transiently expressed at the lamellipodium (leading edge), forming a haptotactic gradient to promote front-rear epithelial polarization and directional cell migration. However, the precise role of laminins in epithelial cell polarization is not well understood. We have generated a stable knock down of the α3 subunit of LM-332 (LMα3 KD) in MDCK cells using an shRNA retroviral system. LMα3 KD cells plated under subconfluent conditions in serum-containing medium show a compact morphology characterized by a reduction in the cell area and an increase in cell height comaped with control cells. A similar phenotype is observed in control cells in which TGF-β-induced LM-332 production is reduced by a TGF-β receptor inhibitor. Plating suppressed LMα3 cells on LM-332-enriched ECM rescues the compact morphology phenotype, which is consistent with a role of LM-332 in this cell behavior. Time-lapse videomicroscopy analysis shows that control cells initially spread and persistently migrate, while LMα3 KD cells show a defect in spreading as they fail to stabilize their lamellipodium and thus undergoing unproductive cycles of protrusion/retraction. Failure to form stable lamellipodium in the absence of LM-332 is consistent with an attenuation of integrin β1-mediated signaling, as shown by a reduction in FAK and Akt phosphorylation and with a reduction in focal complex size and myosin light-chain phosphorylation. Overall, these data support the hypothesis that LM-332mediated integrin β1 signaling is required for the stabilization of the lamellipodium after initial spreading in MDCK cells to promote persistent directional migration.

(159) Xylosyltransferase and glucuronyltransferase activities of LARGE are required for dystroglycan function

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Alpha-dystroglycan (α-DG) is a highly glycosylated cell surface protein and binds to laminin-G (LG) domain-containing the extracellular matrix (ECM) proteins such as laminin, agrin and neurexin. These interactions are required for a variety of physiological processes, including maintenance of the integrity of the skeletal muscle membrane and the development of the central nervous system. Perturbed glycosylation leads to reduced ligand binding by α-DG and is a common pathologic feature in a class of congenital muscular dystrophies that are often accompanied by brain and eye abnormalities. Although mutations in the LARGE gene and several others whose products are involved in *O*-mannosyl glycan synthesis have been identified in these disorders, the precise activity of LARGE has remained unclear. We now have evidence that pinpoints the enzymatic activity of LARGE and that indicates the basis of binding between LARGE-modified α-DG and ligand. Specifically, using a recombinant form of LARGE lacking the transmembrane region in an in vitro enzymatic assay, we show that LARGE is a bifunctional glycosyltransferase with both xylosyltransferase and glucuronyltransferase activities. Further evidence from MALDI-TOF- and NMR-based analyses reveals that LARGE synthesizes a unique heteropolysaccharide composed of repeating units of (-3Xyl-α1,3GlcAβ1-), and we show that it is this modification that confers the ability to bind LG domain-containing ECM ligands. α-DG is known to bind the LG4–5 domains of the laminin α-chain. Harrison et al. (2007) previously showed by point mutagenesis and crystallography approaches that three surface-exposed basic patches in LG4–5 contribute to binding with α-DG. It is likely that α-DG binds to these basic patches of laminin through electrostatic association, via the negatively charged glycan synthesized by LARGE. This notion is supported by the fact that one of the patches contributes to binding with heparin, which is also composed of acidic sugars GlcA and iduronic acid (Harrison et al. 2007).

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(160) Dual-linked role of integrin αV mediates TGF-β activation and fibronectin fibrillogenesis to regulate laminin-332-mediated repair in MDCK epithelial cells

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TGF-β is secreted by cells in an inactive form (latent TGF-β) through its association with Latency-Associated Protein (LAP). Activation involves binding of LAP by αV integrins, but this has not been clearly demonstrated in kidney epithelial cells. TGF-β has been linked by us and others to epithelial repair, but it also plays a role in fibrosis. Thus, understanding the TGF-β activation machinery in renal epithelial cells is of great importance in epithelial repair after injury. Here, we show that MDCK cells with integrin αV knocked down (αVKD) show a reduction in TGF-β activation, Smad2-mediated laminin (LM)-332 production (critical for directional migration and wound repair) and spreading. Addition of active TGF-β restored normal levels of LM-332 and cell spreading. Control, but not αVKD cells, adhered to LAP in an RGD-dependent fashion, suggesting that LAP is a ligand for integrin αV. In control cells, integrin αVβ3 focally colocalized with LAP in the lamellipodium, but were segregated toward the cell body, suggesting its dissociation. Interestingly, TGF-β accumulated in discrete foci toward the dorsal surface in control cells. However, in αVKD cells resulted in almost perfect colocalization of TGF-β with LAP. This suggests that, in the absence of integrin αV, TGF-β cannot dissociate from LAP, possibly due to lack of mechanical tension. Such mechanical tension might be provided by fibronectin (FN) assembly. In fact, αVKD cells showed less contractility and reduced MLC-II phosphorylation and were unable to assemble fibronectin (FN). FN assembly is dependent on ROCK, and blocking its activity reduced TGF-β activation. In conclusion, mechanical tension provided by integrin αV-mediated FN assembly during cell migration allows integrin αV to also bind to LAP and activate TGF-β to induce LM-332 expression and epithelial repair.

(161) RhoGTPase-mediated LIM kinase regulates collagen deposition and cleft for mation during mouse submandibular gland branching morphogenesis

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Branching morphogenesis is a complex and dynamic process involving several changes in the epithelium, including the dynamic regulation of cytoskeletal tension, epithelial polarity and basement membrane deposition. Here, we report that LIM kinase, a dual-specificity serine/threonine kinase, regulated by Cdc42 and Rac GTPase in salivary glands, may be a master regulator of these cellular processes in developing salivary glands. LIMK I/II function in a myosin-independent pathway to increase cofilin phosphorylation, inactivate it and stabilize F-actin filaments. LIMK I/II also destabilize microtubules (MTs) through interaction with p25a. Interestingly, within 24 h of incubation with salivary gland organ cultures, LIMK I/II siRNAs destabilize clefts, which are indentations of the basement membrane that initiate branching morphogenesis. This was also observed in epithelial mesenchyme-free cultures treated with an LIMK inhibitor (BMS-3) that impacts both the actin- and MT-dependent effects of LIMK, whereas an inhibitor that only affects F-actin organization (BMS-5) prevents initiation of new clefts, but does not destabilize existing clefts. While there was no effect on cell proliferation or apicobasal polarity in the presence of BMS-5, there were changes in cell shape, when comaped with the vehicle control, reducing the area and perimeter of all epithelial cells. BMS-3 drastically reduced epithelial cellular organization, cell–cell adhesions and cell proliferation. Significant changes in basement membrane were also observed to be under the control of LIMK. BMS-5 increased deposition of collagen IV at the periphery and in the lumen of the epithelial tissue, whereas BMS-3 caused aberrant and reduced deposition of collagen IV. The mechanism by which LIMK impacts basement membrane assembly through actin and MT organization during salivary gland branching morphogenesis is currently under investigation.

(162) Protomer and Network Organization of the α6 Chain of Collagen IV

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Collagen IV is a family of 6 chains (α1-α6), which form triple-helical protomers that assemble into supramolecular networks. Two distinct protomers with chain compositions of α121 and α345 have been established. These oligomerize into an α121 and α345 networks, respectively. At the C-terminals, the protomers oligomerize by homo-typic interaction through their trimeric NC1 (noncollagenous) domains, forming α121 and α345 NC1 hexamers, respectively. Their trimertrimer interfaces are stabilized by novel sulfilimine bonds (-S = N-), a covalent cross-link that forms between Met93 and Hyl211. A third protomer, with a composition of α556 has been proposed, based on limited immunochemical analyses, that assembles hetero-typically with a α121 protomer forming an α1256 NC1 hexamer at the trimer-trimer interface. In this study, we investigated the protomer and network organization of this putative α556 protomer and the α1256 NC1 hexamer by determining the chain identity of NC1 domains that are cross-linked by sulfilimine bonds. High-resolution mass spectrometry analyses of tryptic peptides derived from α1256 NC1 hexamers revealed that sulfilimine bonds specifically cross-link α1 to α5 and α2 to α6 NC1 domains, thus providing the spatial orientation between interacting NC1 trimers. Using this information, we constructed a 3D homology model in which the α565 NC1 trimer shows a good chemical and structural complementarity to the α121 NC1 trimer. Our studies provide the first chemical evidence for an α556 protomer and the heterotypic interaction

between the α565 protomer and the α121 protomer. Moreover, our findings, in conjunction with our previous studies, establish that the six collagen chains are organized into three canonical protomers α121, α345 and α556 forming three distinct networks: α121, α345 and α121-α565, each of which is stabilized by sulfilimine bonds between their C-terminal NC1 domains.

(163) Repository of recombinant expression constructs for mammalian glycosylation enzymes: Baculovirus vectors for glycosyltransferase and glycoside hydrolase production in insect cells

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Mammals encode 204 glycosyltransferase (GT) and 75 glycoside hydrolase (GH) family members that assemble and degrade the diverse array of glycans attached to intracellular and secreted proteins and lipids, which have critical roles in biological recognition. To enable structural and functional studies on these enzymes, we are assembling a repository of recombinant plasmids and baculovirus expression vectors encoding all known human GTs and GHs for production of these proteins in HEK293 or insect cells, respectively. These eukaryotic hosts are expected to provide the appropriate chaperones and post-translational processing machinery needed for proper folding and enzymatic activity. Our original baculovirus vector design for the expression of GT's with *N*-terminal transmembrane domains involved using the polyhedrin promoter to express the affinity tagged, secreted GT ectodomains and involved replacing each GT cytoplasmic tail and transmembrane domain with a signal peptide, 8× HIS and Strep-tag II affinity tags, followed by the Tobacco Etch Virus (TEV) protease cleavage site and the GT stem and catalytic domains. Our original baculovirus vector design for GH expression involved using the polyhedrin promoter to express the full-length protein with a C-terminal TEV cleavage site followed by the 8X HIS and Strep-tag II affinity tags. During the course of this project, we also constructed four other baculovirus vectors designed to express GTs with different transcriptional promoters and/or purification tags for comparative purposes. This poster summarizes the results obtained to date using the original baculovirus vectors to express 194 human GTs and 81 GHs in the baculovirus/insect cell system. It also compares production and secretion levels obtained with a test set of 11 GTs expressed using the original and new vectors. Interestingly, no clear trend emerged from this latter analysis of the impact of using different promoters and purification tags on GT expression and secretion.

(164) Enhancing β1,4-galactosyltransferase and α2,6-sialyltransferase activity bydomain swapping and site-directed mutagenesis

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β1,4-galactosyltransferase (B4GALT1) and α2,6-sialyltransferase (ST6GAL1) are Golgi-resident Type II transmembrane enzymes that are partially secreted due to proteolytic cleavage of their stem domains. We hypothesized that intracellular B4GALT1 and ST6GAL1 activity levels could be increased by replacing their cytoplasmic tail / transmembrane domain / stem (CTS) regions with the CTS of human α1,3fucosyltransferase VII, which is not subject to proteolytic cleavage (El-Battari *et al*, Glycobiology 2003). We tested this hypothesis by expressing chimeric and native forms of B4GALT1 and ST6GAL1 in insect cells, which lack both activities and therefore provide a sensitive system that can be used to compare the secreted and retained levels of these enzyme activities. Substantial amounts of B4GALT1 and ST6GAL1 activity were secreted when we expressed the native enzymes, but both were greatly reduced and the amounts of intracellular activity were greatly increased when we expressed the chimeras. In the course of these experiments, we also noticed that the original bovine B4GALT1 sequence (Shaper *et al.*, PNAS 1986) encodes a Leu at position 282, whereas Phe is fully conserved at that position in all other B4GALT1 sequences. We hypothesized that B4GALT1 activity might be increased further if we changed this residue to match the consensus. This hypothesis was supported by the results of activity assays, which showed that the Phe282 form of B4GALT1 had several fold higher activity than Leu282. Finally, we found that the native and chimeric forms of B4GALT1 and ST6GAL1 were all localized in the Golgi apparatus when we examined their intracellular distributions by tagging them with fluorescent proteins. We suggest that the mutated B4GALT1, as well as the chimeric B4GALT1 and ST6GAL1, can be used as significantly improved glycoengineering tools to enhance galactosylation and sialylation of recombinant glycoproteins in insect and other systems.

(165) Screening of glycogenes involved in myogenic differentiation of murine satellite cells

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Myogenesis is a complex process including the formation of muscles in embryo, their post-natal growth and regeneration. It corresponds to the differentiation of precursor cells or satellite cells to myotubes. During this process, the cell modifies its surface to promote cell–cell alignment and fusion and myotube interactions. Such a mechanism implicates glycoproteins, glycosaminoglycans and glycolipids. Previous transcriptomic studies used the Taqman low-density array technology and demonstrated that among 375 glyco-genes, only 37 have a high variation in their expression during the differentiation of the myogenic cell line C2C12 (Janot et al. 2009). We used the same approach to follow the glyco-gene expression during the differentiation of murine satellite cells in myotubes. In addition, we also studied glyco-genes expression during the early adipogenic differentiation of satellite cells in order to compare the gene expression during both differentiation pathways. By this new approach, we have depicted glyco-genes only implicated in the myogenic differentiation. Only 31 glyco-genes with an expression variation up to two times fold change seem mainly implicated in myogenic differentiation.

Among them, 14 are common to C2C12 and satellite cell differentiation in myotubes. The 31 glyco-genes were classified according to the biological function of their products. We observed a main group (1/3 of glyco-genes) corresponding to gathering adhesion proteins. In this group, the *Itga11* gene presented the highest overexpression (300 times fold change). A functional approach allowed us to validate this result. Indeed, the addition of antibodies against Itga11 decreased dramatically the cell fusion until 9%. Interestingly, a 6-O-GlcNAc-sulfotransferase gene, encoding for Chst5, presented also a high overexpression (54 times fold change). This result suggested that the keratan sulfates may be implicated in the myogenesis process.

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(166) Gene expression of brain-specific glycosyltransferases and their epigenetic regulation

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Glycosyltransferases play key roles for glycosylation in mammals. Although most glycosyltransferase genes have been cloned so far, it is still unclear how glycosyltransferases are expressed in tissue-specific manners. To clarify the mechanism of tissue-specific glycosyltransferase expression, we employed epigenetic approach. Epigenetics is a gene regulation mechanism which does not rely on genome DNA sequence, such as DNA methylation or post-translational modifications of histone. We analyzed one of brain-specific glycosyltransferase gene, GnT-IX, that is involved in branching of *O*-mannosyl glycan in the brain and found that GnT-IX expression is highly dependent on brain-specific chromatin activation with histone modifications (Kizuka et al. 2011). Conversely, we found that forced chromatin activation induced GnT-IX expression even in non-neural cell lines. In this study, we searched for an epigenetic factor which is involved in tissue-specific chromatin regulation of the GnT-IX gene. We found that several histone deacetylases are specifically involved in the epigenetic silencing of GnT-IX expression. In addition, we analyzed the expression mechanism of another brain-specific glycosyltransferase, ceramide glactosyltransferase (CGT) and found that the expression of CGT is regulated by its unique chromatin status. These results suggest that epigenetic regulation is an essential upstream event for the tissue-specific expression of glycosyltransferase.

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(167) Generation of an inducible cartilage-specific deleter using human aggrecan enhancer/promoter that is trackable in vivo using luciferase

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Chondrocytes constitute the sole cell type in articular cartilage, a tissue that covers the extremities of bones. These cells are also a major cell type in the intervertebral disc that acts as a cushion between the individual skeletal elements in the vertebral column. Collagen type II promoter was used to target these cells for many years since the chondrocyte-specific element in its promoter was characterized (Zhou et al. 1995). However, collagen type II was shown to express in other organs such as the kidney (Kolpakova-Hart et al. 2008) and its major disadvantage is the fact that in adult tissues its transcription is significantly down-regulated. Aggrecan is another major extracellular matrix protein expressed in articular cartilage more robustly than collagen type II. Henry et al. (2009) successfully created a chondrocyte-specific deleter by knocking-in the Cre recombinase gene downstream of the stop codon of the endogenous mouse aggrecan gene. We have utilized a transgenic approach where we have utilized the human aggrecan enhancer/promoter to drive expression of inducible Cre recombinase (Cre-ER^{T2}) followed by an IRES and the luciferase coding sequence, forming a bi-cistronic mRNA in transgenic mice. The properties and efficiency of the inducible Cre recombinase were tested by examining X-gal staining of tissues from embryos as well as adult double transgenic with Rosa26R mice. These mice were injected with the inducer, tamoxifen, at different time points during embryonic development and post-natally. Strong X-gal staining was observed in growth plate and articular cartilage as well as the fibrocartilage of meniscus, trachea and intervertebral discs reproducing the pattern of endogenous aggrecan gene expression. In addition to this mouse being an efficient deleter, the presence of luciferase allows the visualization of aggrecan expression in vivo. This has been tested before or after the induction of osteoarthritis through destabilization of the medial meniscus ligament. We are currently using this deleter to assess the role of human TIMP3 and its class-specific variant in the protection of articular cartilage in surgical model of osteoarthritis

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(168) Repository of recombinant expression constructs for mammalian glycosylationenzymes: production of glycosyltransferases and glycoside hydrolases inmammalian cells

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Glycan biosynthetic and catabolic enzymes play critical roles in encoding the diverse collection of carbohydrate structures involved in biological recognition events in animal systems. Mammalian

glycosyltransferases (GTs) (204 members) and glycoside hydrolases (GHs) (75 members) are responsible for the assembly and degradation of glycan structures attached to intracellular and secreted proteins and lipids, yet the enzymatic, biochemical, and structural characteristics of many of these enzymes are not well understood and most are not available in sufficient quantities for biochemical studies or enzymatic synthesis of glycan structures. Challenges for recombinant production of the glycosylation enzymes arise from general requirements for eukaryotic expression to provide chaperone systems and post-translational modifications necessary for effective folding and function. To address the need for these recombinant products, we established a repository of expression constructs encoding the catalytic domains of all human GTs and GHs for production in HEK293 cells or baculovirus/ insect cells. In mammalian cells, we developed an efficient transient transfection strategy for serum-free HEK293 suspension cultures providing secretion of the recombinant products into the conditioned media as affinity tagged forms. Alternative vector platforms with either short epitope and affinity tags or larger GFP or GFP-Fc fusions provide strategies for protein production, affinity purification, and protein detection and quantitation. Protocols for tag removal and glycan cleavage via combined protease and endoglycosidase digestion have also been developed to produce enzymes compatible with biochemical and structural studies. The poster will summarize the status of construct generation and protein expression in mammalian cells as well as availability of the constructs from the repository. The ultimate goals are to provide recombinant platforms for production of active glycosylation enzymes for use in biochemical, chemo-enzymatic, and structural studies on these critical human proteins. Supported by NIH Grant P41 RR-005351-20S1.

(169) Mutation of the UDP-Galactose Transporter, SLC35A2, Causes a Novel Type ofCongenital Disorder of Glycosylation

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Congenital Disorders of Glycosylation (CDG) are typically inherited autosomal recessive disorders that can be detected by abnormal glycosylation of serum transferrin (Tf).

Here we present a new CDG subtype involving X-linked somatic mosaicism of the Golgi localized UDP-Galactose transporter, *SLC35A2*. The individual has developmental delay, coagulopathy, infantile spasms and a Tf profile showing loss of [Sia +Gal] from multiple branches of complex type N-Glycans. Genetic analysis of primary fibroblasts revealed a mixed population of cells containing either a normal allele or a 120bp deletion within the first exon of *SLC35A2*, while parental and patient DNA from whole blood lacked this deletion. Loss of SLC35A2 resulted in exposure of terminal GlcNAc moieties on cell surface glycoproteins detected by greatly increased binding of the GlcNAc binding lectin GSII (*Griffonia simplicifolia II*). Cell sorting and separation of the two populations showed that cells with the deletion were highly positive for GSII staining, whereas cells with a normal *SLC35A2* had 20 fold less GSII staining. Separated populations demonstrated that the GSII positive population had a significantly reduced level of SLC35A2 UDP-Galactose transport activity. Our results also demonstrate the possibility another UDP-Galactose transporter may exist since most of the complex type N-Glycans still contained galactose.

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(170) The Art and Science Whole Exome Sequencing to Solve Novel GlycosylationDisorders

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Recent advances in DNA sequencing technologies, specifically Whole Exome Sequencing, have helped to identify the genetic causes for many unsolved rare genetic disorders. Yet only a few have involved defects in the various glycosylation pathways. We performed WES on nearly 50 individuals with various forms of Congenital Disorders of Glycosylation (CDG) as confirmed by ESI-MS of serum transferrin. We estimate that only 20% of our unsolved cases are, in fact, due to mutations in already known CDG types. (*SRD5A3, DPAGT1, ALG1, TUSC3*). Our pipeline consists of a standard filtering method (based on Score, % Variant reads, predicted pathogenicity), but because of the unique glycosylation angle, we are able to incorporate an addition layer of filtering based on our understanding of glycobiology. We have identified four new defects using this pipeline strategy. Validation of candidates is performed using gene complementation in combination with two independent biomarkers. The first, Intercellular Adhesion Molecule-1 (ICAM-1), is consistently reduced in CDG patient fibroblasts, and we have shown that gene complementation can restore ICAM-1 to nearly normal levels. The second, ER-Glyc-GFP, an endoplasmic retained GFP containing an engineered N-glycosylation site. When it is occupied by a glycan, steric hindrance destroys the fluorescence. But under hypoglycosylation conditions, the glow returns because the site is unoccupied. With an estimated 1-2% of the genome devoted to glycosylation, we anticipate many new candidate genes to be identified by WES in the coming years. We will discuss the methods and newly discovered defects.

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(171) Whole Exome Sequencing Identifies STT3A as a Novel CDG

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Whole Exome Sequencing has greatly enabled our ability to quickly and affordably solve rare genetic disorders. Using a combination of homozygousity mapping and whole exome sequencing, we report the first individual with a deficiency in the oligosaccharyltransferase (OST) subunit STT3A. The affected male from a consanguineous Middle-Eastern family presented with severe developmental delay, profound hypotonia, microcephaly, abnormal eye movement without tracking and epilepsy. The patient also has a similar, but more mildly affected female sibling. Aligning

regions of homozygousity and exome results revealed 31 novel homozygous mutations in which two genes are involved in glycosylation, *STT3A* and *GALNT8*. Since the affected sib had a type I transferrin pattern consistent with loss of entire N-glycan chains, the Golgi localized *GALNT8* was unlikely to be the defect. However, Sanger sequencing confirmed the presence of the c.1878 T > C mutation in STT3A that results in a p.Val626Ala. This mutation was absent from 1138 Middle-Eastern Exomes and 6500 Exomes of European and African-American origin. The p. Val626Ala mutation reduces the stability STT3A resulting in a several fold decrease in steady state STT3A content. Pulse-labeling analysis of patient fibroblasts showed hypoglycosylation of two glycoproteins, preprosaposin and progranulin, that are known substrates of the STT3A isoform of the OST. The hypoglycosylation defect of the STT3A p.Val626Ala mutation could be replicated in HeLa cells that had been treated with siRNA for STT3A and then transformed with a siRNA resistant construct of the STT3A p.Val626Ala allele. Thus far genetic disorders have been identified in 4 of the OST subunits, *MAGT1, TUSC3, DDOST, STT3A*.

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(172) Actin regulates the acquisition of a myofibroblastic phenotype by passaged chondrocytes

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Articular cartilage lacks the ability to repair itself and when damaged progresses to the point where total joint replacement is necessary. Unfortunately, these synthetic replacements will ultimately fail. Biological repair of damaged cartilage is limited by access to a large number of cells capable of producing and accumulating the matrix which mimics native articular cartilage. Expansion of cell numbers can be achieved by serial passage in monolayer culture, however, this results in cell phenotype change. During passaging chondrocytes spread, acquire actin stress fibers and synthesize type I collagen and tenascin C. Though these features are indicative of dedifferentiation, the actual cell phenotype has not been fully characterized. Furthermore the mechanism leading to this phenotypic change has not been elucidated. The hypothesis of this study is that passaged chondrocytes become myofibroblastic-like and actin regulates this change. Our studies revealed that passaged bovine chondrocytes had a lower proportion of globular to filamentous (g-/f-) actin when comaped with primary (unpassaged) cells. This correlated with elevated levels of type I collagen, tenascin C, smooth muscle α actin and transgelin mRNA. Culturing passaged chondrocytes within stress-relaxed collagen gels caused significant gel contraction comaped with primary chondrocytes. These features are characteristic of myofibroblasts. Treatment of passaged cells grown in monolayer to actin depolymerization agent, latrunculin B, significantly increased the proportion of g-/f-actin which correlated with lower levels of type I collagen, smooth muscle α actin, and transgelin mRNA and loss of the ability to contract collagen gels. These findings suggest a role for actin in the regulation of myofibroblastic-like phenotype during dedifferentiation. A further understanding in the mechanisms modulating dedifferentiation may result in the development of ways to facilitate the expression of the chondrocyte phenotype during/after chondrocyte passaging which will facilitate the generation of sufficient cells for biological repair.

(173) Epigenetic activation of mouse brain ganglioside synthase genes

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Gangliosides are sialic acid-containing glycosphingolipids abundant in the central nervous tissues. The quantity and the expression pattern of gangliosides in the mammalian brain change drastically during early development and are mainly regulated through the stage-specific expression of ganglioside synthase genes. It is still unclear, however, how the transcriptional activation of ganglioside synthase genes is regulated during development. Previously, we demonstrated that histone acetylation could mediate the activation of ganglioside synthase genes during brain development (Suzuki et al. 2011). In the present study, we have further investigated the epigenetic regulation of two key regulatory glycosyltransferases, *N*acetylgalactosaminyltransferase I (GA2/GM2/GD2/GT2 synthase) and sialyltransferase II (GD3 synthase) in neural stem cells (NSCs) derived from mouse embryonic brains. The chromatin immunoprecipitation assay showed that the genomic region around the transcriptional start site of both genes was selectively associated with active chromatin histone marks, which were correlated with their mRNA expression levels during normal brain development and also in vitro neuronal differentiation. Indeed, the association of trans-activation factors with these genomic regions was increased in NSCs whose histone deacetylase activity had been inhibited by drugs or siRNA silencing. Surprisingly, the neuronal maturation of NSC could be induced by the addition of exogenous ganglioside GM1, which in turn gave rise to changes of the chromatin status of ganglioside synthase as well as neuronogenesis genes. This observation suggests a novel role of the GM1 residing on the nuclear envelope for maintaining the ultrastructure of a subnuclear domain. These findings indicate a feedback pathway between the ganglioside expression level and the epigenetic activation. Our studies thus provide a novel molecular mechanism by which ganglioside synthase genes are regulated through epigenetic control during brain development.

Reference

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(174) Promoting human mesenchymal stem cell growth on silk-based biomaterial with bound recombinant collagen proteins

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Collagen-like proteins in the bacteria *S. pyogenes* have been shown to adopt a typical triple-helix structure with a stability similar to that of animal collagens even though they lack post-translational proline hydroxylation. Expression of bacterial collagen-like proteins in *E. coli* allows large scale recombinant protein production and easy modification through molecular biology techniques, thus providing a superior model system for the studies of collagen. To create biomaterials, the collagen domain was flanked by a C-terminal repetitive silk consensus sequence $(GAGAGS)_n$ which we hypothesized might allow integration into solid silk materials. Many forms of silk biomaterials have been designed for a wide range of applications in tissue engineering, but silk itself lacks biological activity to efficiently retain and interact with cells and other ECM components, an activity which can be provided by the collagen domain. Purified chimeric collagen-silk protein was shown to still form a stable triple-helix and was observed to now bind specifically to silk films and scaffolds, indicating the ability of the $(GAGAGS)_n$ sequence to recognize this motif in silk materials. The binding affinity could be controlled by varying the number of repeats of the silk sequence in the chimeric protein. The *S. pyogenes* collagen-like domain is considered a "blank slate" with no known biological activity, but activities were added by introducing well-established fibronectin and integrin binding sequences from mammalian collagens into the collagen domain. Silk scaffolds containing recombinant collagen-silk proteins with fibronectin and integrin binding domains were found to promote hMSC adhesion and proliferation in cell culture. We anticipate that these silk-binding collagen-like proteins could functionalize silk to regulate stem cell growth and differentiation. Such collagen-silk materials may eventually lead to the creation of simple, inexpensive and biocompatible artificial ECM in the future.

(175) Using Molecular Mechanics to Predict Bulk Material Properties of Fibronectin Fibers

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The structural proteins of the extracellular matrix (ECM) form fibers with finely tuned mechanical properties matched to the time scales of cell traction forces. Insights into the molecular origins of these properties offer a deeper understanding of the complex relationship between the cellular environment and cell behavior. Several proteins such as fibronectin (Fn) and fibrin undergo molecular conformational changes that extend the proteins and are believed to be a major contributor to the extensibility of bulk fibers. The dynamics of these conformational changes has been thoroughly explored since the advent of single molecule force spectroscopy and molecular dynamics simulations but remarkably, these data have not been rigorously applied to the understanding of the time-dependent mechanics of bulk ECM fibers. Using the tools of single molecule analysis extrapolated to molecular networks combined with measurements of protein density within fibers, we have examined the influence of molecular properties on the dynamic mechanical properties of Fn fibers. Fibers were simulated as molecular strands with architectures that promote either equal or disparate molecular loading under conditions of constant extension rate or constant applied force. Measurements of protein concentration within micron scale fibers using deep ultraviolet transmission microscopy allowed the simulations to be scaled appropriately for comparison to in vitro measurements of fiber mechanics as well as providing estimates of fiber porosity and water content. Comparing the properties predicted by single molecule measurements to in vitro measurements of Fn fibers showed that domain unfolding is sufficient to predict the high extensibility and non-linear stiffness of Fn fibers with surprising accuracy, with disparately loaded fibers providing the best fit to experiment. This work shows the promise of this microstructural modeling approach, which is generally applicable to other ECM fibers and could be further expanded to tissue scale by incorporating these simulated fibers into three dimensional network models.

(176) Biomechanical Imaging: mapping the mechanical properties of the cell

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Integrated mechanical responses of the cell including spreading, contraction, division, migration, apoptosis and mechanotransduction depend on the mechanical properties of the cell. Most microrheological techniques can provide only local measurements of mechanical properties and thus are not representative of whole cell behavior. Other techniques, such as atomic force microscopy, can generate detailed maps of cell stiffness, but are time consuming and can only probe the cell surface. Biomechanical imaging is a novel method that creates a virtually instantaneous, detailed map of internal cell shear modulus and prestress distribution. NIH3T3 fibroblasts were cultured on a soft (4–10 kPa) polyacrylamide gel micropatterned with 2 μm Alexa-633 labeled fibronectin dots on a regularly spaced 6 μm grid. Fluorescent 500 nm nanospheres were phagocytized by the cell before the substratum was deformed uniaxially to 4–10% strain. Dot displacements were used to calculate cell traction forces, while internalized nanospheres were used to measure intracellular displacements. Together, these measurements did not exceed 30 s. By comparing changes in cell traction forces and nanosphere positions before and during uniaxial deformation, a map of the cell's shear modulus distribution was obtained using finite element analysis. The cell was modeled as an incompressible, isotropic but highly inhomogeneous, linearly elastic body of uniform thickness under plane-stress conditions. We solved the inverse problem for shear modulus distribution by iteratively minimizing an internal displacement data-matching function. Using the obtained shear modulus distribution and traction force data, we generated a map of cell prestress. All the retained measured displacements were matched to within 0.1 μm, which was within estimates of experimental error. The calculated range of the shear modulus values (0.1–2 kPa) matched those found with other techniques. Our study showed that biomechanical imaging can provide detailed maps of cellular shear modulus and prestress distributions.

(177) Novel mechanism for localized delivery of osteoinductive peptides, protein-releasing nanocages and gene delivery vectors on bone graft materials for enhanced osteoregeneration

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Hydroxyapatite (HA) makes up the mineral component of bone, and synthetic HA biomaterials are widely used in clinical dentistry and orthopedics. Although HA is highly osteoconductive, osteoinductivity is limited. In this study, we developed a method for anchoring osteoinductive factors onto multiple types of HA biomaterials, as well as the human allograft bone, with the goal of enhancing the osteoregenerative potential of these graft substrata. Specifically, an HA-binding domain, heptaglutamate (E7), modeled on bone-binding domains within native proteins, was attached to peptides derived from the osteoinductive proteins, collagen I and bone morphogenic protein-2 (BMP-2). Addition of the E7 domain to osteoinductive peptides resulted in 45× greater peptide density and retention in vitro, and importantly, E7 peptides were retained on implanted HA and allograft bone for at least 2 months in vivo. Greater peptide density via E7-directed coupling was effective in stimulating osteoblastic differentiation of mesenchymal stem cells and significantly greater bone formation on HA materials implanted into rat tibiae. Additionally, we exploited the polyglutamate–HA interaction as a mechanism to tether protein delivery nanocages and gene delivery vectors to the surface of synthetic HA and allograft bone. Incorporation of polyglutamate domains into the capsids of both P22 nanocages and adeno-associated virus (AAV) significantly increased the number of cages and viral particles initially bound to, and retained on, HA. More efficient coupling of polyglutamate AAV vs wild-type AAV on HA substrata led to 12-fold greater delivery of the luciferase gene to target cells. Although these studies used model cargo molecules (e.g. luciferase), we are currently engineering polyglutamate-modified P22 cages and AAV to deliver BMP-2 protein and gene, respectively. In sum, the polyglutamate–HA tethering mechanism is broadly applicable for the delivery of many types of bioactive peptides, as well as vehicles for protein and gene delivery, on a multitude of clinical bone graft products to improve osteoregeneration.

(178) Visualizing Molecular Forces Across Vinculin in Living Cells

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In vivo cells adhere to the deformable extracellular matrix (ECM) that is both a source of applied forces and a means of mechanical support. Cells detect and interpret mechanical signals, such as force and rigidity, from the ECM through mechanotransduction. This process is central to cell migration, tissue organization and many disease states. While the connections between cells and the ECM, mediated by structures called focal adhesions (FAs), are primary determinants of mechanotransduction, the molecular mechanisms mediating this process are largely unknown. Progress has been limited by an inability to measure dynamic forces across proteins in living cells. Therefore, we have developed an experimentally calibrated Forster resonance energy transfer (FRET)-based biosensor that measures forces across specific proteins with pico-Newton sensitivity. The sensor has been applied to vinculin, a critical linker protein in the connections between the integrins and actin filaments whose recruitment to FAs is force-dependent. We show that vinculin recruitment to FAs and force transmission across vinculin are regulated separately. Highest tension across vinculin is associated with adhesion assembly and enlargement. Conversely, vinculin is under low force in disassembling or sliding FAs at the trailing edge of migrating cells. Furthermore, vinculin is required for stabilizing adhesions under force. These data reveal an unexpected regulatory mechanism in which the ability of vinculin to bear tension determines whether adhesions assemble or disassemble under applied force. Current efforts focus on identifying critical regulators of molecular tension across vinculin in dynamic FAs. Preliminarily, we have identified Srcfamily kinases as critical regulators of molecular tension. Treatment with the Src-family kinase inhibitor PP2 and a non-phosphorylatable mutation of a Src phosphorylation site in the vinculin tension sensor (Y1065F) alter the tension across vinculin in FAs. These data suggest both biochemical regulation and cellular force-generation play a significant role in determining the molecular tension across vinculin.

(179) Biomedical engineering: An approach that can be effectively used not only to improve, but also to probe insect protein glycosylation pathways

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The introduction of insect cell systems as hosts for recombinant glycoprotein production during the 1980s injected a "need to know" factor that intensified interest in insect protein glycosylation pathways. Insect glycobiologists expected that insect cells would probably *N*-glycosylate newly synthesized proteins, but would not process the *N*-glycans to complex, terminally sialylated structures. The long-held view that insects lacked sialic acid metabolism was one fundamental basis for this limitation, but the data were sparse. Nevertheless, these expectations were confirmed and extended in a multitude of new reports as insect cells became commonly used as platforms for recombinant glycoprotein production. These results underscored a major evolutionary difference between insects and higher eukaryotes and predicted that recombinant *N* glycoproteins produced in insect systems might not be structurally authentic. In fact, they predicted that complex terminally sialylated *N*-glycans on native mammalian glycoproteins would be replaced by paucimannosidic structures when produced in insect systems. This revealed a major weakness of insect glycoprotein production platforms when one stopped to consider that complex, terminally sialylated *N*-glycans play important roles in glycoprotein function. This problem prompted my group to develop biomedical engineering approaches and to

use them to "humanize" insect protein *N* glycosylation pathways. These increasingly sophisticated efforts continue to yield improved, transgenic insect cell lines with the ability to produce complex, terminally sialylated *N* glycans. In addition, we find that biomedical engineering approaches can be used to effectively probe endogenous insect cell glycoprotein processing functions, which are not as simple as anticipated. Our recent biomedical engineering results, together with data from molecular genetic approaches, contribute to the growing recognition that insects have a restricted capacity for endogenous glycoprotein sialylation. Furthermore, these findings led us to discover significant biochemical and cell biological differences between certain mammalian and insect enzymes involved in sialic acid production and utilization. We believe that these differences reveal post-translational mechanisms underlying the restricted nature of sialic acid metabolism in insect systems.

(180) The importance of sodium bicarbonate/CO_2 culture for chondrocyte proliferation in monolayer

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Introduction: Chondrocytes cultured in a flow bioreactor, produce significantly more extracellular matrix when bicarbonate ($NaHCO_3$) is added to the culture media (1). The aim of this study was to determine the effect of bicarbonate is to increase cell proliferation or to increase matrix production per cell. Chondrocytes were isolated from metacarpal joints of 18–24-month steers by enzyme digestion. Cells were seeded in monolayer in 25 cm^2 flasks (1.6 x 10^5 cells) for a total of 7 days. The Ham's F12 media, with 20% FBS, 100 µg/mL ascorbate was changed every 24 h. The media was either supplemented with 35mM HEPES (starting pH 7.3, AIR) or with 20mM HEPES + 14mM $NaHCO_3$ (starting pH 7.3, 5% CO_2) 37°C, 95% relative humidity. After 2 days, half the flasks from the AIR condition were moved to CO2 (supplemented with NaHCO3 media), and half of the CO2 flasks were moved to AIR (without NaHCO $_3$media). Flasks were cultured on a shaker at low speed or under static conditions. Cells were counted and extracellular pH was measured every 24 hours, during the 7-day culture period. Proliferation was substantially higher (3–4-fold increase) in flasks left in the CO2 incubator, and in flasks removed from the AIR incubator to the CO2 incubator. Interestingly, proliferation was similar in both static and mixed systems

Discussion: We found that whether the system was well mixed with no acid gradient or static which allowed the presence of an acid gradient, cells cultured in a CO2 incubator proliferated much more rapidly than those cultured in air. Hence, CO2/bicarbonate culture rather than extracellular pH was important in driving cell proliferation and hence increasing matrix production in a bioreactor.

Reference

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(181) Organotypic 3D-culture of uterine fibroid cells

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Fibroids, benign tumors of the myometrium, grow mainly by accumulation of large amounts of altered collagen. Fibroid cell monolayer cultures (2D) do not reflect the complexity of these tumors. Our objective was to grow organotypic 3-dimensional fibroids in culture that are more representative of human uterine fibroids producing and accumulating the extracellular matrix. Immortalized fibroid cells were cultured on polylactic acid (PLA) scaffolds. We compared static seeding onto scaffolds in cell culture plates with dynamic seeding in a suspension culture system using the Rotary Cell Culture System bioreactor. After 7 days, cell numbers in the scaffolds were assessed by colorimetric (MTS) assay: Dynamic seeding using the bioreactor was more effective and scaffolds contained twice as many cells as statically seeded scaffolds. Subsequently, dynamically seeded 3D-scaffolds were cultured for 3 weeks in the bioreactor and comaped with cells grown in monolayers (2D). Samples were collected weekly to assess cell numbers, collect RNA and stained with Masson-Trichrome to detect collagen.Cell numbers increased until week 2 then remained stable in the scaffolds but tended to decrease in the 2Dcultures. High-quality RNA was extracted from the PLA scaffolds and RT-PCR analysis showed that mRNAs for TGFB3, COL1A1 and COL3A1 were expressed in 3D-cultures and did not change considerably over time. In 2D cultures, expression of TGFB3 and COL1A1 mRNA tended to decrease after week 1 while COL3A1 was highest at week 3. Masson-Trichome staining showed cells grown on scaffolds contained more collagen than cells grown in 2D. This study is the first step in creating an organotypic 3D-model that can be utilized to study the pathophysiology of uterine fibroids in-vitro. There are no universally accepted small animal models for uterine fibroids. Our 3D-fibroids can ultimately be transplanted into mice and provide a replicable model system to study medical treatments for fibroids. Funding: Charles Hammond Research Fund, Department of Obstetrics and Gynecology, Duke University.

(182) 3D mapping of complex flow fields in biomaterials

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Current approaches to growing tissues in three dimensions (3D) for regenerative medicine require transport of nutrients and growth factors to cells in a seeded scaffold. Mechanical forces such as fluid shear have been shown to enhance cell growth and differentiation, yet our knowledge of its local magnitude and its effect on cells in 3D is limited due to lack of precise flow measurements within biomaterial scaffolds. Here, we show that phase-contrast nuclear magnetic resonance (NMR) can be used to measure microflows in porous biomaterials and can successfully generate 3D maps of flow, pressure, shear and permeability throughout the scaffold in real time. We find that the visualization of complex fluid dynamics in three dimensions can be used to monitor changes in flow patterns resulting from the hydrodynamic properties of the material. The technique also enables non-invasive observation of

any structural changes to the biomaterial under flow such as compression and/or material erosion. Ultimately, these flow maps can be employed to optimize biomaterial properties with respect to flow and mass transport and enable investigation of cell responses under precisely measured flow dynamics in 3D scaffolds.

(183) Organization of Myocardial Matrix Collagens and Elastins of Heart Failure Patients after Mechanical Circulatory Assist

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Introduction: Mechanical circulatory assist (MCA) is increasingly important in treatment of heart failure (HF) and dramatically reduces myocardial loading. While MCA may normalize certain parameters, including myocyte size and neurohormonal activation, conflicting data exist regarding its impact on extracellular matrix (ECM) remodeling and myocardial fibrosis; little is known about biomechanical loading effects on structural organization of ECM constituents in the myocardium. We evaluated the structure of collagens and elastins in patients before and after MCA treatment in the setting of endstage HF by directly imaging unstained human myocardium using second harmonic generation (SHG) and multiphoton excited fluorescence (MPEF). Left ventricular (LV) apical tissue cores removed during device implantation and corresponding LV free wall (LVFW) sections from explanted hearts obtained at the time of transplantation were examined. Tissues from eight HF patients were analyzed. LV apical and LVFW samples from 10 non-transplantable normal human hearts were used as controls. Frozen tissue samples were sectioned at 60 microns for SHG and MPEF imaging of collagens and elastins, respectively. Spectral analysis confirmed identification of ECM constituents. Fibril size, quantity and orientation of collagens and elastins were characterized and quantitatively analyzed using 3D stack images. Western blot (WB) confirmed relative expression of matrix constituents. Myocardial collagen was elevated in pre-MCA samples relative to normal ($P < 0.05$), and levels were normalized following treatment. MCA reduced the myocardial elastin volume fraction from 17.3 to 12.4% ($P < 0.01$). Pre-MCA endstage HF samples were characterized by increased ECM deposition and smaller fibril diameter. Treatment with MCA increased fibril size and reduced overall ECM fiber content. The structure, as well as overall quantity, of ECM constituents affects the biomechanical properties of myocardium. Novel imaging strategies employing minimally manipulated biological specimens can shed light on the contribution of ECM protein structure to remodeling processes in HF.

(184) Laminin active peptide-polysaccharide matrices for tissue engineering

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Biomaterials that provide a support or scaffold for tissue formation play key roles in virtually tissue engineering approaches. The extracellular matrix (ECM) components including laminin, collagen and fibronectin and their active peptides are potential candidates for affording the cell binding activities to materials. Laminins are a major component of the basement membrane, a thin ECM, and have diverse biological activities. Our goal is to identify active sequences from laminins and to use the biologically active peptides for biomaterials as a synthetic basement membrane. We already have identified various biologically active peptides from laminins using more than 3000 synthetic peptides. These peptides recognized various cellular receptors and have the potential ability to serve as bio-adhesiveness for tissue engineering. We prepared peptide-polysaccharide matrices using three laminin active peptides, A99 (AGTFALRGDNPQG, bind to integrin αvβ3), AG73 (RKRLQVQLSIRT, bind to syndecans) and EF1zz (ATLQLQEGRLHFXFDLGKGR, X = Nle, bind to integrin α2β1) and three polysaccharides, chitosan, alginate and agarose, and examined their biological activities. Most of the peptide-polysaccharide matrices showed cell attachment activity and neurite outgrowth activity. The morphological appearance of the attached cells was found to depend on the peptides and physical properties of the polysaccharides. Further, the mixed AG73/EF1zz-chitosan matrices mimicked the cell adhesion of a multifunctional protein, suggesting that the mixed peptide-polysaccharide matrices are useful as a multifunctional biomaterial for tissue engineering. These results suggest that the receptor type of ligands and physical properties of scaffolds are critical for cellular functions. The peptide-polysaccharide matrices have a potential to be used as a synthetic basement membrane for tissue engineering.

(185) Cyclic tensile strain inhibits interleukin-1β and tumor ncerosis factor-α-induced aggrecanase in human chondrosarcoma cell line OUMS-27 by stretch-activated channles

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Osteoarthritis is a disease of cartilage destruction caused by mechanical loads and inflammatory cytokine-induced extracellular matrix (ECM) destruction. Aggrecan is a major component of the cartilage ECM. We investigated the cellular response in OUMS-27 to a cyclic tensile stress (CTS) on the expression of aggrecanases (ADAMTS species). Furthermore, OUMS-27 cells were treated stretch-activated channel inhibitor Gadolinium. The effects of CTS on the expression of interleukin (IL)-1β (10 ng/ml) and tumor ncerosis factor (TNF)-a (10 ng/ml) induced aggrecanase in OUMS-27 were studied by a reverse transcription real-time PCR. After gadolinium treatment, OUMS-27 was treated IL-1b and TNF-a with or without CTS. 10% CTS reduced on the expression of IL-1β (10 ng/ml) and TNF-a (10 ng/ml) induced ADAMTS9 mRNA expression in OUMS-27. Gadolinium treatment blocked CTS-induced aggrecanase expression reduction. A mechanical

stress reduces inflammatory cytokine-induced aggrecanase expression. The molecular mechanisms involved in this process were not clear, but the mechanochemical signal was transduced through stretch-activated channels in chondrocyte.

(186) Actomyosin cytoskeletal organization distinguishes lobopodia from lamellipodia

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Three-dimensional (3D) cell migration through chemically and structurally diverse environments is an important component of embryogenesis, immune surveillance and wound healing. The regulation of cellular force production (mechanotransduction) by cell–matrix adhesions can control how cells move. For example, the rigidity of the 2D extracellular matrix regulates cellular contractility to control the speed of cell migration, while the elastic behavior of the 3D matrix governs the mechanical mode of membrane protrusion. Cells in non-linear elastic environments use actin polymerization to extend small, fan-shaped lamellipodia, while cells in linear elastic materials are characterized by blunt, cylindrical lobopodia. To determine how the linear elastic 3D extracellular matrix regulates mechanotransduction to control the mode of leading-edge protrusion, we investigated the function of the actomyosin machinery during lamellipodia- and lobopodia-based migration. In non-linear elastic 3D collagen, PIP3 and active Rac1 and Cdc42 were targeted to the leading edge, consistent with 2D lamellipodia-based migration. In contrast, cells migrating inside linear elastic dermal explants and the cell-derived matrix (CDM) were characterized by non-polarized Rac1, Cdc42 and PIP3 signaling. Despite these differences in polarized signaling, 3D lamellipodia and lobopodia protruded with a similar speed and persistence, and RhoA activity remained relatively uniform within cells using either mode of migration. Significantly, myosin IIA localization was different in lobopodia comaped with lamellipodia. Myosin IIA was localized along distinct peripheral F-actin fibers in lobopodia, while in lamellipodial cells, it was organized in a periodic pattern associated with cortical F-actin beneath the plasma membrane. Switching fibroblasts from lobopodia-to lamellipodia-based migration by inhibiting cellular contractility and mechanotransduction corresponded with the loss of GFP-myosin light chain from the peripheral fibrillar structures. Thus, lobopodia are distinguished by distinct myosin IIA-positive structures that may act to generate the intracellular force necessary for this mechanical mode of lamellipodia-independent 3D migration.

(187) Delivery of platelet-derived growth factor from Bone–Mimetic electrospun matrices as a chemotactic factor for mesenchymal stem cells

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Although bone has a dramatic capacity for regeneration, certain injuries, pathologies and procedures present defects that are unable to heal properly and require surgical intervention to induce and support osteoregeneration. Autograft, healthy bone harvested from the patient, is often required in order to fill the defect. Many researchers have turned toward the use of engineered biomaterials to replace the painful procedure of autografting. Utilizing the process of electrospinning, our lab has developed a bone-like matrix consisting of composite nanofibers of the mechanically stable polymer polycaprolactone (PCL), and the natural bone matrix molecules type-I collagen (col) and hydroxyapatite (HA) nanocrystals. Previously, we have shown that PCL/col/HA matrices support greater mesenchymal stem cell (MSC) adhesion, proliferation and activation of integrin-related signaling cascades in comparison with electrospun PCL alone. In this study, we evaluated the potential of our bone-mimetic matrices (BMMs) to stimulate the recruitment of MSCs, the multipotent bone progenitor cells within bone marrow, through the sustained delivery of platelet-derived growth factor-BB (PDGF-BB). Specifically, we found that BMMs adsorbed, and subsequently released, significantly greater amounts of PDGF-BB over 8 weeks comaped with PCL scaffolds. Released PDGF-BB retained its bioactivity, stimulating significant MSC migration in Boyden chamber assays. Additionally, we developed a more stringent model of MSC migration, involving greater dilution of the released PDGF over a larger distance than the standard assays by placing BMMs +PDGF 1.5 cm away from a confluent MSC front. A significantly greater number of cells were observed migrating beyond the initial front, toward the PDGF gradient created by the scaffold than in control wells. Collectively, these results suggest that the inclusion of the bone matrix molecules collagen I and HA within electrospun scaffolds not only enhances MSC adhesion and survival, but also serves to concentrate PDGF-BB from solution, leading to sustained local delivery and MSC chemotaxis.

(188) Long-term traction force measurements for composite extracellular matrix patterns

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Cellular contractility has become an important focus of contemporary studies on complex cellular processes such as cancer progression, epithelial to mesenchymal transition and differentiation. Due to the complex and orthogonal nature of the numerous signals presented by the extracellular matrix (ECM) to cells, tools that are able to independently control cell shape, substratum rigidity, spacing and presentation of multiple cell adhesion molecules and cellular strain while measuring cellular traction forces (CTFs) would promote a more complete understanding of the mechanobiology of these processes. We have developed such a technique for measuring the real-time CTFs of individual and groups of cells on polyacrylamide hydrogels for at least 2 weeks with independent control of cell shape and patterns of multiple adhesion molecules. A microcontact printing procedure was used to create a grid pattern of fluorescently labeled cell adhesion molecules that are 2 μm in diameter and 5 μm center-to-center on a glass substratum. A novel two-step process was developed to produce a dual pattern of aligned dots corresponding to spatially distinct cell adhesion molecules, such as fibronectin and E-cadherin, which could be imaged with separate fluorescent channels. This pattern was

transferred to a soft polyacrylamide hydrogel with NHS-acrylic acid ester to form a covalent bond between amines on the cell adhesion molecules and the hydrogel. CTFs could then be calculated by determining the displacement of the fluorescent protein markers from their initial positions of the grid pattern by referencing the positions of the undeformed markers. Finally, we showed that cells could be imaged for up to 2 weeks on this pattern, demonstrating the feasibility of this tool for measuring long-term processes such as stem cell differentiation.

(189) Cell–cell and cell–matrix Interactions on Biomimetic 3D Scaffolds

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An in-depth understanding of the interactions between cells and three-dimensional (3D) matrices (scaffolds) is pivotal to the development of novel biomaterials for tissue regeneration. However, it remains a challenge to find suitable biomimetic substratums and tools to observe cell–material and cell–cell interactions on 3D matrices. In the present study, we developed biomimetic nanofibrous 3D gelatin scaffolds (3D-NF-GS) and utilized confocal microscopy combined with a quantitative analysis approach to explore cell–matrix and cell–cell interactions on the 3D-NF-GS. The 3DNF-GS (prepared by a thermally induced phase separation technique combined with a particle leaching method) mimic both the physical architecture and chemical composition of natural collagen. Both human gingival fibroblasts (HGFs) and osteoblasts were used to examine how cells adhere, proliferate, migration and matrix deposition on the 3D-NF-GS. Integrin b1, phosphor-paxillin and vinculin were used to detect cells responses to the nanofibrous architecture of 3D-NF-GS. Collagen I matrix production by both cell types on the 3D-NF-GS was visualized and quantified using a novel approach incorporating TRITC label in the 3D-NF-GS. Based on the confocal microscopy, this work has developed qualitative and quantitative methods to study cell–matrix and cell–cell interactions on biomimetic 3D matrices, which provides valuable insights for the development of appropriate scaffolds for tissue regeneration.

(190) Deciphering sulfation code of chondroitin sulfate that regulate neuronal directionality and path finding

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Chondroitin sulfate proteoglycans (CSPGs) are among the most important biomolecules in the central nervous system (CNS), providing both permissive and inhibitory influences to the axonal growth. CSPGs are up-regulated at the scar site in spinal cord injuries and limit the regenerative capacity of the adult CNS. Conversely, CSPGs also periodically support neuronal growth, plasticity and regeneration. This duality of CSPGs is understood to result from the variation in their sulfation patterns. Therefore, a thorough understanding of the sulfation–function relationship in CSPGs is a topic of great biological and clinical interest. In this study, we conducted a systematic study on the response of hippocampal neurons to different cell choice substratums containing CS (CS-A, CS-B, CS-C, CS-D and CS-E) chains found in the mammalian brain. We observed that neuronal growth is strongly dependent on the type of sulfation pattern of CS. By precisely controlling CS spatial presentation, we show that specific sulfations of the CS can control the directionality as well as growth preference of elongating neurons. Further, we created novel CS-GAG-based interfaces for neuronal guidance by utilizing a combination of permissive and inhibitory CS-GAGs. The insights obtained from this study reveal that the structural basis behind CS mediated neuronal growth and pathfinding and connects the sulfation pattern of CS with their functional manifestations.

(191) Cell aspect ratio alters stem cell lineage

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Adult mesenchymal stem cells (MSCs) "feel" the stiffness of their environment and differentiate in response to it; thus aberrant stiffness resulting from fibrosis in muscle dystrophies could misdirect MSCs into the wrong lineage. Conversely, MSCs have been shown to respond in a myosin-dependent manner to adipogenic and osteogenic media when cell spread area changes from 10^3 to 10^4 μm^2 or when cultured in specific shapes, e.g. circles vs rectangles and polygons, respectively. When both cues are present in a disparate fashion, e.g. highly elongated cells similar to muscle, despite the presence of an abnormally stiff microenvironment, we hypothesized that a myosin contraction-dependent balance could induce a subset of MSCs to differentiate in to a muscle-like phenotype, despite residing in a dystrophic-like stiffness. To regulate MSC morphology, we patterned fibronectin in shapes of varying aspect ratios but the common area on polyacrylamide substratums of known stiffness. MSCs spread to the patterns and localized their focal adhesions in a stiffness- and a shape-dependent manner. Using traction force microscopy, we found that strain energy from cell-generated forces scaled with stiffness, but decreased as a function of cell elongation with isotropic cell patterns producing the highest contractile energy in contrast to our hypothesis. Muscle-specific myosin heavy chain (mMHC), an indicator of early muscle differentiation, also was expressed in a stiffness- and an elongation-dependent manner. On muscle-like stiffness of 11 kPa, elongated cells, i.e. 10:1 and 15:1 patterns, expressed mMHC most strongly. In contrast to osteogenic-like matrices of 34 kPa, highest MHC expression corresponded to isotropic and circle shaped cells. These shape- and stiffness-dependent lineage changes with muscle markers and contractility-based observations suggest that muscle induction may be possible in non-permissive stiffer environments and could prove beneficial to treat fibrotic muscle diseases.

(192) Innovative approaches to study the effect of cell geometry on cell behavior reveala role for plasma membrane lipid dynamics

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Microcontact printing as an approach to produce functionalized surfaces that can confine cells into specific sizes and shapes was introduced over a decade ago (Chen 1997). Since then, this technique has been used in numerous studies and has revealed that cell size and geometry regulate many important physiological processes such as migration, apoptosis, proliferation and differentiation (Thery 2010). However, the downstream effects of cell geometry on cell signaling are still largely unknown. We hypothesized that events at the plasma membrane level may play a key role in the signal transduction of geometrical cues. In order to explore this idea, several cutting-edge imaging and analysis techniques were combined including Total Internal Reflection and Fluorescence-Lifetime Imaging Microscopy as well as high-resolution Raman Spectroscopy Mapping. Our results show a previously unknown dependence of membrane dynamics and lipid rafts formation on cell geometry in mesenchymal stem cells, which correlates with changes in cell contractility and cytoskeleton arrangement. The plasma membranes of round cells formed less lipid rafts and had different lipid compositions and dynamics comaped with cells confined into a square or triangular shape with exactly the same cell area but higher cell contractility. Next, we focused on protein kinase B (Akt), a key signaling molecule involved in cell survival and proliferation known to be regulated by its association with lipid rafts. We report for the first time that Akt protein expression and phosphorylation is regulated by changes in lipid rafts formation dictated by cell contractility. Triangular and square cells presented a significant increase in Akt activation and to nearly 2-fold increase in Akt abundance on the plasma membrane. Given the broad regulatory role of Akt, we believe that this novel mechanism of Akt activation might be involved in many key processes from cell differentiation to cancer development and angiogenesis.

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(193) The interplay of duro- and hapto-taxis in regulating stem cell state

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Anchorage-dependent cells can sense and respond to the extracellular matrix (ECM) properties, e.g. stiffness and ligand density, but gradients of these cues are often found in vivo via normal tissue variation or pathological conditions, such as the post-infarct myocardial scar which is several fold stiffer as well as compositionally different from healthy tissue. We have previously shown that mesenchymal stem cell (MSC) migration and differentiation are regulated by 2D substratum stiffness in vitro, but stiffness directed differentiation, i.e. durotaxis, may be additionally accompanied by haptotactic ligand gradients as a result of increased localized matrix secretion. To better understand how corresponding and opposing gradients regulate MSC behavior, tunable gradients of stiffness and RGD surface concentration were constructed using density gradient multilayer polymerization, a technique that utilizes phase separation between liquids of varying density to create layers of distinct composition. Stiffness gradients were obtained by applying a UV photomask in order to activate a photoinitiator gradient. Ligand gradients were obtained by varying the concentration of acrylated-PEG-RGD in each hydrogel layer. Both gradients were overlayed to investigate the effect of corresponding and opposing haptotactic and durotactic gradients on MSC behavior. These data indicate that MSC migration and subsequent differentiation can be regulated by a variety of ECM stimuli in addition to growth factor-mediated pathways.

(194) Extracellular Matrix Hydrogels Prepared from Porcine Dermis and UrinaryBladder: Structure, Mechanics and In Vivo Remodeling

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Hydrogels prepared from the extracellular matrix (ECM) of decellularized tissues have the potential advantage of combining the favorable remodeling characteristics of ECM scaffolds with the ease of injectable delivery. The ECM of every tissue possesses a unique composition that may affect the physical and biological properties of an ECM hydrogel. Differences in native ECM composition may be compounded by the necessary variation in processing methods required to adequately decellularize each tissue. The present study characterized the structure, mechanics, in vitro cell behavior and in vivo remodeling outcome of hydrogels prepared from porcine dermal ECM (D-ECM) and urinary bladder matrix (UBM) scaffolds. Both ECM scaffolds were enzymatically solubilized with pepsin and then brought to physiologic conditions to induce gelation. ECM hydrogel structure consisted of randomly oriented fibers, with a smaller pore size for DECM hydrogels comaped with UBM. Rheological analysis showed that D-ECM hydrogels had a greater storage modulus and formed more quickly than UBM. D-ECM hydrogels had a greater fraction of soluble collagen, but a lower fraction of sulfated glycosaminoglycans than UBM. In vitro cell behavior also varied between ECM hydrogels as fibroblasts infiltrated further into UBM hydrogels and resulted in greater hydrogel contraction than D-ECM. In vivo, both D-ECM and UBM hydrogels were rapidly infiltrated by CD68+ macrophages in a rat skeletal muscle defect. Both hydrogels had degraded after 35 days and were replaced with islands of muscle cells expressing fast or slow myosin heavy chain. Myogenic quantification showed greater myogenesis for UBM hydrogels than

D-ECM, while both were greater comaped with unrepaired defects. In conclusion, D-ECM hydrogels were more mechanically robust than UBM, which corresponded to less in vitro cell infiltration and hydrogel contraction for D-ECM hydrogels. Though D-ECM hydrogels had superior mechanical characteristics in vitro, UBM hydrogels promoted greater myogenesis in a skeletal muscle defect indicating that there are inherent differences in their composition that affect ECM hydrogel remodeling.

(195) Temporally regulated mechanotransduction controls stem cell cardiomyogenesis

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As cells migrate and differentiate throughout development, they secrete and assemble the extracellular matrix (ECM), giving rise to time-dependent, tissue-specific stiffness, i.e. cardiac muscle originates from soft mesoderm, <500 Pa (Pascal; a unit of stiffness), and stiffens 10-fold as the myocardium matures. When mimicked in vitro with a hyaluronic acid (HA) hydrogel, myocardial matrix stiffening enhanced cardiac-specific gene expression and myofibril organization in immature, isolated pre-cardiac mesoderm. With less specified mouse embryonic stem cells (mESCs), mechanical cues alone were insufficient to induce cardiogenesis, but a combination of precisely-timed developmentally-relevant chemical cues (e.g. BMP4, Activin A and DKK-1) and the presentation of dynamic matrix mechanical cues at the cardiac mesoderm induction stage enhanced maturation of mESC-derived cardiomyocytes vs growth factors alone. While active mechanotransduction aided maturation, the specific proteins responsible for responding to time-dependent stiffness remain unknown. In order to assess matrix-mediated mechanotransduction across cells of varying myocardial commitment, we examined the expression and phosphorylation state of 800+ protein kinases, as well as the organization of mechanosensitive proteins, e.g. focal adhesion kinase (FAK) and vinculin, of pre-cardiac mesoderm or mESC-derived cardiomyocytes plated on matrices with either dynamic or static cardiac tissue-specific stiffness. Microarray analysis of protein kinases showed differential expression as a function of mechanics, confirmed by ratiometric western blotting. Many focal adhesion proteins exhibited time-dependent up-regulation on dynamic vs static matrices, including CAS, FAK, Paxillin and Src. These data indicate that mechanically driven maturation is at least partially achieved via active mechanosensing at focal adhesions. Identifying mechanosensitive pathways that are active in cardiomyogenesis can lead to a better understanding of how stem cell differentiation and development are mediated by extracellular matrix properties.

(196) Magnetically attachable stencils: Non-destructive analysis of migration reveals a context-dependent contribution of the underlying matrix

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To accurately evaluate the contribution of an underlying substratum to cell motility in complex cellular environments, we developed a novel migration assay using magnetically attachable stencils (MAts). When attached to a culture surface, MAts create a defined void in the cell monolayer without disrupting the cells or damaging the underlying substratum. Quantitative analysis of migration into this void reveals the substrate's contribution to migration. The magnetically guided placement of a microfabricated stencil allows for full experimental control of the substratum on which migration is analyzed. MAts enable the evaluation of any intact, defined matrix and make it possible to analyze migration on unique surfaces such as micropatterned proteins, nano-textured surfaces and pliable hydrogels. These studies reveal that mechanical disruption, including the damage that occurs during wound-healing assays, diminishes migration and confounds analysis of individual cell behavior. Analysis of migration on increasingly complex biomaterials reveals that the contribution of the underlying matrix depends not only on its molecular composition but also its organization and the context in which it is presented. Interestingly, using MAts to spatially and temporally separate matrix adhesion revealed a biological hysteresis not previously seen during cell migration.

(197) Kindlin-2 is a mechanosensitive regulator of the cardiac fibroblast differentiation

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Cardiac fibroblasts are able to respond to the mechanical stress and to differentiate into myofibroblasts. Myofibroblasts are highly contractile cells that are responsible for the tissue repair after the myocardial infarction. In a pathological situation, excessive differentiation of the cardiac fibroblasts results in cardiac fibrosis. Mechanical signals in fibrosis result from the local ECM stiffening. Integrin receptors are involved at the initial sensing step at the site of ECM binding. Integrin adaptors are the main candidates for an intracellular transmission of extracellular cues. Kindlin-2 is a recently characterized b1 and b3 integrins intracellular adaptor. Our findings suggest that cardiac fibroblast differentiation is controlled by kindlin-2 in a mechanosensitive way. We observed that kindlin-2 is highly expressed in cardiac fibroblasts. Immunlocalization demonstrated the accumulation of kindlin-2 in focal adhesions of the primary human cardiac fibroblast. Kindlin-2 is up-regulated during myofibroblast activation in conditions of experimentally induced rat heart fibrosis. Furthermore, we subjected primary human cardiac fibroblasts to different mechanical conditions that model the long-term and short-term mechanical stress that cardiac fibroblasts experience during the tissue repair and fibrosis. Kindlin-2 levels were increased in primary human cardiac fibroblasts cultured on fibrosis-stiff silicone substrata in compare with fibroblasts cultured on soft substratums; moreover, the levels of the kindlin-2 and differentiation marker αSMA were co-regulated. The short-term mechanical stimulation of the cardiac fibroblasts was performed by fibronectin-coated ferromagnetic microbeads or by stretching the cells on deformable silicone

membranes. It results in a portion of kindlin-2 translocating from the focal adhesions to the nucleus. Down-regulation kindlin-2 in primary cardiac fibroblasts affected the myofibroblast differentiation by reducing the levels of αSMA. Taken together, our observations show that mechanical stress controls the expression and localization of kindlin-2 in cardiac fibroblasts. Hence, kindlin-2 is novel mechanosensor within the myocardium that contributes to the myofibroblast differentiation.

(198) The Role of SDF-1α and ECM Crosstalk in Directing Neural Stem Cell Recruitment following Neural Injury

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Nearly 1.7 million U.S. citizens sustain a traumatic brain injury (TBI) annually, with more than 50,000 resulting in death. After TBI, pro-regenerative signaling coincides with the injury sequelae to recruit a variety of stem cells to the injury site. Of notable interest is the potential role of cross-talk between the injury-induced cytokine, stromal cell-derived factor 1α (SDF-1α), and the surrounding extracellular matrix (ECM) in directing endogenous neural stem/progenitor cell (NPSC) fate after injury. Therefore, the objective of this study was to investigate the influence of ECM-SDF-1α cross-talk on NPSC behavior. NPSCs obtained from the developing germinal eminence of E14.5 C57BL/6 mice were cultured in neurospheres and plated on ECM-coated plates (groups: poly-L-lysine, fibronectin, laminin, Matrigel; 6 or 0.5 μg/cm^2 vitronectin) with or without SDF-1α (1 μg/mL). Migration was tracked via phase contrast imaging for 4 days; statistical significance was analyzed using a two-way ANOVA with a Bonferroni post hoc test. Differentiation was assessed using immunocytochemistry for NPSCs (nestin), oligodendrocytes (O4), astrocytes (GFAP) and neurons (β III tubulin) then imaged with confocal microscopy (Leica TCS SP2; n = 6 per group). At 1 and 4 days post-plating, migration was significantly dependent on ECM substratum with laminin and Matrigel supporting the greatest migration regardless of SDF-1α supplementation. Although moderate levels of neuronal differentiation were observed in the basal medium on laminin and Matrigel, we observed two phenomena in the SDF-1α groups. First, neuronal differentiation was enhanced in the presence of SDF-1α. Second, newly differentiated neurons appeared to extend further outside of the central spheroid in the presence of SDF-1α. SDF-1α appears to play a critical role in guiding the differentiation of NPSCs towards a neuronal phenotype and may also have an effect on the migration of newly differentiated neurons. Further analysis is underway to elucidate the relationship between SDF-1α, ECM, migration and differentiation.

(199) Matrix-based regulation of adipose-derived stem cell improves myotubeformation and reduces lineage plasticity

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ECM stiffness and cell contractility are potent regulators of stem cell differentiation, but complete myogenic differentiation of bone marrow-derived stem cells (BMSCs) has not been observed. A direct comparison with adipose-derived stem cells (ASCs) demonstrates that ASCs do not just reflect the qualitative stiffness sensitivity of BMSCs, they exceed BMSC myogenic capacity (40-fold higher myogenic marker expression), express the appropriate temporal sequence of muscle transcriptional regulators, and for the first time, show that 2% of ASCs can fuse and form myotubes on matrices that mimic skeletal muscle stiffness, e.g. 10 kPa; this is 10-fold higher than chemical induction. BMSCs have never been shown to fuse. ASC fusion rate can be enhanced 2-fold by increasing contractility via lysophosphatidic acid or by better aligning ASCs on the mechanically patterned matrix (alternating neurogenic and myogenic stiffness) that mimics spatial stiffness distributions in innervated skeletal muscle. The ASC fusion rate can also be suppressed via blebbistatin or siRNA for α 5 or V integrin to block ASC mechanosensing. Successful stem cell-based therapies will require acclimating cells to the abnormally stiff ECM of muscular dystrophy while inducing and/or maintaining myogenesis, fusion and dystrophin delivery; the most promising observation about ASCs fused via matrix stiffness is that fused ASC-derived myotubes do not exhibit lineage plasticity when replated onto matrix mimicking a dystrophic muscle, which has been a significant impediment to the successful translation of adult stem cells in muscle diseases. Our efforts to develop tissue-engineered musculoskeletal systems hinge on the understanding gained here about the extracellular niche, so that we may overcome fibrosis and regenerate muscle using injected ASC-derived muscle.

(200) Adhesive heterogeneity within the stem cell niche promotes differentiation

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Most cell culture environments are uniformly adhesive, but that does not match the heterogeneous adhesivity found in native ECM, such as a fibronectin-rich matrix in which many stem cells reside during differentiation. When examining the adhesivity of the fibronectin matrix, we noted that surface intra-ligand spacing ranged up to 30 μm with an average of 3 ± 1 μm. These adhesive regions were also finite in size, creating small ligand-rich areas. Using copolymer mixtures of fibronectin-binding [polyacrylic acid (PAA)-polystyrene (PS)] and non-binding components [polyethylene oxide (PEO)-PS], we created a 3-dimensional niche that could be tuned to be more or less adhesive and directly determine to what extend fibronectin adhesion mediates stem cell lineage expression. We first confirmed that our niche contained fibronectin- and non-fibronectin-containing domains, which were 0.1 μm^2 in size and spaced 0.6 ± 0.2 μm apart when the niche was composed of 75% PEO-PS and 25% PAA-PS. While spacing was 5-fold smaller in the engineered niche, spacing heterogeneity was similar at 33% deviation. Two stem cell sources, i.e. primary isolated human mesenchymal stem cells (hMSCs) and human embryonic stem cells differentiated into mesenchymal progenitors (hES-MP),

cultured on this niche expressed the most robust vinculin-containing adhesions comaped with niche with more or less fibronectin. qPCR microarray data indicated that these two cell sources underwent fibronectin-containing domain-dependent differentiation: hMSCs became adipogenic and hES-MPs became osteogenic absent specific chemical induction. Thus, adhesive domains mimicking native ECM induce stem cells to differentiate and should be used in future regenerative strategies.

(201) Combinatorial effects of RGD signaling and tunable matrix stiffness on the differentiation of human primary cells in a 3D environment

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It is well recognized that the topography, stiffness and organization of the extracellular matrix (ECM)-binding proteins can trigger intra-cellular signal cascades. However, our understanding of how ECM and cell-scaffold mechanics affect intrinsic and extrinsic signaling is poorly understood. To date, efforts to interpret the relation between mechanical cues and biological functions have primarily employed 2-D models. The extrapolation of findings from 2-D systems to in vivo situations is challenging as cells in vivo experience a hierarchical 3-D environment. We recently developed a novel family of injectable hydrogel whose shear modulus can be precisely varied to match tissues ranging for neuronal to cancellous bone. By modifying the polymer backbone or gel environment with peptide motifs and proteins that interact with cells, the cross-talk between cell-receptor mediated cues and mechanical environment prescribed by the hydrogel can be investigated in a 3-D model. We have been studying the effect of RGD-mediated signaling in conjunction with matrix stiffness in the differentiation of stem cells and maintenance of phenotype, in the presence and the absence of exogenous stimulants. Our findings suggest that matrix stiffness and receptor mediated signaling can manifest as synergistic and antithetical signals depending on the cell type. Using this strategy the criteria and conditions for the differentiation and organization of endothelial cells and chondrocytes have been elucidated.

(202) The basement membrane of hair follicle stem cells is specialized with EGFL6, which interacts with a unique sensory nerve complex, the piloneural complex

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A special sensory nerve complex, the piloneural complex, is localized at the upper bulge of mouse hair follicles where hair follicle stem cells reside. This neural connection causes hair follicles to be more than simple skin appendages, but rather unique sensory organs. In addition, the piloneural complex is involved in upper bulge hair follicle stem cell maintenance. Although this precise neural patterning seems essential for proper sensory functions and stem cell regulation in the bulge, the underlying mechanisms are entirely unknown. We found that the upper bulge basement membrane is specialized with EGFL6, a family member of nephronectin. High-resolution 3D imaging of hair follicles revealed that EGFL6 is deposited as longitudinal parallel stripes at 2 μm regular intervals. These EGFL6 stripes interdigitate with longitudinal parallel axons of the piloneural complex, which also show 2 μm regular intervals. The axons do not interact with EGFL6 directly. Instead, non-myelinating Schwann cells surrounding the axon endings interact with the EGFL6 stripes. We are currently investigating the role of EGFL6 in mediating the cross-talk between hair follicle stem cells and the piloneural complex, using several in vitro assay systems and gene-targeting mouse models.

(203) Comparison of Human Pluripotent Stem Cells Cultured on Matrigel. And Recombinant Human Vitronectin using TeSR.-E8. low-Protein Medium

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TeSR-E8 has recently been introduced as a simplified, low-protein version of the commercial media formulations mTeSR™1 and TeSR™2 for human pluripotent stem cell (hPSC) maintenance. This medium was developed by STEMCELL Technologies based on the formulation known as E8, which was recently reported by the Thomson lab (Chen et al., 2011). E8 was developed via the pairwise removal of extraneous additives from the TeSR. core media formulation, resulting in a low-protein medium with a minimum set of components. We tested the performance of TeSR-E8 with two different substratums, either Matrigel. or recombinant human Vitronectin (rhVitronectin) using multiple hPSC lines; our results showed that we could routinely achieve a high expansion of H9 hPSC in TeSR-E8 with either Matrigel. (8 ± 2-fold; mean ± SD, $n = 9$ passages) or rhVitronectin (11 ± 3-fold; $n = 9$ passages), and similar results were obtained with other hPSC lines. To enable the success of achieving long-term cultures reproducibly, it was critical that key steps in the passaging protocol were tightly regulated. For example, improper handling techniques such as incomplete dissociation or low plating density during passaging resulted in poor plating efficiencies and/or increased differentiation. We also investigated the effects of transitioning hPSCs routinely cultured in TeSR-E8 on Matrigel. to the alternate rhVitronectin substratum, by assessing pluripotency and cell expansion rates obtained for the passages immediately following transition. We found that there was little change in cell expansion rates upon transitioning hPSC from Matrigel. to rhVitronectin, although spontaneous differentiation was observed to be slightly higher in the passages immediately following transition. As a preliminary assessment of cell function, embryoid bodies were successfully generated from cells grown on both substratums. Overall, our findings demonstrate that TeSR-E8, a low-protein formulation, offers an effective alternate to TeSR. media when used with rhVitronectin or Matrigel. substratums.

(204) 3-O-sulfated heparan sulfate increases epithelial progenitor cell proliferation

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During organogenesis a rapid expansion of the epithelial progenitor pool is required for growth and morphogenesis. Kit and Fgfr2b signaling maintain and expand the progenitor pool in branching epithelial organs. Elucidating the cellular mechanisms that induce rapid expansion of epithelial progenitors is crucial to understand organogenesis and to expand progenitors for regeneration. Since heparan sulfate (HS) is required for Fgfr2b function we hypothesized that specific HS synthesized by Kit + progenitors may control their expansion. Kit + epithelial cells were isolated from fetal salivary glands and the HS biosynthetic enzymes were analyzed. Surprisingly, the enzymes that generate 3-*O*-sulfated heparan sulfate (3-*O*-HS) were specifically and highly expressed in Kit + progenitors and Fgfr2b-signaling rapidly increases their expression. Using recombinant enzymes to specifically modify HS we show that 3-*O*-HS increases Fgfr2b signaling and the number of Kit + progenitors. Alternatively, reducing Kit signaling decreases 3-O-sulfotransferase expression and organogenesis. Thus 3-*O* sulfated HS increases Fgfr2b and Kit signaling that feeds back to increase HS biosynthesis providing a rapid response mechanism to modify HS structures and control progenitor proliferation in response to a growth factor. The identification of specific HS structures that control localized progenitor cell proliferation will be useful to expand progenitor cells for use in regenerative therapy.

(205) Therapeutic angiogenesis of 3D stem cell cluster formed on FGF-immobilized substratum

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Stem cells are one of the most powerful tools in regeneration medicine. However, many limitations remain regarding the use of stem cells in clinical applications, including poor cell survival and low treatment efficiency. Here, we describe an innovative 3D cell mass (3DCM) culture based on cell adhesion [basic fibroblast growth factor (bFGF)-immobilized substratum] and evaluate the therapeutic efficacy of 3DCMs composed of human adipose-derived stem cells (hASCs) in a mouse model of ischemic limb. For a formation of 3DCM, hASCs were split and seeded on MBP-bFGF-coated 24-well plates. The expression profiles of angiogenesis-related proteins were assessed by human Angiogenesis Array Kit. 3DCMs were characterized by RT-PCR, western blot analysis, immunofluorescence staining and FACS analysis. To study the direct influence of hypoxia on vascular endothelial growth factor (VEGF) expression in 3DCMs, HIF-1α mRNA-depleted hASCs were prepared by HIF-1α siRNA transfection. Angiogenic growth factor secretion and vasculogenesis from transplanted 3DCMs were confirmed by immunofluorescence staining. The 3DCMs released various angiogenic factors (e.g. VEGF, interleukin-8, bFGF) and differentiated into endothelial and smooth muscle cells within 3 days in normal medium. HIF-1α and VEGF expression was remarkably reduced in HIF-1α-depleted hASCs compared with their expression in cells transfected with control siRNA. Moreover, human endothelial cell marker expression appeared to be down-regulated in HIF-1α siRNA-transfected cells comaped with control siRNA-transfected cells. Finally, the 3DCMs formed mature vasculature and provided better treatment efficiency than monolayer-cultured hASCs in a mouse hindlimb ischemia model as well as in a gel-assisted vasculogenesis assay. This study demonstrates that 3DCM culture promotes the efficient vascular differentiation of stem cells and 3DCM transplantation results in vascular regeneration through the direct therapeutic effects of the injected cells. This is the first study to show the direct formation of vasculature by mesenchymal stem cells in a clinical model.

(206) Extracellular Matrix Dynamics of 3D Pluripotent Stem Cell Morphogenic Environments

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Pluripotent stem cells (PSCs) can differentiate in suspension as 3D multicellular aggregates referred to as embryoid bodies (EBs), which mimic aspects of embryonic development including the formation of complex extracellular matrices (ECM). Differentiation of EBs can be modulated by various culture parameters (e.g. soluble media, pO_2, hydrodynamics) and EBs offer some unique advantages over 2D culture methods, including the ability to recapitulate embryonic morphogenesis and the native structure of developing tissues. Thus, our objective was to evaluate endogenous ECM expression within 3D PSC aggregates undergoing differentiation using well-defined culture environments. Homogeneous populations of EBs were formed in PDMS microwells (1000 ESCs/EB) and cultured for up to several weeks in suspension culture in different soluble media (+/− serum or +BMP-4), oxygen concentrations (3 or 20%) and/or hydrodynamic conditions (static or rotary orbital culture). At different stages of EB differentiation, gene expression analysis was performed for ECM and growth factor (GF) genes, histological and immunostaining analyses were conducted for specific ECM proteins and glycosaminoglycans (GAGs), and ELISAs were performed to quantify the amounts of specific growth factors. In general, pluripotent GF expression decreased prior to the onset of ECM expression and GAG accumulation within EBs during the first 7–10 days of differentiation. Hyaluronan was among the earliest ECM molecules detected in within EBs and found to be most abundant in regions where mesenchymal populations of cells first appeared. Significant changes in many GFs and ECM proteins were observed by removing serum from the culture media, as well as by hypoxic culture conditions and the introduction of hydrodynamic forces created by rotary orbital culture. Overall, these results indicate that the ECM composition of 3D PSC microenvironments can be modulated by different environmental culture parameters and suggest how endogenous ECM dynamics may be used to direct PSC differentiation to specific lineages.

(207) Changes in Glycan-related Gene Transcripts Following Human Embryonic StemCell Differentiation into Cell Types Derived from Ectoderm, Mesoderm or EndodermLineages

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During vertebrate development, glycan structures on secreted and cell surface glycoconjugates are known to be highly regulated in abundance and are believed to play many important roles including modulation of cell signaling, adhesion, and pattern formation. In order to model and probe these glycan structural changes in a developmental context, we are monitoring the levels of transcripts encoding all glycan-related genes (>800 target genes) in various stages of human embryonic stem cell (hESC) development to determine the extent to which the glycan structural changes can be predicted at the gene expression level. In the present study, we are using a quantitative real-time PCR (qRT-PCR) platform to determine changes in the steady-state transcript abundances for genes involved in the biosynthesis, catabolism and remodeling of glycoconjugates in undifferentiated, pluripotent hESCs and populations of these cells that have been differentiated into neural crest (NC), smooth muscle (SM) and pancreatic endoderm (PE). Each of these differentiated cell types arise from a different germ layer. NC is derived from ectoderm, SM from mesoderm and PE from definitive endoderm. Preliminary analysis of these differentiated cell lines revealed a significant number of transcripts that increase or decrease >10-fold compared to the undifferentiated hESC samples. The large changes in transcript abundances following differentiation seem to occur more often in genes encoding proteins involved in terminal sugar modification like sialyltransferase or galactosyltransferase family members. A statistical analysis on multiple biological replicates of each cell type will be used to determine significant changes between transcripts for undifferentiated and differentiated cell types and will be used to identify potential lineage specific differences. We previously analyzed Islet1+ hESCs, which are an intermediate differentiation stage, between undifferentiated and SM, in the mesoderm lineage. A comparison between hESC, Islet1+ and SM will provide insight into the progression of glycan-related gene expression during cellular development within a single lineage. Collectively, this data will be used to make predictions about changes in glycosylation during differentiation of hESCs, which will be compared to glycan structural profiles. (Supported by NIH grants RR018502 and GM103490)

(208) The role of α2,3-6-linked NeuAc and galactose binding protein in iPS-MBMC pluripotence and differentiation

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Induced pluripotent stem cells (iPSs) are somatic cells that have been reprogramed to a pluripotent state via introduction of defined transcription factors. iPSs are a potential valuable resource for regenerative medicine, but whether iPS are identical to embryonic stem cells (ESCs) remains unclear. Cell surface glycans have been used to characterize human ESCs (hESCs) and monitor their differentiation. It was recently demonstrated that the hESC changes their glycophenotype when they differentiated in neural precursor, presenting β-Gal as terminal saccharide on cell surface. The presence of high levels of β-Gal in differentiated hESCs raises the question whether this phenomenon occurs only as a result of differentiation or the appearance of these saccharide epitopes are directly involved in this process. In this study, we manipulate the presence of α2,3 and α2,6 neuraminic acid on the surface of hESCs and iPSs from menstrual blood-derived mesenchymal cells. By using specific neuraminidase we observed that the remotion of sialic acid promotes changes in cell morphology, induces cell detachment from colony and enhances expression of genes related to ectoderm differentiation. In agreement, we demonstrated by immunofluorescence that cells that became PNA positive co-localizes with ectoderm marker β-3 tubulin but not with mesoderm or endoderm markers. Further flowcytometry analysis demonstrated that iPSs and hESCs present galactose binding molecules rather than α2,3- or α2,6-sialyllactose. Thus, emerging terminal Gal epitopes could be involved in the differentiation of iPSs and H9 cells to ectoderm precursor through the attachment of Gal-binding proteins. Together, our results show for the first time strong evidences that the presence of sialic acid is crucial to maintain embryonic cell pluripotency and therefore that modulation of sialic acid biosynthesis and its addition to glycoconjugates emerge as a mechanism that may regulates stem cell differentiation.

(209) Fibrillin-1 assemblies regulate mesenchymal stem cell performance

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Fibrillin-1 assemblies calibrate TGFβ and BMP bioavailability thereby regulating multiple stages of bone formation, from mesenchymal stem cell (MSC) lineage determination to extracellular matrix mineralization. Abnormally high TGFβ and BMP signaling causes bone loss in 3-month-old Fbn1 mutant mice by increasing bone resorption through up-regulation of RANKL expression in osteoblasts. Additionally, calvarial osteoblasts deficient for fibrillin-1 or Fbn1-silenced osteoprogenitor cell lines differentiated more rapidly and yield more minerals nodules than the wild-type (WT) counterparts. To refine the onset of the osteoblast defect, Fbn1 was inactivated in skeletal progenitor cells or pre-osteoblasts precursor by crossing $Fbn1^{Lox/-}$mice with Prx1-Cre or Osx-Cre transgenic mice respectively. Whereas bone mass was reduced to the same extent in 3--month-old $Fbn1^{Prx-/-}$ and $Fbn1^{Osx-/-}$ mice, bone loss was further diminished only in 6-month-old $Fbn1^{Prx-/-}$ mice. To assess whether an additional cell abnormality characterizes older $Fbn1^{Prx-/-}$ mice, colony forming unit-fibroblast (CFU-F) efficiency assays were performed on bone marrow-derived MSCs of 3- and 6-month-old WT, $Fbn1^{Prx-/-}$ and $Fbn1^{Osx-/-}$ mice. While CFU-F numbers were comparable between 3- and 6-month-old WT and $Fbn1^{Osx-/-}$ samples, those of 3- and 6-month-old $Fbn1^{Prx-/-}$ MSCs were respectively more

and fewer than WT. Hence, premature depletion of osteoprogenitor cells exacerbates osteoclast-driven osteopenia in ageing Fbn1 mutant mice. Substantial expansion of the hematopoietic compartment together with bone fat depletion further suggested that fibrillin-1 might be a component of marrow niche that couples MSC and HSC differentiation, a notion further supported by the finding of Fbn1 expression in and fibrillin-1 deposition around Nestin$^+$ niche cells.

(210) Stem cell-derived matrix is necessary to switch from a pluripotent to lineage-specified state

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While differentiation methods often rely exclusively on growth factors to direct mouse embryonic stem cell (mESC) fate, the ESC niche also contains the fibrillar extracellular matrix (ECM) proteins, including fibronectin (FN), collagens and laminins. Many soluble factors used in ESC differentiation are known to increase ECM expression, e.g. activin A and FN, yet the ECM's ability to direct ESC fate alone is not well understood and likely occurs at points during development. We examined whether ECM proteins were necessary and/or sufficient to direct mESC differentiation. mESCs, grown for 6 days as embryoid bodies under differentiating conditions in the absence of serum FN, maintained expression of pluripotency markers and decreased expression of lineage differentiation markers. Embryoid bodies also showed a spatiotemporal correlation between expression of FN and GATA4, a marker for differentiation and an inverse correlation between FN and Nanog, a pluripotency marker. Maintenance of mESC pluripotency with leukemia inhibitory factor (LIF) inhibited fibrillar matrix production, but in inductive conditions on gelatin, mESCs created a fibrillar ECM containing FN and laminin. The mESC-derived matrix is unlike that derived from conventional fibroblasts, which create ECM lacking laminin. Careful analysis of the mESC-derived matrix, however, indicated lineage-specific differences that perhaps help to drive differentiation in vivo: endoderm growth factors activin A and Wnt3a causes mESCs to produce more ECM with a slightly different composition from mesodermal conditions using BMP4. Naïve mESCs cultured on the mESC endoderm-derived matrix surprisingly showed enhanced differentiation into neural progenitors rather than endoderm, but when cultured with endodermal growth factors, endoderm fate was preferred; these data indicate that some lineages require matrix and soluble cues, whereas the matrix is sufficient for others. When taken together, these data imply that FN is necessary for mESC differentiation and that a fibrillar ECM can enhance and/or direct differentiation.

(211) Identification of the fucosylated glycoproteins potentially involved in pluripotency regulation using lectin pulldown-coupled proteomic analysis

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We have identified the fucose-specific lectin UEA-I as the pluripotency-associated lectin that can be used to effectively identify and isolate viable human pluripotent stem cells (hPSCs). Although it is known that the binding activity of UEA-I to hPSCs likely results from the expression of certain fucosylated glycoproteins, the identities of these glycoproteins and their potential functions involved in the regulation of pluripotency in human cells remain to be defined.

Using UEA-I and antibodies targeting pluripotency-associated antigens, we monitored the dynamic changes of fucosylation marks and cellular pluripotency in cells during reprograming and differentiation. Among four cell surface biomarkers for pluripotency that we tested, UEA-I was the first one showing in the somatic cells which were reprogramed to acquire pluripotency. Despite a rapid down-regulation of POU5F1 at the early stage of directed neuronal differentiation in human embryonic stem cells (hESCs), the cells retained UEA-I reactivity and slowly lost it throughout the differentiation. These results suggest the fucosylation marks recognized by UEA-I in hPSCs may be functionally important for cells to establish and sustain pluripotency.

Using UEA-I pulldown-coupled protemoics analysis, we identified 37 hydrophobic proteins that are potentially fucosylated and commonly present in hESCs and iPS cells (iPSCs). Immunoprecipitation were used to isolate the identified proteins from hPSCs and somatic cells to confirm the fucosylation of these proteins using UEA-I-mediated blotting. Among the 37 hits, 14 proteins were suggested as membrane-associated proteins by gene ontology analysis. Several of these proteins have been related to the regulation of many physiological events, including cell cycle, cell differentiation and embryonic development.

Our results show that protein fucosylation recognized by UEA-I commonly occurs in hESCs and iPSCs and suggest that this particular type of protein glycosylation may have functional significance in the regulation of cellular pluripotency.

(212) Soluble Thy-1 promotes migration of BM-MSCs into fibrotic lungs ex vivo

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Bone marrow (BM)-derived mesenchymal stem cells (MSCs) participate in tissue remodeling in idiopathic pulmonary fibrosis (IPF); however, mechanisms and factors that determine the homing of MSCs remain unclear. Thy-1 is a cell surface glycoprotein which is implicated in the extravasation of neutrophils and other myeloid cells; we previously demonstrated that soluble Thy-1 (sThy-1) is antifibrotic in vivo and in vitro via interaction with β_5 intergin. We hypothesized that sThy-1 could facilitate the recruitment of MSCs to fibrotic tissue. To this end, precision-cut lung slices (PCLS, 200 mm) were processed from αSMA-RFP mouse lungs on day 7 after orotracheal bleomycin (BL, 4 U/kg)

instillation using a microsprayer. A transwell migration assay was performed with PCLS on the upper surface of 24-well transwell inserts (polycarbonate membrane with 8 μm pores) and MSCs (2×10^4/well, traced with CellTrace^{TM}CFSE, 5 μM/ml, green) seeded on the lower surface, with rhThy-1 (10, 100 and 1000 ng/ml) added to the upper chamber. At 8 h culture following set-up, PCLS were fixed with 10% formalin and 30% sucrose OCT embedding/cryosectioning for microscopy examination. With this ex vivo model, we have found that sThy-1 significantly enhances the transmigration of MSCs into fibrotic tissue in a dose-dependent manner. Most MSCs which migrated into lungs were localized in vasculature, with some incorporated with damaged epithelia and myofibroblasts in fibroblastic foci. Blocking rhThy-1 by neutralizing antibody fully prevented, while either anti-integrin β_5 (1000 ng/mL) or anti-syndecan4 (1000 ng/mL) partially abolished, rhThy-1induced MSCs migration. These data indicate that sThy-1 facilitates MSCs migration via interaction with both integrin and syndecan4 thus promoting MSCs homing in the remodeling phase of lung fibrosis (supported by NIH #HL082818).

(213) Heterotrimerization domain of collagen IX determines chain stagger in triple helix

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Precise mapping and unraveling a mechanism of interaction or degradation of a certain type of collagen triple helix requires a generation of a short and stable collagenous fragment. It is a great challenge especially for a class of heterotrimeric collagens, where chain composition and register (stagger) are important factors. To date, no such system has been reported that can be efficiently used to generate a natural collagenous fragment with exact chain composition and determined stagger. Recently, we demonstrated that the NC2 domain (only 35–50 residues) of FACIT collagens is a potent trimerization domain. Moreover, the NC2 domain of collagen IX provides an efficient selection and heterotrimerization of three distinct chains. Now, we explored the ability of the NC2 domain of collagen IX to determine the chain register within the triple helix. We generated three possible sequence combinations (α1α1α2, α1α2α1, α2α1α1) of a type I collagen fragment attached to the NC2 domain. The fragment of interest is the binding region for the von Willebrand factor A3 domain. In addition, two control combinations were produced, homotrimers of (α1) or (α2). For heterotrimeric constructs, α1α1α2 demonstrated a higher melting temperature than the other two. Binding experiments with the von Willebrand factor A3 domain revealed the homotrimer of (α1) as the strongest binding construct, whereas the homotrimer of (α2)showed no binding. For heterotrimers, α1α1α2 was found to be the strongest binding construct. Differences in thermal stability and binding to the A3 domain unambiguously demonstrate that the NC2 domain of collagen IX determines not only the chain composition but also the chain register in the adjacent triple helix. The system we developed is unique and directly applicable for producing any natural triple helical fragment of any collagen type or developing toolkits of heterotrimeric collagens, such as types I, IV, V, VI, IX and XI.

(214) Modulation of MUC1-associated sialyl Lewis a in normal prostatic RWPE-1 cells by epigenetic regulation of the B3GALT1 gene

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Sialyl Lewis a (sLe^a) and sialyl Lewis x (sLe^x) are selectin ligands involved in leukocyte trafficking and frequently found in metastatic cancer cells. Biosynthesis of these selectin ligands occurs by the sequential action of several glycosyltransferases in the Golgi apparatus. Previously, we reported that the synthesis of MUC1-associated sLe^x was regulated by DNA methylation of the α2-3 sialyltransferase 6 (*ST3GAL6*) gene (Int J Biochem Cell Biol. 43: 586, 2011). In this study, we examine a possible epigenetic regulation of sLe^a by treating human prostatic cells including immortalized normal cells and prostatic cancer cells with several histone deacetylase inhibitors, including valproic acid, tricostatin A and suberoylanilide hydroxamic acid (SAHA). We found that SAHA was the only one that enhanced the production of sLe^a in normal prostatic cells. Employing siRNA technology and co-immunoprecitation, we found that the sLe^a was decorated on MUC1, and up-regulation of *B3GALT1* gene was responsible for the production of sLe^a. We further showed that acetylation of histone-3 and histone-4 was up-regulated by SAHA treatment, suggesting their involvement in the activation of *B3GALT1* gene. We conclude that SAHA treatment increases MUC1-associated sLe^a by stimulating the *B3GALT1* gene via histone modification. SAHA induction of this metastasis-promoting selectin ligand in normal prostatic cells should be considered a potentially serious side effect of this drug recently approved by the US Food and Drug Administration. (The work is supported by grants from VA Merit Award 1I1BX000985, NIH 1R21HL097238 and Nebraska LB506.)

(215) Distinct Functions for Bromide and Chloride During Basement Membrane Assembly

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Collagen IV networks are critical to the stability of basement membranes. These networks contain sulfilimine bonds that covalently cross-link the NC1 interfaces of adjoining collagen IV protomers. Bond formation occurs within basement membranes as the enzymatic product of peroxidasin with the mechanism utilizing hypohalous acids as chemical intermediates. Within this reaction, we have now discovered that bromide and chloride have distinct roles in the formation of collagen IV networks. Mechanistic studies in the matrix demonstrated that peroxidasin forms sulflimine bonds via its halogenation cycle, oxidizing bromide ions into hypobromous acid which then catalyze bond formation. In contrast to the role of bromide during bond formation, chloride has emerged as a critical element during the process of assembling

NC1 domains into a suitable substratum for peroxidasin. Altogether, the reaction hinges on the sequential action of both halides. Functionally, the bonds were experimentally shown to reinforce the NC1 hexamer substratum. Thus, sulfilimine bonds are key structural elements of collagen IV networks, forming through the separate and coordinated activity of bromide and chloride ions. This advancement elevates the field of basement membrane biology by highlighting the unique extracellular enzymology and chemistry occurring within matrices.

(216) Shedding of GPI-anchored proteins by a novel GPI-cleaving enzyme

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Glycosylphosphatidylinositol (GPI) anchoring of proteins is a conserved post-translational modification in eukaryotes. It has been suggested that GPI anchors act as functional molecules regulating trafficking and membrane localization of the modified proteins. One of the characteristic features of GPI-anchored proteins is that these proteins are releasable from the cell membrane by cleaving GPI moieties. There are several GPI-cleaving enzymes. Here, we identified and characterized a novel GPI-cleaving enzyme. We previously reported that PGAP3 is required for the removal of an unsaturated fatty acid from GPI lipid at the Golgi apparatus, which is essential for GPI fatty acid remodeling. PGAP3 belongs to a transmembrane hydrolase superfamily. By homology to PGAP3, an uncharacterized protein named PGAP6 was found in the superfamily. PGAP6 is a transmembrane protein conserved among metazoa and was mainly localized at the cell surface. In PGAP6 overexpressing cells, surface expressions of several GPI-anchored proteins including CD55, CD59 and Sca-1 were significantly decreased. These results suggest that PGAP6 is a novel GPI-cleaving enzyme at the cell surface. We are investigating its biochemical and physiological functions.

(217) O-glycosylation in Spodoptera frugiperda (Sf9) and Trichoplusia ni (Hi-5) insect celllines is complex and include abundant hexuronic acid (Sf9 and Hi-5) and *O*-linked phosphocholine (Sf9)

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We have investigated the *O*-glycome of mucin-type proteins produced in *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (Hi-5) insect cell lines, which are commonly used for recombinant protein production with the baculovirus expression system. The P-selectin glycoprotein ligand-1/mouse IgG_{2b} (PSGL-1/mIgG_{2b}) fusion protein was secreted to the culture medium and purified with affinity chromatography and gelfiltration. Reduced *O*-glycans released from the fusion protein was analyzed by liquid chromatography mass spectrometry (LC-MS^2). The *O*-glycomes of both cell lines are complex and diverse; a result in contrast to previous investigations that have reported a simple glycosylation pattern. Three GalNAcol core structures, Galβ3GalNAcol, HexA-GalNAcol and HexA-(Fuc-)GalNAcol were found in both cell lines, while a single Fucitol core structure (HexNAc-HexA-Fucitol) was found in Hi-5. Hexuronic acid and terminal HexNAc was found in almost all extended structures (Sf9 and Hi-5). *O*-linked glucuronic acid has previously been reported in *Drosophila melanogaster* embryos, but not in Sf9 and Hi-5. The cell lines produce *O*-glycans substituted with sulfate (Sf9 and Hi-5) and phosphocholine (Sf9). To our knowledge this is the first report of phosphocholine (PC)-substituted *O*-glycans, confirmed with positive and negative mode LC-MS^2 and western blot. In Hi-5, a family of large structures containing a variable number of $(HexNAc\text{-}HexA\text{-}Hex)_n$ repeats, +/− sulfate, were found. No evidence of sialic acid was detected by LC-MS, but low amounts of DMB (1,2-diamino-4,5-methylenedioxybenzene) labeled sialic acid was detected with reverse phase high-performance liquid chromatography (RP-HPLC). The amount was at least 2–3 orders of magnitude lower than any of the other monosaccharides detected, suggesting a limited or absent expression of sialic acid in Sf9 and Hi-5.

(218) Comprehensive Mass Sectrometric Mapping of the Alpha 1(V) Collagen Chain

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The type V collagen α1(V) chain is broadly distributed in $\alpha1(V)_2\alpha2(V)$ heterotrimers that are incorporated into and effect the geometry of type I collagen fibrils and is also found in less characterized $a1(V)_3$ homotrimers. Aberrant expression of either the α1(V) or α2(V) chain can underlie the connective tissue disorder classic Ehlers-Danlos syndrome, whereas autoimmune responses against the α1(V), but not the

α2(V), chain are linked to lung transplant rejection and atherosclerosis. The major collagenous COL1 domain of the α1(V) chain is more extensively post-translationally modified than are those of other fibrillar collagen chains. Such modifications comprise hydroxylated prolines and lysines, the latter of which can be glycosylated. These types of modifications can contribute to the epitopes underlying immune responses against collagenous antigens, and the extensive nature of such modifications may contribute to the unique biological properties of the α1(V) chain. We here use mass spectrometry of high resolution and mass accuracy to map such modifications in the bovine placental α1(V) triple helical COL1 domain and in human recombinant pro-α1(V) procollagen. Findings include the locations of post-translational modifications that vary or are invariant between the bovine tissue α1(V) and human recombinant pro-α1(V) chains, and which may aid in locating and characterizing α1(V) autoimmune epitopes and in providing further insights into col(V) function. Notably, an unexpectedly large number of hydroxyproline residues were mapped to the X positions of COL1 Gly-X-Y triplets, contrary to expectations based on previous amino acid analyses of hydrolyzed α1(V) chains from various tissues. This difference is best explained by the presence of a relatively large number of 3Hydroxyproline sites with less than 100% occupancy, suggesting a previously unknown

mechanism for the differential modification of α1(V) chain and type V collagen properties.

(219) Lysyl oxidase and transglutaminase-dependent collagen fibril formation in chicken embryonic cornea

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Mature cornea consists of orthogonally stacked lamellae formed by thin collagen fibrils evenly spaced in parallel. Lysyl oxidases (LOX) catalyze the deamination of specific (hydroxy)lysyl residues in collagen and elastin which results in aldehyde-derived covalent cross-links, effectively inhibited by ßaminopropio nitrile (ßAPN). Transglutaminases stabilize protein assemblies by γ-glutamyl-ε-lysine cross-links, irreversibly inhibited by a specific inhibitor for tissue transglutaminase (tTG). Here, we have studied the suprastructural organization of matrices deposited by keratocytes with and without cross-link formation.

Collagenous matrices were obtained from 3D-cultures of keratocytes from chicken embryonic cornea. The arrangement of the collagen fibrils and the fibril diameter distribution with and without cross-link formation were analyzed by transmission electron microscopy. LOX activity was inhibited by βAPN and tTG ac tivity was irreversibly inhibited by the peptide Boc-DON-Gln-Ile-Val-OMe. Moreover, keratocyte cultures were analyzed by immuno 14 fluorescence and metabolic labeled (C-prolin) for cross-link characterization.

Typical sheets of orthogonally arranged collagen fibrils were formed by keratocyte cell cultures without βAPN whereas such laminae were not apparent in cultures with βAPN. This suggests that the formation of cross-links is cru cial for tissue-specific matrix organization in chicken cornea. In contrast, the orthogonal arrangement of fibrils was not strongly effected in cultures with the tTG inhibitor. Interest ingly, the collagen fibrils formed were thicker with a clearly visible banding pattern. In the presence of activated tTG, fibrils reconstituted in-vitro from soluble collagens were thinner and formed networks that were absent in controls without tTG. We presume that the stabilization of aggregates formed early is essential for the formation of the typical matrix or ganization of chicken cornea.

The arrangement of collagen fibrils in 3D-cell cultures is very similar to that found in developing mammalian corneal stroma. Thus, LOX-derived cross-link formation ap pears as crucial factor for fibril formation and fibril arrangement into lamellae whereas tTG-derived cross-links are essential for fibril di ameter control.

(220) Disruption of B3gnt2 expression may not be achieved by gene-trapped mutant mice

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Poly-*N*-acetyllactosamine (polyLacNAc) carbohydrate backbone is one of the basic carbohydrate structures found on various glycoproteins, such as erythrocyte band 3 and corneal keratan sulfate proteoglycans. The carbohydrate chains are produced by a cooperative reaction of two distinct enzymes, ß1,4-galactosyltransferases and ß1,3-*N*-acetylglucosaminyltransferases and ß1,3-*N*-acetylglucosaminyltransferase-2 (ß3GnT2) is suggested to be the major ß1,3-*N*-acetylglucosaminyltransferase for polyLacNAc synthesis. Mutant mice having retroviral insertion on a gene for ß3GnT2 enzyme, *B3gnt2*, have analyzed for biological function of ß3GnT2 and polyLacNAc chain in vivo. However, genuine *B3gnt2* knockout mice have not been produced yet. To investigate biological function of polyLacNAc chain in mice, we obtained a knockout-first vector for *B3gnt2* from International Knockout Mouse Consortium and produced two mutant mouse lines, one is a gene trapped mutant mouse line and the other is a systemic gene knockout mouse line. Using bacterial β-galactosidase gene (*lacZ*) as a reporter gene, we observed localization of the cells that originally express *B3gnt2* in the mutant mice. We found that *lacZ* is expressed in various tissues such as brain, liver and kidney, in both mutant mice, indicating ubiquitous expression of *B3gnt2*. Interestingly, we observed that the expression level of the reporter gene is weaker in tissues of heterozygous gene trapped mutant mice ($B3gnt2^{+/trap}$) than heterozygous gene knockout mice ($B3gnt2^{+/-}$). We also found that *lacZ* expression pattern between $B3gnt2^{+/trap}$ and $B3gnt2^{+/-}$ is largely different in some tissues such as the brain, suggesting the presence of multiple alternative splicing forms of *B3gnt2* mRNA. By RT–PCR, we detected mRNA containing an exogenous gene element originated from a gene trap cassette at 5' untranslated region of intact *B3gnt2* mRNA in $B3gnt2^{+/trap}$ mice. This result indicated that functional *B3gnt2* mRNA may be expressed from the gene-trapped mutant *B3gnt2* allele.

(221) Analysis of endopeptidase reactions involved in ERAD

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ER quality control (ERQC) is a protein surveillance system, which scrutinizes the folding status of newly synthesized proteins in the ER (Raasi and Wolf 2007). In this system, aberrant proteins, which fail to form a functional folding/complex, will be degraded by a degradation pathway called ER-associated degradation (ERAD). The cytoplasmic peptide:*N*-glycanase (PNGase) is a deglycosylating enzyme that is involved in the ERAD and releases *N*-glycans from misfolded glycoproteins. We have been analyzing the PNGase-dependent ERAD pathway in *Saccharomyces cerevisiae* (Hosomi et al. 2010). However, the intact de-*N*-glycosylated form was not detected for many *N*glycosylated ERAD substrata even when proteasomal activity was impaired, making us wonder how important the PNGase-mediated deglycosylation in ERAD process would be. CPY* (CPY-star) is a mutant of a vacuolar protease (carboxypeptidase Y) and has been extensively used as a model ERAD substrate glycoprotein in yeast. In a previous study, a deglycosylated form of CPY* was not detected, while delay in degradation was observed (SuZuki et al. 2000). In order to analyze the degradation intermediates of CPY*, two different tags on N and C terminus were added on

CPY* (FLAG-CPY*-HA). By anti-tag antibody, we successfully detected several degradation intermediates. Interestingly, a fragment was identified to be *N*-glycosylated in a strain without PNGase, while it was deglycosylated in the presence of this enzyme. This result clearly indicated that PNGase is also involved in degradation of CPY*, but an endoprotease activity prevented us from detecting the intact de-*N*-glycosylated form. In this presentation, we will report the analysis and characterization of novel endopeptidase reactions involved in ERAD process.

References

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(222) Evolutionary origins of (GPP)n 3-hydroxyproline formation in fibrillar collagen

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Approximately half of all proline residues in fibrillar collagen are hydroxylated. The predominant form is 4-hydroxyproline, which plays a functional role in triple helix folding and stability. A minor form of the modification, 3-hydroxyproline, is still without a clear function. Using peptide mass spectrometry, we have recently been able to reveal several previously unknown molecular sites of 3-hydroxyproline in fibrillar collagen. In fibril-forming A-clade collagen chains, four new 3-hydroxyproline sites were found [A2, A3, A4 and $(GPP)_n$] in addition to the A1 site at P986. The C-terminal $(GPP)_n$ motif can contain as many as five consecutive GPP triplets each with the potential for 3-hydroxylation. To assess, the evolutionary origins of the $(GPP)_n$ as a substratum, we surveyed the pattern of 3-hydroxyproline occupancy from early chordates through amphibians, birds and mammals. We examined multiple tissue sources for type I collagen (tendon, bone and skin) and type II collagen (cartilage and notochord) homologs. Type II collagen was included in our study as the known fibrillar collagen genes of pre-vertebrates (lamprey and sea quirts) have sequence features resembling *COL2A1*. It appeared from our mass spectral analysis of extant animal tissues that the $(GPP)_n$ as a substratum for 3-hydroxylation was peculiar to vertebrate fibrillar collagen. Indeed of the species tested, birds were evolutionarily the earliest to reveal 3-hydroxyproline in the $(GPP)_n$ motif with an average of one residue per $(GPP)_n$. In higher vertebrates (mouse, bovine and human), as many as five 3-hydroxyproline residues per $(GPP)_n$ motif were found, with an average of two residues per $(GPP)_n$ motif. Notably, in type I collagen from all species tested the modification exhibited clear tissue specificity, with 3-hydroxyproline occurring almost exclusively in tendon. The structural significance is unclear but the level of 3-hydroxylation seems to have increased as tendons evolved. Post-translational modifications, particularly cross-linking, are believed to be important in modulating the unique tissue-specific properties of type I collagens. The present results support a concept that 3-hydroxyproline residues contribute fundamentally to collagen structure and the diversification of connective tissues.

(223) Novel insights into diabetic bone complications

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Diabetes doubles the risk of bone fracture. Organic and inorganic components of the bone matrix determine bone strength, and poor quality of bone in diabetes is independent of bone mineral density. Studies indicated that in diabetes glycation of collagen, the most abundant protein in the bone matrix prompts the abnormal arrangement of collagen molecules leading to fragile bones. Moreover, diabetic bone osteopenia is attributed to lower enzymatic collagen cross-links. What remains unknown is whether diabetes down-regulates lysyl oxidase (LOX), which is made by bone forming cells (osteoblasts), consequently reducing enzymatic collagen cross-links. We used primary calvarial rodent osteoblasts to examine collagen and glycated collagen regulation of LOX. Our findings indicate that collagen up-regulates LOX in osteoblasts, while glycated collagen fails to induce LOX. To determine the mechanism of collagen up-regulation of LOX, we investigated roles for collagen receptors, namely integrins and discoidin domain-receptor 2 (DDR2). Inhibitor and knockdown studies suggest that collagen up-regulates LOX through DDR2, and independent of integrins. Additionally, we assessed diabetes regulation of LOX in a mouse calvarial bone-healing model. Our in vivo experiments show that diabetes up-regulates LOX mRNA, protein and enzyme activity in partially healed bone (day 7). To further examine factors resulting in up-regulation of LOX in diabetes, we performed histological analysis. Our observations after 7 and 14 days of bone healing suggest that hematomas, which form in the initial stage of bone healing, do not resolve in diabetic mice. Because hematomas are a rich source of growth factors, we suspect that unresolved hematomas in diabetic healing bone may promote cell proliferation at the expense of osteoblast differentiation and mineralization.

(224) Important but over looked: Silicon control of the extracellular matrix

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Alterations in the composition of and an imbalance in the metabolism of the extracellular matrix (ECM) in connective tissues (CTs) are associated with many pathological conditions such as impaired tissue regeneration, osteoporosis, arteriosclerosis and some types of cancers. Adequate nutrition that provides essential trace elements is crucial in maintaining ECM homeostasis, although this relationship is not fully understood. Silicon (Si), a trace element abundant in our diet, has been shown to be beneficial for maintaining CT health. However, despite 40 years of research since the first scientific evidence, the mechanism of action of Si on CTs still eludes researchers. Here, the effects of the biologically prevalent form of Si (orthosilicic acid) on ECM synthesis and stabilization were investigated in vitro. Experiments showed a significant effect of Si on the post-

translational maturation of collagen type I in human dermal fibroblast cells. We hypothesized that silicon modulates the aggregation of procollagen type I that is secreted into the cell culture media, regulating its self-assembly into collagen fibrils. Indeed, gene expression profile analysis of dermal fibroblasts treated with Si suggested that pathways related to collagen fibrillogenesis were affected. In addition, using in vitro wound healing assays, we showed that Si increased the proliferative rate of dermal fibroblast cells. In conclusion, these findings suggest that Si has a significant regulatory effect on the proliferation of dermal fibroblast cells and the maturation of collagen type I in their ECM. Therefore, Si may influence collagen type I fibril formation at the onset of the extracellular matrix development in connective tissues and or during tissue repair.

(225) Identifying bottlenecks in efforts to glycoengineer insect cells for humanized Nglycan processing

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Sf9 is an insect cell line that is widely used as a host for recombinant glycoprotein production by baculovirus expression vectors. We have isolated glycoengineered Sf9 cell derivatives that express mammalian glycosyltransferase genes and produce recombinant glycoproteins with humanized *N*glycans. However, we have not evaluated their efficiencies of humanized *N*-glycan processing. To begin this analysis, we examined the electrophoretic mobilities of HIS-tagged human erythropoietin (HIS-hEPO) expressed in Sf9 and several of its glycoengineered derivatives. We found that SfSWT-1 cells produced HIS-hEPO with the tightest banding pattern and slowest electrophoretic mobility, suggesting these cells provided the most efficient and extensive *N*-glycan processing. The fact that SfSWT-1 was the only cell line glycoengineered to express human β1,2-*N*-acetylglucosaminyltransferase I (MGAT1) further suggested that this enzyme might represent a bottleneck in our Sf9 glycoengineering effort. We tested this hypothesis by examining the mobility of HIS-hEPO produced by glycoengineered cell lines with or without MGAT1 co-expression and found that MGAT1 had little or no impact in any cell line tested. To examine other potential bottlenecks, we co-expressed HIS-hEPO with various combinations of late-acting glycosyltransferases, including MGAT1, MGAT2, B4GALT1, and ST6GAL1, in SfSWT-6 cells. These cells produce a high mobility form of HIS-hEPO, indicating they provide a low efficiency of humanized *N*-glycan processing. B4GALT1 had the most significant impact, suggesting this enzyme is a major bottleneck. This hypothesis was supported by the fact that co-expressing HIS-hEPO with each of several different engineered, hyperactive forms of B4GALT1 decreased its electrophoretic mobility. Furthermore, co-expression with the most hyperactive B4GALT1 increased the proportion of terminally galactosylated *N*-glycans on HIS-hEPO from 6% to 56%, as determined by MALDI-TOF/ MS. These results identify B4GALT1 as a major bottleneck in efforts to glycoengineer Sf9 cells and that it can be overcome using engineered, hyperactive forms of this enzyme.

(226) Disruption of Col2a1 pre-mRNA alternative splicing affects matrix assembly and post-natal bone development

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The aim of this study is to decipher the biological significance of the developmentally-regulated Col2a1 pre-mRNA splicing event in skeletogenesis. It is known that exon 2-containing mRNA isoforms (IIA) are generated by chondroprogenitor cells while differentiated chondrocytes synthesize mRNA isoforms devoid of exon 2 (IIB). To address why this splicing switch occurs, a novel *Col2a1* knock-in mouse model was generated that expresses only the IIA isoform throughout all stages of pre- and post-natal development. Surprisingly, mis-expression of the IIA collagen isoform is apparently well-tolerated since both heterozygous (ki/+) and homozygous (ki/ki) mice are viable with no overt phenotype. However, closer examination reveals significant phenotypic changes in the extracellular matrix of cartilage and trabecular bone in ki/+ and ki/ki mice when comaped with wild type. Specifically, the ultrastructure of the collagen fibrillar network in 1-month articular cartilage and trabecular bone shows a "denser" matrix containing thicker, abnormal-shaped fibrils and potential fibril fusions. Western blot analysis reveals that, although unusual, these fibrils are stabilized by cartilage-typic cross-links. In addition, micro-Ct analysis shows increased trabecular and cortical bone mass in ki/+ and ki/ki tissue at 4 months (more significantly in ki/+). Preliminary three-point bending tests reveals lower energy-to-fracture values for ki/+ 4-month femora, indicating a potential material defect that may be linked to the abnormal collagen fibrillar matrix. From the data acquired so far, we have developed a hypothesis that the reason for the abnormal fibrillar matrix and altered bone mass/quality in ki/+ and ki/ki tissue is due to the fact that type XI collagen is also altered in this mouse model. This is because the α 3 chain of type XI collagen and the α 1 chain of type II collagen are both encoded by the *Col2a1* gene. We therefore predict that the normal function of type XI collagen in regulating collagen fibrillogenesis is altered due to mis-expression of the IIA exon 2-encoded cysteine-rich globular domain in the amino propeptide of the α3 (XI) chain. Future studies will confirm this interesting concept and potentially reveal an unexplored role for type XI collagen in post-natal bone development.

(227) The *Neisseria meningitidis* serogroup X xcbA gene product catalyses synthesis of the bacterial capsular polysaccharide

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Neisseria meningitidis is one of the leading causes of bacterial meningitis. The *N. meningitidis* serogroups A, B, C, Y, W135 and more recently, X are responsible for most cases of disease. Currently available conjugate vaccines for serogroups A, C, Y and W135 have been shown to be safe and effective. Since there is no

vaccine against serogroup X and given its recent emergence as a cause of large outbreaks of disease, it is expected to be a major contributor to future epidemics. We expect that an understanding of the biosynthesis of the serogroup X capsular polysaccharide would provide useful tools for understanding the disease and for vaccine production. Two groups have predicted that the protein encoded by the serogroup X gene *xcbA*, is the glycosyltransferase responsible for synthesis of the capsular polysaccharide (a1®4)-linked *N*-acetyl-Dglucosamine -1-phosphate. We cloned and expressed *N. meningitidis xcbA* (NmX *xcbA*) in *E. coli* and determined its ability to catalyze formation of the capsular polysaccharide. Cytosolic preparations of NmX *xcbA* encoding *E. coli* had glycosyltransferase activity as determined by incorporation of radiolabel from UDP-[^{14}C]-GlcNAc into a polymer. This enzymatic activity was inhibited by tunicamycin in a dose-dependent manner. We detected capsular polysaccharide in membrane and cytosol fractions of NmX *xcbA* expressing *E. coli*, in ELISA assays using serogroup X capsule-specific antiserum. This demonstrates that the XcbA product is the serogroup X capsule polysaccharide and suggests that transfer of the NmX *xcbA* gene alone to *E.coli* is sufficient for serogroup X capsule polysaccharide production. Given that capsular polysaccharide extraction directly from *N. meningitidis* serogroup X tends to give low yields, *E.coli* may provide an attractive alternative for serogroup X capsule expression for vaccine production. Together these results demonstrate that NmX *xcbA* encodes the glycosyltransferase responsible for synthesis of the *N. meningitidis* group X capsular polysaccharide.

(228) A putative polypeptide *N*-acetylgalactosaminyltransferase/WBSCR17 regulates cell adhesion and endocytic pathways in HEK293T cells

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Mucin-type O-glycosylation is a common post-translational modification of membrane and secreted proteins and is important for various physiological activities such as protection of epithelium, cell adhesion and control of immune systems. The biosynthesis of mucin-type carbohydrates is initiated by a large family of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAc-T), which constitutes 20 isozymes in mammals. We previously identified one of the genes, which is designated Williams–Beuren syndrome chromosome region 17 (WBSCR17) as it is located in the chromosomal flanking region of the Williams–Beuren syndrome deletion. Recent genome-scale analysis of HEK293T cells treated with a high concentration of GlcNAc demonstrated that WBSCR17 is one of the up-regulated genes possibly involved in endocytosis (Lau et al. 2008). To elucidate its roles in more detail, we first biochemically characterized it and demonstrated that it was localized mainly in the Golgi apparatus in COS7 cells, as is the case for the other GalNAc-Ts. Assay of recombinant WBSCR17 expressed in insect cells showed very low activity toward typical mucinpeptide substratums. We then suppressed the expression of endogenous WBSCR17 in HEK293T cells using siRNAs and observed phenotypic changes of the knockdown cells with reduced lamellipodium formation, altered *O*-glycan profiles and unusual accumulation of glycoconjugates in the late endosomes/lysosomes. Analyses of endocytic pathways revealed that macropinocytosis, but neither clathrin-nor caveolin-dependent endocytosis, was elevated in the knockdown cells. The overexpression of recombinant WBSCR17 stimulated lamellipodium formation, altered O-glycosylation and inhibited macropinocytosis. WBSCR17 therefore plays important roles in lamellipodium formation and the regulation of macropinocytosis as well as lysosomes. Our data provide the first implication that WBSCR17 is involved in the control of the dynamic membrane trafficking through macropinocytosis, in response to the nutrient concentration as exemplified by environmental GlcNAc.

Reference

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(229) Use of experimentally controlled quaternary structure mutants to probe regulatorymodes of UDP-glucose dehydrogenase

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UDP-glucose Dehydrogenase (UGDH) catalyzes two successive oxidations of UDP-glucose to form UDP-glucuronate, a precursor to glycosaminoglycan and heparan sulfate production, steroid detoxification and hyaluronan biosynthesis. Targeted disruption of UGDH in model organisms leads to impaired heart valve development, and specific point mutations in the UGDH coding sequence are associated with congenital cardiac valve malformation in humans. In the current study, we recreated these clinically observed mutations, R141C and E416D and investigated their impact on UGDH quaternary structure, protein stability, and enzymatic activity in vitro. Characterization of the mutant recombinant enzymes purified from *E.coli* revealed modest alterations in the enzymatic activity of the mutants, and a significant reduction in the half-life of enzyme activity at 37°C. Furthermore, the quaternary structure of both mutants, normally hexameric, was destabilized to favor the dimeric species, and the intrinsic thermal stability of the R141C mutant was highly compromised. These observations suggest that the quaternary assembly of the enzyme is essential for optimal activity. To test this, we further investigated parameters influencing the assembly of the hexameric enzyme, which is typically observed as a trimer of dimers, by designing a series of point mutants whose quaternary structure could be experimentally controlled. Using available UGDH crystal structures to guide design, we engineered mutations within dimer-dimer interfaces to generate an inducible hexamer and an obligate dimer form of UGDH. Comparison of kinetic and thermal stability parameters among the hexameric wild-type enzyme and the engineered mutants revealed intrinsic structurally-dependent properties that support a model in which controlled assembly of the hexamer is a novel and critical feature in the mechanism of UGDH regulation.

(230) Consequences of Suppressing Expression of the R789C and R992C Collagen II Mutants in Cell-Based Models

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Mutations in *COL2A1* are associated with disorders characterized by alterations of skeletal development. Single amino acid substitutions in procollagen II frequently decrease the thermostability of mutant collagen molecules and alter their ability to assemble into homotypic and heterotypic fibrils. Moreover, misfolded thermolabile mutants are poorly secreted into the extracellular space, so they have a tendency to be excessively accumulated in cells, thereby causing endoplasmic reticulum stress and apoptosis. Therapeutic approaches have been proposed to counterbalance the pathological consequences of mutations in collagen genes. These main approaches include gene therapy by suppressing the mutant allele and cell therapy by delivering cells expressing the wild-type collagen variant. To date, these approaches have been relatively ineffective due to the inability to selectively suppress mutant alleles and failure to deliver a sufficient number of cells expressing wild-type collagen to the affected tissue. Still, we have yet to define the minimal conditions for such therapies that have to be reached to allow for extracellular matrix remodeling and cellular recovery from stress. Here, we employed a controlled tetracycline-inducible system for the expression of the thermolabile R789C or R992C collagen II mutants in cell-based constructs, allowing us to decrease the production of the mutant protein by 25, 50, 75 or 100% with respect to the initial production of those mutants. Analyses of selected intracellular and extracellular parameters have demonstrated that affected cell-based experimental systems are able to recover only when 100% expression of mutant collagens is shut off, but not if 25% or more mutant molecules persist in the analyzed system. Our data suggest that successful therapies for diseases caused by the presence of thermolabile collagen mutants may depend on the complete elimination of those mutants rather than on their partial reduction. Supported by NIH R01AR049537.

(231) Fibrillin-2 microfibril assembly depends on fibronectin fibrillogenesis and is suppressed by ADAMTSL2

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Fibrillin microfibrils are homo- or heteropolymeric structures of 10–12 nm diameter in the extracellular space. They can be comprised of three fibrillins in humans and two in mice. Microfibrils have a structural function and a major role in growth factor regulation. Fibrillin-1 and fibrillin-2 appear to have differential effects on TGFb and BMP activity. Therefore, mechanisms that regulate their relative proportions in microfibrils are crucial in morphogenesis. The process of fibrillin-1 assembly into microfibrils has been extensively studied owing to *FBN1* mutations in Marfan syndrome. In contrast, less is known about fibrillin-2 assembly and the mechanisms regulating this process. Here, we studied fibrillin-2 assembly with an antibody specifically recognizing fibrillin-2 in wild-type (Wt), fibrillin-1-deficient, fibronectin-deficient mouse fibroblasts. The process of fibrillin-2 assembly is essentially similar in wild-type and *Fbn1*-deficient. Wt cells show strong intracellular staining for fibrillin-1 and fibrillin-2 during the first 24–48 h. By 48 h, they begin to assembly patches of immunoreactive fibrillin-2 on the cell surface. With continuing culture duration, both the cell surface and intracellular staining disappear and are replaced by short linear immunostained structures aligned with fibronectin fibrils. These fibrillin-2 microfibrils lengthen and co-localize extensively with fibronectin. When fibronectin assembly was inhibited by FUD in either *Wt* or *Fbn1*-deficient fibroblasts, fibrillin-2 assembly diminished. *Fn* knockout fibroblasts cultured without exogenous fibronectin, assembled neither fibrillin-1 nor fibrillin-2 into microfibrils. ADAMTSL2, a secreted extracellular matrix protein that interacts with LTBP1 and fibrillin-1, enhanced fibrillin-1 microfibril biogenesis in cultured fibroblasts, but considerably reduced fibrillin-2 assembly. Both N- and C-terminal recombinant fibrillin-2 fragments interact with ADAMTSL2 in surface plasmon resonance with K_D of 16.3 nM and 35.2 nM, respectively. These findings demonstrate that cell-mediated incorporation of fibrillin-1 and fibrillin-2 into microfibrils is essentially similar and indiscriminate and occurs in discrete steps, with an absolute requirement for fibronectin. However, locally expressed molecules, like ADAMTSL2, may modulate fibrillin assembly favoring fibrillin-1 incorporation into microfibrils in certain tissues or at distinct developmental stages.

(232) Chondroitin sulfate synthase-2/chondroitin polymerizing factor: Characterization and impact on chondroitin sulfate biosynthesis

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To date, six glycosyltransferases for chondroitin sulfate synthesis have been identified, and the complex of chondroitin sulfate synthase-1 (CSS1)/chondroitin synthase-1 (ChSy-1) and chondroitin sulfate synthase-2 (CSS2)/chondroitin polymerizing factor (ChPF) is assumed to play a major role in CS biosynthesis. We found an alternative splice variant of mouse CSS2 in a database, which lacks the N-terminal transmembrane domain, contrasting to the original CSS2 and investigated the roles of these CSS2 forms. Both the original enzyme and the splice variant, designated CSS2A and CSS2B, respectively, were expressed at different levels and ratios in tissues. Western blot analysis of cultured

mouse embryonic fibroblasts confirmed that both enzymes were actually synthesized as proteins and were localized in both the endoplasmic reticulum and the Golgi apparatus. The pull-down assay revealed that either of CSS2A, CSS2B and CSS1/ChSy-1 heterogenously and homogeneously interacts with each other, suggesting that they form a complex of multimers. In vitro glycosyltransferase assays demonstrated a reduced glucuronyltransferase activity in CSS2B and no polymerizing activity in CSS2B co-expressed with CSS1 in contrast to CSS2A co-expressed with CSS1. Radiolabeling analysis of cultured COS-7 cells overexpressing each variant revealed that whereas CSS2A facilitated CS biosynthesis, CSS2B inhibited it. Molecular modeling of CSS2A and CSS2B provided support for their properties. Then, we generated and analyzed $CSS2^{-/-}$ mice. Although they were viable and fertile, exhibiting no overt morphological abnormalities or osteoarthritis, their cartilage contained CS chains with a shorter length and at a similar number to wild type. Further analysis using $CSS2^{-/-}$ chondrocyte culture systems, together with siRNA of CSS1, revealed that CSS2 mainly participated in the extension, whereas CSS1 participated in both the extension and the initiation. Our study demonstrates the distinct function of CSS1 and CSS2, providing a clue in the elucidation of the mechanism of CS biosynthesis.

(233) A minimalist fibril: a collagen-like fibril assembly of a designed triple helix with periodic sequences for the charged and the hydrophobic residues but no Hyp.

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The detailed molecular recognition mechanism guiding the well known self-assembly of the D-periodic fibrils of fibrillar collagens has long confounded the researchers. Here, we characterize the self-assembly of a recombinant triple helix that resembles the fibrillogenesis of collagen in several major aspects.

This triple helix, designated peptide 242, consists of 417 amino acids in length and is expressed in *E. coli* using an artificial gene construct; it forms stable triple helix with a melting temperature of 42°C. Upon increases of pH and temperature (26–37°C), the 242 self-assembles into long, smooth fibers with the typical cross-strait banding patters of fibrillar collagens under electron microscope, except with a *D-periodicity* of 33 nm. The 242 is designed to have three 120 residue repeating units with identical amino acid sequence which we believe instigated the 33 nm-D-staggering arrangement in the fibril; each repeating unit is 33 nm in length once adapted to the triple helix conformation. Furthermore, like collagen fibrillogenesis, this fibril forming process is a reversible process proceeds spontaneously from the triple helix state. Since the peptide 242 contains neither Hyp nor "telopeptides". Two factors implicated in the fibrillogenesis of collagen, its fibril assembly has thus, established a set of minimal requirements for the triple helices to self-associate laterally to form a superastructure with a high level of regularity. Mutagenesis and biophysical studies are underway to further define the specific molecular interactions that determine the structural specificity of the fibrils.

(234) Are N-glycosylation sites always fully occupied?

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N-glycosylation is often considered "irreversible" once glycoproteins are expressed on the cell surface or are secreted. However, the addition of a dolichol-linked *N*-glycan precursor (GlcManGlcNAc) onto a nascent peptide is a solely enzyme-catalyzed and non-template driven process. The likelihood and efficiency of this enzymatic reaction could be impacted by local factors. Current glycoproteomic techniques have identified numerous N-glycosylation sites from a wide range of samples. Unfortunately, little attention is paid to whether a glycosylation site is fully occupied or not, particularly in a complex biological sample. In an attempt to investigate the existence of partially occupied sites, three pairs of urine and plasma samples from different healthy individuals were fully de-*N*-glycosylated by PNGase F in $H^{18}O$ environment and followed by proteolytic digestion. The digested peptides were characterized by the standard shotgun-based proteomics pipeline. Sites were identified by the presence of an N-glycosylation consensus motif, in which asparagine was characterized as an ^{18}O-incorporated deamidation product: aspartic acid. A total of 1073 non-redundant N-glycosylation sites were identified, in which 925 were derived from urine samples and 319 from plasma samples. Importantly, the non-glycosylated forms (native asparagine) of more than 200 sites were unambiguously detected, confirming the co-existences of glycosylated and non-glycosylated variants in vivo. Furthermore, within a specific glycoprotein, certain sites were found to have a higher predilection for partial occupancy over others. This is the first large-scale demonstration of the presence of partially occupied N-glycosylation sites in complex mixtures, further indicating that N-glycosylations sites are not always fully occupied. The detection of these partially occupied sites will advance our understanding of this complex biosynthetic process.

(235) Tissue Homeostasis and Leukocyte Immune Responses in Animals are Regulatedby Density Dependent Lectin-Glycan Interactions

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We have recently introduced the concept that animal tissues regulate the expression of cell surface glycans and endogenous lectins to maintain homeostasis (1). Metabolic and environmental effects are known to change the density of cell surface glycans on specific glycoproteins and levels of lectins that bind and cross-link them, with concomitant changes in the activities of the receptors (2). The migration and innate and adaptive immune responses of leukocytes also rely on lectin-glycan interactions (3). Recent evidence indicates that the immunosuppressive activity of tumor cells may be due to secretion of galectins including galectin-3 in the tumor microenvironment, which up-regulates the density of LacNAc epitopes on the surface glycans of invading cytotoxic T-lymphocytes (4). This appears to increase cross-linking of glycoprotein

receptors by galectin-3 with concommitant down regulation of the T-cell receptor on the T-cells. The reduced cytotoxic activity of the T-cells can be reversed by addition of galectin-3 ligands such as LacNAc or citrus pectin. Hence, both tissue homeostasis and leukocyte immune responses in animals appear to utilize lectin-glycan density dependent interactions for integrated cellular responses.

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(236) Role of cell surface lysosome-associated membrane protein-1 (LAMP1) in metastasis

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Expression of β1,6 branched *N*-oligosaccharides on cell surface glycoproteins correlates with invasive and metastatic properties of several human and murine tumors and cancer cell lines. The terminal substitutions on β1,6 branch appear to provide ligands for several endogenous lectins, and the highly branched large structures appear to modulate the structure and function of proteins that carry them. Some of the carrier proteins identified include cell adhesion molecules like cadherins, integrins, CD44 and growth factor receptors like EGFR which are critical from invasion and metastasis point of view. Another interesting molecule that carries these oligosaccharides is Lysosome-Associated Membrane Protein-1 (LAMP1), a highly glycosylated protein that lines the lysosomal membranes, but gets translocated to surface of activated granulocytes/macrophages and tumor cells, in a metastatic potential-dependent manner. Both activated granulocytes/macrophages and metastatic cells use similar invasive mechanisms to colonize the target organ. Each LAMP1 molecule carries highly branched 17–19 *N*-oligosaccharides with different terminal substitutions providing potential ligands for endogenous lectins like Eselectin and galectin-3. It has also been shown to interact with extracellular matrix (ECM) and basement membrane (BM) components. Surface expression of LAMP1 and β1,6 branched *N*-oligosaccharides on low- (B16F1) and high- (B16F10) metastatic melanoma variants was found to correlate with their metastatic potential and treatment of B16F10 cells with anti-LAMP1 antibodies resulted in significantly reduced adhesion and experimental metastasis of these cells. Stable clones of i) B16F1 cells transduced with mutant LAMP1 (defective in lysosomal targeting) which show several fold higher surface expression of LAMP1 when comaped with even B16F10 cells and ii) B16F10 cells in which LAMP1 expression has been down-regulated using shRNAs against LAMP1, are being used to investigate the role of both the protein LAMP1 and its associated carbohydrates in different steps of metastasis.

(237) Altered *O*-glycan expression in tumor glycoproteins: genetics and consequences

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The molecular mechanisms underlying altered glycosylation in tumor cells are poorly understood. A common tumor-associated carbohydrate antigen expressed by a wide range of different cancers is the Tn (GalNAcα1-Ser/Thr), and in some cases it sialylated derivative Sialyl Tn (STn) (NeuAcα 2-6GalNAcα1-Ser/Thr). Our studies and others show that the Tn antigen commonly appears at an early stage of cancer and is associated with poor prognosis and tumor metastasis. However, the genetic basis for Tn/STn antigen expression is unclear. We recently found that expression of the Tn/STn antigens can arise from loss-of-function of T-synthase, required for normal core 1 *O*-glycan biosynthesis, due to its incorrect folding as a result of compromised expression of its specific molecular chaperone Cosmc, which is encoded by an X-linked (Xq24) gene. Acquired mutations or loss-ofheterozygosity in *Cosmc* are observed in some human tumors as well as human tumor cell lines. This presentation will focus on the molecular events in humans and animals that can cause expression of the Tn antigen, and the consequences of Tn antigen expression on glycoprotein structure and function, as well as on tumorigenesis.

(238) Heterogeneity in cell–matrix adhesion as an indicator of a metastatic state

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Cancer cells have great genetic diversity, which may be reflected in the variation in metastatic potential. As it has been difficult to determine a comprehensive set of genetic markers to define a metastasizing cell population, we propose to examine a common behavior, i.e. cell–matrix adhesion, that may be differentially regulated in metastasizing vs non-metastasizing cancer cells. To assess cell–matrix adhesion strength in a heterogeneous population of cancer cells, we employed a spinning disc device where cells adhering to matrix-coated substratums were exposed to radially dependent shear. Within a cell population, those at the center or edge experience low or high matrix detachment forces, respectively. Exposure to acute shear for a highly metastatic cancer cell line, i.e. MDA-MB-231, resulted in a broadly distributed population of cells unlike the sigmoidal, homogeneous shear response observed from non-metastatic, non-malignant or somatic cell lines, MCF7, MCF10A, NIH 3T3 fibroblasts, respectively. This suggests that adhesion strength differences might scale with cell state with the most metastatic cells coming from high-shear regions. Magnesium concentration differences have been reported between stroma and tumors, and it is also involved in regulating integrin activation.

While there was an up to 2-fold increase in adhesion strength for MCF7 or MCF10A cells in the presence of 0.5 mM magnesium, MDA-MB-231 cells exhibited a 10-fold increase in adhesion strength and also developed sigmoidal, homogeneous shear response when exposed to 0.5 mM magnesium. Finally, matrix stiffness difference between softer stroma and stiffer tumors implies that invasive cells must be well adherent on softer stroma to extravasate. Non-malignant and non-metastatic cells preferentially attached to stiffer matrix, and while the majority of metastatic cells detached on the soft matrix, cell attachment strength was broadly distributed again with 15–20% of cells remaining attached. These findings suggest a potential mechanism to differentially regulate metastasizing cell adhesion and also support using population-based adhesion assays to assess adhesion heterogeneity and select those cells that may have the highest metastatic potential.

(239) Expression of β1,3- *N*-acetylglucosaminyltransferase genes in human cancer cell line SW620 and NCI-H23 treated with bosutinib, a Src kinase inhibitor

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SRC is a well-known oncogene, which has been implicated in pathways regulating cell proliferation, angiogenesis, invasion and metastasis. In our previous studies, we had generated a mouse cell line expressing v-src and examined the expression of the β1,3-*N*-acetylglucosamonyltransferase-2(β3GnT2) and β1,3-Nacetyl-glucosamonyltransferase-8 (β3GnT8) genes in both wild-type NIH/3T3 and the stably transfected cell lines. We had found that the cell lines with overexpressed SRC had a higher level of β3GnT8 transcripts than the normal NIH/3T3 cell line. But the expression ofβ3GnT2 was unchanged. To further study the regulatory role of SRC on the β3GnT genes, we had studied the expressions of all nine different β3GnTs in the human colon cancer SW620 cells and in the human lung cancer NCI-H23 by treating the cells with bosutinib, a SRC inhibitor. The real-time qPCR results showed that when the cancer cell lines were treated with bosutinib, the expression of the β3GnT3, β3GnT6 and β3GnT8 in SW620 and the expression of theβ3GnT2, β3GnT3, β3GnT4, β3GnT5, β3GnT6 and β3GnT8 in NCI-H23 were also affected. Furthermore, the FACS analysis revealed that the surface expression of poly- *N*-acetyllactosamine was altered by the SRC inhibitor, which was consistent with the results from the gene expression studies. As a summary, we had demonstrated that several β3GnT genes were under the control of SRC pathway in human cancer cell lines. And surface expression of poly-*N*-acetyllactosamine was also altered in human cancer cells under the treatment the Src inhibitor.

(240) Soluble form of luthern/B-CAM in plasma as a potential maker for hepatocellular carcinoma

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The lutheran (Lu) blood group glycoprotein is an immunoglobulin superfamily transmembrane protein in which the extracellular domain contains one variable, one constant-1 and three intermediate immunoglobulin-like domains, V-C1-I-I-I. The extracellular domain of Lu specifically binds to laminin α5, a major component of basement membranes in various tissues. A splice variant of Lu known as basal cell adhesion molecule (B-CAM) has the same extracellular and transmembrane domains as Lu, but it lacks the COOH-terminal 40 amino acids of the cytoplasmic tail. Although Lu has been studied as the antigen of the Lu blood group system and in the context of sickle cell disease, B-CAM was identified as up-regulated antigen in carcinoma tissues. Our previous study showed that Lu/B-CAM was cleaved by MT1-MMP and shed from the cell surface of epidermoid carcinoma. In this study, we examined the soluble form of Lu/B-CAM in culture media and plasma of hepatocellular carcinoma (HCC). Two HCC cell lines, HepG2 and HuH-7, released the soluble form of Lu/B-CAM into culture media. We also examined the soluble form of Lu/B-CAM in the plasma of mice bearing HuH-7 cells. The concentration of Lu/B-CAM released in mice plasma was correlated with tumor size. Moreover, the soluble form of Lu/B-CAM in the plasma of HCC patients was significantly decreased after the resection of the tumor. These results suggest that the soluble form of Lu/B-CAM serves as a novel marker for HCC. Immunochemical studies also showed that, although the expression of Lu/B-CAM was mostly observed in HCC, MT1-MMP was not always expressed in tumor tissues, suggesting another mechanism to release the soluble form of Lu/B-CAM from cell surface. Biochemical studies showed that the soluble form of Lu/B-CAM in the culture media was precipitated into exosome fraction. A part of Lu/B-CAM in the plasma of HCC patients may be also released as exosomes.

(241) Different expression profile of glycosphingolipids in human breast cancer stem cells vs non-cancer stem sells

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Previous studies have demonstrated that expression levels of certain glycosphingolipids (GSLs) are correlated with various cellular phenotypes and that some GSLs are functionally involved in cell signaling. Recent studies have shown a drastic change of GSL expression pattern during differentiation of human embryonic stem cells into different cell lineages (Liang YJ, et al. PNAS, 2010 and Liang YJ, et al. Stem Cells, 2011).

Here, we report different expression profiles of GSLs in cancer stem cells (CSCs) and non-CSCs. CSCs are a small cell population in cancer cells and have characteristic features like resistance to chemotherapy drugs and capability of initiating tumor growth. We used immortalized human mammary epithelium cells transfected with a transcription factor, Twist (HMLE/Twist-ER). The cells express Twist after induction and that induces epithelial mesenchymal transition, resulting in acquisition of breast CSC phenotype, which is detected as CD44high/CD24low cells. We compared GSLs expression between CSC and non-CSC populations. GSLs were extracted and analyzed with HPTLC-orcinol staining, HPTLC-immunostaining and mass spectrometry. We found that 1) Fuc-(n)Lc4 is significantly reduced in CSC, 2) GSL with formula Hex(3)-Cer, probably Gb3Cer, is abundant in non-CSC, but absent in CSC, 3) GD1 is greatly increased in CSC and 4) Lower expression of GM3 and higher expression of GM2 in CSC. We are in process of analysising gene expression levels of various glycosyltransferases by Q-RTPCR in order to identify the responsible glycosyltransferases that cause differential GSL expression between CSC and non-CSC populations. Based on our gene expression analyses, we will further investigate the functional role of these differentially expressed GSLs to maintain CSC or non-CSC phenotype using gene transfection or gene knock-down approach. These data should be useful as potential marker molecules for breast CSC or for phenotype reversion from CSC to non-CSC.

(242) Decorin induces PGC-1α and mitostatin to regulate mitochondrial respiratory complexes in breast carcinoma cells

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Tumor metabolism is substantially altered relative to that of a normal, quiescent cell in order to maintain the rapid proliferative state required for tumorigenesis. Tumor cell mitochondria are emerging as key biosynthetic hubs to provide essential macromolecules necessary for cancer progression and angiogenesis. We discovered that soluble decorin attenuated several mitochondrial respiratory chain complexes in triple-negative MDA-MD-231 mammary carcinoma cells. This activity was mediated by and dependent on Met, the receptor for hepatocyte growth factor and one of the major targets of decorin. Moreover, we found a rapid and dynamic interplay between PGC-1α, a key regulator of mitochondrial biogenesis and the decorin-induced tumor suppressor gene mitostatin, a mitochondrial-associated membrane protein. This biological interplay between PGC-1α and mitostatin led to stabilization of mitostatin mRNA and concurrent accumulation of mitostatin protein. Further, depletion of PGC-1α via siRNA markedly abrogated stabilization of mitostatin mRNA and protein. Utilizing RNA immunoprecipitation, which is the RNA analog to chromatin immunoprecipitation, we were able to immunoprecipitate all PGC-1α containing ribonucleoprotein complexes formed upon decorin treatment and found that PGC-1α physically bound to mitostatin mRNA, thus providing a mechanistic basis for this rapid stabilization. Paradoxically, this process was orchestrated by Met signaling, insofar as blocking the Met tyrosine kinase or depleting Met abrogated this response. Importantly, the biological output of decorin-induced mitostatin via positive regulation of PGC-1α played a key role in decorin-mediated degradation of respiratory complexes. Indeed, knockdown of mitostatin was able to block the ability of decorin to suppress the respiratory complexes. Further, this knockdown resulted in an increase in VEGFA and compromised decorin-evoked VEGFA suppression. Collectively, these data provide a completely novel role for decorin as a negative regulator of tumor mitochondrial activity and vis-à-vis tumor angiogenesis in mammary carcinoma cells.

(243) Heparan sulfate proteoglycans: Regulators of oncogenic signaling in brain cancer

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Glioblastoma (GBM) is the most common malignant primary brain tumor of adults with a mean survival of less than 2 years. The disease is characterized by diffuse invasion within the adjacent non-neoplastic brain and the abnormal activation of receptor tyrosine kinase (RTK) signaling pathways. Indeed, the most frequent alteration in GBM is the amplification of EGFR. During normal development, heparan sulfate proteoglycans (HSPG) and cell adhesion molecules (CAMs) help regulate cell signaling and cell migration. Our lab has shown that the extracellular sulfatase SULF2, which modifies HSPGs at the cell surface, regulates PDGFRα signaling and GBM growth (Phillips et al. 2012). We hypothesize that HSPGs and CAMs in the tumor microenvironment are important regulators of cell signaling and invasion in GBM. In the current study, we investigate the relationship between RTK signaling profile and expression of HSPGs, HSPG modifying enzymes and CAMs in primary human GBM tumors and human GBM xenografts. In GBM, we find increased expression of a number of HSPG core proteins, including syndecans and glypicans, and of HSPG modifying enzymes such as SULF1 and SULF2. Interestingly, the pattern of the expression of HSPG and CAM-related genes and the pattern of RTK activity vary between tumors with different molecular phenotypes. These studies support the concept that GBM is a molecularly heterogeneous disease and identify specific HPSGs and HSPG-modifying enzymes that may help drive oncogenic signaling within distinct subtypes of GBM.

(244) Heparanase enhances the insulin receptor signaling pathway to activate ERK inmultiple myeloma

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ERK signaling regulates proliferation, survival, drug resistance and angiogenesis in myeloma. Although the mechanisms regulating ERK activation in myeloma are not fully understood, we previously demonstrated that ERK phosphorylation is elevated by heparanase, an enzyme associated with aggressive behavior of many cancers. In this study, myeloma cell lines expressing either high or low levels of heparanase were utilized to determine how

heparanase stimulates ERK signaling. We discovered that the insulin receptor was abundant on cells expressing either high or low levels of heparanase, but the receptor was highly phosphorylated in heparanase-high cells compared with heparanase-low cells. In addition, protein kinase C activity was elevated in heparanase-high cells and this enhanced expression of insulin receptor substratum-1 (IRS-1), the principle intracellular substratum for phosphorylation by the insulin receptor. Blocking insulin receptor function with antibody or knockdown of IRS-1 expression using shRNA diminished heparanase-mediated ERK activation in the tumor cells. In addition, up-regulation of the insulin signaling pathway by heparanase and the resulting ERK activation were dependent on heparanase retaining its enzyme activity. These results reveal a novel mechanism whereby heparanase enhances the activation of the insulin receptor signaling pathway leading to ERK activation and the modulation of myeloma behavior.

(245) Glycosylation of laminin receptor integrin (α3β1) regulate their association withtetraspanin CD151 and thus motility/invasion on basement membrane component (matrigel)

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Invasion is the key requirement for metastasis. For invasion, tumor cells modulate their adhesion and spreading to the matrix, regulate matrix degradation and movement. All these processes are mediated by cell surface molecules. Invasive tumors show several cell surface modifications. Expression of β1,6-branched N-oligosaccharides is one such consistent modification associated with invasion. Both invasive tumor cells as well as normal cells involved in invasive functions like trophoblast cells during implantation, activated granulocytes and macrophages during inflammation and even endothelial cells during angiogenesis express them. Using the B16 murine melanoma invasive variants, we have earlier shown that β1,6 branched N-oligosaccharides on B16 melanoma cells regulate motility in exactly the opposite manner on the extracellular matrix (ECM) and Basement Membrane (BM) components, it promotes motility on ECM (fibronectin) but reduces it on BM (laminin/matrigel). To understand the significance of these observations in context of invasion, we demonstrate that altered motility on the ECM and BM component is not due to the alteration in surface expression of integrin receptors for fibronectin (α5β1) and laminin (α3β1). Both α-and β subunits of above integrin receptors on B16BL6 cells carry these oligosaccharides and their presence on these receptors enhanced spreading and haptotactic motility on fibronectin but they were attenuated on matrigel. Tetraspanins are the major modulators of integrin's function. Expression of most tetraspanins, particularly CD82 and CD151, did not correlate with invasiveness of B16 melanoma cell lines. CD151 is a major tetraspanin that regulates the function of laminin receptors. We demonstrate that glycosylation on laminin receptor α3β1 impeded its association with CD151 and thus modulate cell spreading and motility on matrigel in such a way that it facilitates invasion. This suggests that tumor cells probably regulate their motility in a context (ECM or BM)-dependent manner to enhance their invasiveness.

(246) NK-derived galectin-1 is involved in lysis of low-sialylated 3-MCA-induced fibrosarcoma tumor cells

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Galectin-1, an endogenous glycan-binding protein affected by sialic acid residues on glycans, has been mainly determined to possess immunoregulatory activities in cancer models, wherein tumor susceptibility to galectin-1 was mostly elucidated following its exogenous administration. Nevertheless, limited information is available on tumor regression mediated by galectin-1, which is produced by innate immune cells. Recently, we showed that low-immunogenic 3-methylcholantrane (3-MCA)-induced fibrosarcoma cells arise in BALB/c mice express a high level of sialic acids and develop aggressive tumors in secondary recipients. Removing the sialic-acids from those cells by sialidase transformed them into NK-susceptible cells that overexpress galectin-1. Here, we show preliminary data of galectin-1 overexpressed in co-cultures with spleen cells. In addition, we show for the first time that galectin-1 is up-regulated within NK cells in co-cultures of tumor variants bearing less sialic acids and spleen cells. Our data further demonstrates the production of galectin-1 by tumor and NK cells in co-cultures, which induces death of low-sialylated tumor cell variants but inhibited in the presence of β-lactose. Accordingly, NK-derived galectin-1 may be considered to contribute to the immune surveillance mechanisms occurring in animal models. Decoding the effects of tumor sialylation on the roles galectin-1 plays in regressive tumor microenvironment will be of particular benefit in the development of novel therapies to treat cancer.

(247) Impact of hyaluronan turnover on vesicle trafficking and exosome biogenesis

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Hyaluronan is an extracellular glycosaminoglycan, synthesis and degradation of which are controlled by hyaluronan synthases (HAS) and hyaluronidases (Hyal), respectively. Co-overexpression of the HAS3 and Hyal1 isozymes in prostate cancer cell lines accelerates tumorigenesis and lymphatic metastasis in mice as a result of Hyal1-induced cell motility and altered surface presentation of adhesion receptors. Products of concurrent HAS3 and Hyal1 action engage intracellular signaling pathways through specific receptors such as CD44 and subsequent receptor-mediated endocytosis. Therefore, we examined the general impact of Hyal1 on vesicle trafficking as a possible mechanism for Hyal1-stimulated motility. Overexpression of Hyal1 significantly increased receptor-mediated hyaluronan uptake by prostate tumor cell lines. The intracellular hyaluronan accumulation was the result of accelerated vesicle trafficking. Internalized hyaluronan co-localized with Hyal1, with a small fraction detected in lysosomes and the majority accumulated in non-lysosomal vesicles and the endocytic recycling complex. Hyal1 is both a lysosomal and an extracellular enzyme, so we additionally examined the relationship between intracellular vesicular trafficking and extracellular

levels of Hyal1. Differential fractionation of conditioned media to yield microvesicular, exosomal and soluble extracts revealed Hyal1 present in all fractions, with enrichment in exosomes. Chemical modulation of vesicle trafficking was used to dissect the specific trafficking processes required for Hyal1 secretion and identify which aspects of trafficking were impacted by Hyal1 expression. In addition, we examined the effects of differentially fractionated conditioned media on proliferation of hyaluronan-producing tumor cells and prostate stromal cells. Since tumor exosomes may have a role in metastatic target tissue preparation, our results suggest that Hyal1 modulation of exosome secretion or targeting may be a novel mechanism by which Hyal1 accelerates metastasis.

(248) Shed syndecan-1 translocates to the nucleus of tumor and host cells

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Multiple myeloma is a disseminated malignancy of plasma cells that originates within the bone marrow. It is the second most prevalent hematological malignancy in the USA. Myeloma cells express high levels of the cell surface proteoglycan syndecan-1. Syndecan-1 regulates cross-talk between tumor and host cells by acting as a co-receptor for growth factors, chemokines and other regulatory proteins that drive tumor growth and progression. Syndecan-1 is shed from the cell surface and concentrates within the tumor microenvironment. Shed syndecan-1 can accumulate to high levels in the serum of myeloma patients and is an indicator of poor prognosis. Recently, our lab has shown that syndecan-1 in the nucleus alters histone acetyltransferase (HAT) activity thereby regulating gene expression. Knowing syndecan-1 is present in the nucleus, we aimed to determine whether shed syndecan-1 can translocate to the nucleus of cells as well. To investigate this, we used ARH-77 cells, a human lymphoblastoid cell line that lacks endogenous expression of syndecan-1. The ARH-77 cells were grown in the presence of medium containing either murine or human shed syndecan-1. Remarkably, shed syndecan-1 translocated to the nucleus of ARH-77 cells as confirmed by western blotting and confocal imaging. Moreover, exogenous shed syndecan-1 added to myeloma cells (CAG) or bone marrow-derived stromal cells (ST2) also translocated to the nucleus. Additionally, we found that shed syndecan-1 translocation to the nucleus is dependent on the presence of intact glycosaminoglycan chains on its core protein. These data indicate that shed syndecan-1 is taken up by tumor and host cells where it may participate in shuttling regulatory proteins bound to its heparan sulfate chains to the nucleus. Because nuclear syndecan-1 has been shown to down-regulate HAT activity, shed syndecan-1 that translocates to the nucleus likely impacts gene expression. Therapeutic regulation of syndecan-1 translocation to the nucleus represents a novel treatment strategy to control myeloma.

(249) Integrin α4 Mediates Neuroblastoma Dissemination

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Neuroblastoma (NB) is a childhood cancer arising from neural crest cells. Despite aggressive therapy, NB with distant metastasis is associated with an overall survival rate of less than 30%. Thus, NB metastasis poses a significant obstacle in achieving remission. NB adhesion receptors in the integrin family impact cell adhesion, migration, proliferation and survival. Integrin α4, a fibronectin, osteopontin and VCAM-1 receptor typically found on leukocytes, is essential for neural crest cell motility during development. Here, we show that reconstitution of α4 expression in human and murine neuroblastoma cell lines selectively enhances in vitro interaction with fibronectin, osteopontin and VCAM-1 and increases cell migration. In vivo α4 expression enhanced experimental metastasis in orthotopic and syngeneic tumor models, reconstituting a pattern of organ involvement similar to that seen in patients. Importantly, the matrix adhesive function of integrin α4 alone was insufficient, but required, for this effect since antagonism of integrin α4 adhesion blocked metastasis. The results implicate integrin α4 as a potential target for the control of NB progression. Clinical approaches previously used to target α4 in multiple sclerosis might therefore be considered for this aggressive malignancy.

(250) Targeted drug delivery to tumor vasculature by the IF7 peptide in mouse gliomamodel

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Although glioma cells cultured in vitro are responsive to several anti-cancer drugs, it is very difficult to treat glioma in vivo due to blood-brain barrier (BBB). Chemotherapeutics injected to blood circulation do not pass from blood circulation to brain stroma due to tight junctions between endothelial cells. In the previous study, we identified a carbohydrate mimetic peptide, designated as IF7, that homes tumor vasculature (Hatakeyama et al. 2011). IF7 binds to annexin A1, which is expressed specifically on the endothelial cell surface of malignant tumors. We showed that IF7 conjugated anti-cancer drug SN38 (IF7-SN38) suppressed growth of the colon, melanoma, breast, prostate and lung tumor models in the mouse. Since previous study suggested that IF7 is transported across endothelial cells through endocytotic pathway, we hypothesized that IF7 can deliver anti-cancer drug to brain stroma overcoming BBB. In this study, we produced glioma tumors in mouse brains by intracranial injections of C6 cells (Suzuki-Anekoji et al. 2011). To determine targeting of IF7 peptide to C6 glioma tumors, IF7 tagged by fluorescent Alexa 488 (IF7-A488) was injected intravenously. IF7-A488 targeted tumor vasculature within minutes, penetrated through endothelial cell wall and spread to the outer edge of glioma sphere. The control peptide RQ7 conjugated A488 did not target to the tumor vasculature. Then we implanted luciferase-expressing C6 (C6-Luc) cells in mouse brains. Growth of C6-Luc tumor was monitored by Xenogen IVIS imager. Daily intravenous injection of 3.6 μmol/kg of IF7-SN38 reduced size of C6-Luc glioma, while the control peptide RQ7 conjugated SN38 did not. The mouse brain specimen treated by IF7-SN38 showed enlarged and necrotic tumor vasculature, and

apoptotic death of surrounding glioma cells. These results strongly encourage clinical application of IF7-SN38 to glioma tumors.

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(251) The roles of melanoma-associated protein (CSPG4) and perlecan (HSPG2) in cancer, potential targets for drug therapy

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Cancer metastasis is characterized by the spread of cancer cells to distant organs and tissues, which relies on changes in cell–cell and cell–extracellular matrix (ECM) interactions. Proteoglycans are major components of the ECM and cell surface, where they have been shown to mediate some of these cell–ECM interactions. Hence, changes in the expression levels and structures of these complex molecules are likely to affect cancer cell progression. Studies have shown that both perlecan, an extracellular heparan sulfate proteoglycan and a chondroitin sulfate proteoglycan 4 (CSPG4), a cell surface proteoglycan and thought to be decorated with chondroitin sulfate, are capable of modulating the metastatic potential of malignant cells making them attractive potential immunotherapeutic targets to control metastasis. Thus, this study aims to investigate the expression levels and structures of CSPG4 and perlecan produced by the human colon carcinoma cancer cell line, WiDr, to assess their glycosaminoglycan structure. WiDr cells expressed CSPG4 on their cell surface, which was not secreted into the ECM, while perlecan was produced and secreted into the culture medium but not laid down in the ECM. We hypothesize that perlecan and CSPG4 work cooperatively interacting with integrins and the surrounding environment to instruct cells to adhere, migrate and proliferate. In WiDr cells, perlecan was colocalized with the α2β1 integrin, while CSPG4 colocalized with the β1 integrin chain but not the α2 component. Scratch assays that were used as model of cell expansion have shown that anti-integrin β1 antibodies reduced cell expansion on both tissue culture polystyrene (TCPS) and collagen-coated TCPS. Interestingly, anti-CSPG4 monoclonal antibodies (clone 9.2.27) were able to reduce cell expansion on type I collagen-coated TCPS, but not TCPS suggesting a link between the substrata that the cells are exposed to, the expression of CSPG4 and specific integrins. We plan to investigate the role of glycosminoglycans decorating proteoglycans and whether these carbohydrate structures modulate the interactions with the integrins, affecting cell adhesion.

(252) Expression of core 2 β-1,6-*N*-acetylglucosaminyltransferase is an independent risk factor for PSA recurrence after radical prostatectomy and closely associated with aggressive potential

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To avoid over treatment of early stage prostate cancer (PC) is a major concern. It is very important to discover a novel biomarker for malignant potential of PC. Core2 β-1,6-*N*-acetylglucosaminyltransferase (C2GnT) is an glycosyltransferase which forms core2 branching *O*-glycan on various glycoproteins. C2GnT expressions have been associated with malignant potential of the cololectal, lung, testicular and bladder cancer. (EMBO J, 2011). We examined whether this is the case with PC. To determine the role of C2GnT in progression of PC, LNCaP cells which do not express C2GnT were transfected with pcDNA3-C2GnT, called LNCaP-C2GnT. These cells were determined adherent activity on prostate stromal cells (PrSc), enhancement of growth factor expressions under the co-incubation LNCaP and PrSc, and tumor formation activity in nude mice orthotopic inoculation system. To determine the correlation of C2GnT expression with pathological parameters and biochemical outcome after radical prostatectomy, paraffin-embedded specimens of 67 patients with PC were immunohistochemically examined for C2GnT expression using the antiC2GnT polyclonal antibody. Five times number of LNCaP-C2GnT was adhered to PrSc comaped with mock. Under the co-incubation condition, HGF, VEGF, FGF and KGF were significantly increased at LNCaP-C2GnT ($P < 0.05$). In the orthotopic tumor formation assay, LNCaP-C2GnT was formed tumor bigger than mock ($P < 0.05$). The C2GnT-positive rate in pT2 (47.7%) was significantly lower than that in pT3 (96.0%; $P < 0.0001$). C2GnT-negative patients showed significantly longer PSA-free survival than C2GnT-positive patients. These results suggest that expression of C2GnT on prostate cancer correlate positively with the malignant potential of prostate cancer. Further clinical trial is necessary to determine the implication of C2GnT as a novel biomarker of prostate cancer.

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(253) Antitumor effects of bio-active components from *Laminaria japonica* on S180-bearing mice

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Fucoidan was extracted from *Laminaria japonica* with enzymatic hydrolysis method. Fucoxanthin was extracted with alcohol and then purified by MgO-kieselguhr column chromatography. The antitumor activities of fucoidan and fucoxanthin from *Laminaria japonica* were studied in this article. Cyclophosphamide is positive control and distilled water is negative control. S-bearing mice were used as animal model. The changes of Stumor weight in vivo, the changes of the viscera index and the changes of MDA content and SOD activity of liver and serum in S_{180}-bearing mice were investigated when different dosage fucoidan (100, 500, 1000 mg/kg.d)

and fucoxanthin (5, 10, 20 mg/kg.d) were administered orally. The histological changes of tumor were observed by optical microscope. It was shown that both of fucoidan and fucoxanthin can inhibit tumor growth markedly in S_{180}-bearing mice compared with negative control group. The inhibition rates of fucoidan were 25.5, 36.4 and 41.8%, respectively. While the inhibition rates of fucoxanthin were 41.4, 58.1 and 62.8%, respectively. The antitumor effect of fucoxanthin was better than fucoidan in vivo. Both of them can decrease MDA content and increase SOD activity of liver and serum in S_{180}-bearing mice. They also can increase thymus index and spleen index of S_{180}-bearing mice which showed they could enhance the immune function. The mechanisms of their antitumor maybe concerned with their antioxidant effect and effect of immune modulation. It is known that fucoxanthin has very strong antioxidant activity and it can protect organs from oxidative damage, so it also has significant antitumor activity in vivo.*Keywords: Laminaria japonica* / fucoidan / fucoxanthin / antitumor / S_{180}

(254) N-acetylglucosaminyltransferase IVa regulates metastatic potential of mouse hepatocarcinoma cells through glycosylation of CD147.

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N-acetylglucosaminyltransferase (GnT)-IV a is a key enzyme that catalyzes the formation of the GlcNAC β1-4 branch on the core structure of complex N-Glycans,which is the common substrate for other N-acetylglucosaminyltransferases, such as GnT-III and GnT-V. Our recent study indicates that the expression of GnT-IVa in Hca-F cells was much higher than that in Hepa1-6 cells, these two mouse hepatocarcinoma cell lines have high and no metastatic potential in lymph nodes respectively. To investigate the effects of GnT-IVa on the metastasis of hepatocarcinoma, exogenous GnT-IVa was introduced into Hepa1-6 cells, and on the other hand, the expression of GnT-IVa was down-regulated in Hca-F cells. The engineered overexpression of GnT-IVa in Hepa1-6 cells increased the antennary branches of complex N-glycans and reduced bisecting branches in vitro and in vivo, which leads to the increase in migration and metastatic capability of hepatocarcinoma cells. Conversely, down-regulated expression of GnT-IVa in Hca-F cells showed reduced tetra-antennary branches of N-Glycans, and significantly decreased the migration and metastatic capability. Furthermore, we found that the regulated GnT-IVa converts the heterogeneous N-glycosylated forms of CD147 in Hepa1-6 and Hca-F cells, and significantly changed the antennary oligosaccharide structures on CD147. These results suggest that GnT-IVa could be acting as a key role in migration and metastasis of mouse hepatocarcinoma cells through altering the glycosylation of CD147. These findings should be valuable in delineating the important function of GnT-IVa during the process of hepatocarcinoma growth and metastasis.

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(255) The lectin domain of the ppGalNAc-T glycosyltransferases acts as a switch, further controlling glycopeptide substratum glycosylation in an N- or C-direction

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Mucin type O-glycosylation begins with the transfer of GalNAc to Ser/Thr residues of proteins. It is initiated by a large family (20) of ppGalNAc-T glycosyltransferases. Members show varying preferences for peptide characteristics including neighboring glycosylation, charge and composition. These transferases play a role in development, tumorgenesis and possibly signaling. Structurally, ppGalNAc-Ts consist of an N-terminal catalytic domain linked via a short linker to a C-terminal ricin like lectin domain. The properties of both domains are not well understood but are believed to have unique functions in glycosylation site recognition. In order to characterize their substratum specificities, we have reported a series of oriented random peptide and glycopeptide substratums for quantifying transferase-specific catalytic domain preferences (2011, J Biol Chem. 286:14493). We now extend our studies to investigate the function of the lectin domain, using a series of random glycopeptides containing N- or C-terminal placed Thr-*O*-GalNAc. These substratums will determine the effects of GalNAc placement on transferase specificity and determine the optimal separation between the initial Thr-*O*-GalNAc and the glycosylation site of the catalytic domain. Our results suggest that ppGalNAc T1, T2, T14 exhibit preferences for a random glycopeptide that contains a C-terminal placed Thr-*O*-GalNAc, while T3 and T6 display preferences for a random glycopeptide with an N-terminal placed Thr-*O*-GalNAc. T5 and T12 display approximately equal preferences for an Nand C-terminal placed Thr-*O*-GalNAc. With this work, we believe that we have now uncovered another level of control of mucin-type O-glycosylation that will further advance the field and our understanding of the regulation of this type of modification. Transferases were generous gifts of the following labs: L Tabak, NIH, H.Clausen, University of Copenhagen, K Moreman, University of Georgia, and D Jarvis, University of Wyoming. Supported by NIH grant R01 CA078834.

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(256) A glycopeptide approach for the site-specific characterization of O-linked glycosylation of human synovial lubricin by CID and ETD

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The joint-related diseases such as RA (rheumatoid arthritis) and OA (osteoarthritis) as well joint injury are suggested to be associated with altered composition of the synovial fluid and increased degradation and inflammation of the articular cartilage proteins from that of normal joint. A decrease in expression and increase in degradation of lubricin, accelerating the destruction of the joints aggravating the disease, has been suggested during advanced stages of RA and OA, indicating that lubricin may be a good joint-specific biomarker for early diagnosis of the disease. The site-specific structural characterization of mucin-type *O*-linked glycopeptide/glycoproteins such as lubricin is a challenging task due to heterogeneity, lack of consensus sequence and limited access to proteolytic sites often associated with the high density of *O*-glycan core structures. That lubricin is a mucin molecule that is substantiated when it is treated with sialidase A and *O*-glycanase, which reduced the size to the size of protein without post-translational modification (148 kDa), indicating that *O*-linked oligosaccharides mainly consists of core 1 structures. In this study, an efficient ETD-MS^2 approach has been developed which identified 26 differently *O*-glycosylated peptide in the mucin domain of synovial lubricin from an allergic arthritis patient. In addition to detection of mono sialylated and non-sialylated core 1 *O*-linked glycopeptides, a small proportion of di-sialylated core 1 and non-sialylated core 2 containing glycopeptides were detected. The data suggest that both the threonine in the tandem repeat of EPAPTTPK can be glycosylated. The identification of core 2 glycopeptides confirmed the presence of core 2 structures in human synovial lubricin indicating that in addition to lubrication (in particular core 1), lubricin glycosylation also may have an immunological impact. This was substantiated with that recombinant lubricin expressing only core 1 structures did not bind recombinant L-selectin, while lubricin isolated from synovial fluid did.

(257) Chemical biology approaches reveal insights into mechanism of heparan sulfate biosynthetic enzyme action

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Heparan sulfate (HS) is a linear heavily *O*- and *N*-sulfated polysaccharide present on cell surfaces and within the extracellular matrix. HS mediates several processes including inflammation, anti coagulation and cell adhesion through HS–protein complexes. The HS-protein interactions are based on the HS structural heterogeneity and diverse sulfation patterns which are controlled by a group of HS biosynthetic enzymes. Although the enzymatic activity of HS biosynthetic enzymes is well known, the importance and relative contribution of hexuronic and glucosamine residues at the active site for enzyme action is unclear. In this study, we utilize synthetic probes and stable isotopes to define the dynamics of enzyme action. The results of this study also provide new insights into the assembly mechanism of heparan sulfate and heparin chains.

(258) Elucidating the Complexity of Heparin Oligosaccharides using Heparin Lyase Enzymes and Mass Spectrometry

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Heparin and Heparan Sulfate (HS) are linear sulfated polysaccharides located on cell surface membranes and in extracellular matrices in most animal tissues. Heparin/HS chains consist of repeating disaccharide units of [(GlcA4 β(1-4)GlcNAcα(1-4)] with poly-disperse sulfation, *N*-acetylation and uronosyl epimerization. The structural elucidation of such complex and diverse polysaccharides is an exceptionally challenging task and cannot be accomplished without enzyme tools. Three heparinase enzymes are known to cleave heparin and HS chains selectively, via an elimination mechanism. Heparinase I cleaves highly sulfated heparin/HS chains, heparinase III cleaves less sulfated HS chains, while heparinase II cleaves domains of both high and low sulfation on both heparin and HS. This poster describes enzymatic and chromatographic methods used to create heparin oligosaccharides of specific size, as well as mass spectrometric methods in order to analyze the complexities of such molecules. Heparin oligosaccharides are first created using Heparin lyase enzymes cloned from *Bacteroides eggerthii*. The degree of enzymatic activity and heparin depolymerization was achieved by reading absorbance at 232nm. The isolation and purification of size-specific heparin oligosaccharides was performed using a streamlined series of column chromatography techniques. Following isolation and purification of heparin/HS oligosaccharides, the most common method of downstream analysis is MS. However, heparin/HS can be difficult to analyze by MS without using finely tuned ESI parameters to reduce or eliminate in-source fragmentation of sulfated oligosaccharides. Thus, highly sulfated heparin oligosaccharides with defined structure, are used to optimize the ESI tuning parameters in various mass spectrometers. This work shows how improved elucidation of Heparin/HS can only be accomplished by creating oligosaccharide intermediates with a diverse set of tools such as Heparinase enzymes.

(259) Quantifying Individual Glycoforms in Complex Glycomics Mixtures

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Glycan structures and sites of glycosylation locations have been shown to change with the state/condition of the cell in which the proteins are produced. For example, it has been known for over 20 years that tumor cells attach different glycans than the corresponding "normal" cells from the same tissue/organ. A particularly challenging aspect in glycomics is the quantitation of individual glycans present in an isomeric mixture of glycoforms, i.e., glycans with the same composition that differ in their linkages and/or branching. We have overcome the resolution aspect of this challenge by devising an LC separation strategy that permits the resolution of individual glycans. This strategy uses a new fused core HILIC packing material to resolve glycans tagged on their

reducing termini. As an example of these separations, we are able to baseline resolve the two triantennerary glycans released from bovine fetuin whose only difference is one of the galactose residue being attached by either a 1-4 or a 1-3 linkage. Relative quantitation is provided by the addition of a mixture of isotopically labeled glycoproteins as the first step in the sample workflow. The use of these standards provided excellent accuracy and precisions within 20% (RSD), comaped with almost >100% RSD with non-isotopic standards. The focus of this presentation will be to highlight these techniques, to demonstrate their utility for the relative quantification of glycomic mixtures and discuss the extension of this approach to provide absolute glycan quantitation.

(260) Neurofascin 186 is *O*-mannosylated within and outside of the mucin domain

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Protein-O-mannosylation is an important modification in mammals and deficiencies thereof lead to a variety of severe phenotypes. Although it has already been shown that the amount of *O*-mannosyl glycans in the brain is very high, only very few proteins have been identified as *O*-mannosylated. We have developed a gel-based global *O*-glycomics method with which we could show that the relative proportion of *O*-mannosylated proteins rises to comparatively high levels in the mouse brain proteome in the dependence of increasing protein masses. In order to identify other *O*-mannosylated proteins, we enriched GlcNAc and NeuAc carrying proteins from mouse brain lysate using agarose bound wheat germ agglutinin. These proteins were further fractionated by several complementary methods, and by the analysis of the *O*-glycans of the resulting fractions, we identified some other proteins which could serve as a target for the protein-*O*-mannosyltransferases. Among these so far neurofascin was chosen for further analysis as a recombinant probe (mucin domain) and as an endogenous protein from the mouse brain. Mass spectrometric data for both proteins confirmed that neurofascin186 is indeed *O*-mannosylated. Glycopeptide analysis by liquid chromatography-tandem mass spectrometry allowed for the identification of some of the O-mannosylation sites, which are not restricted to the mucin domain, but were found also within N-terminal IgG and fibronectin domains of the protein.

(261) Improvements on the DMB-HPLC method for sensitive and specific detection and quantitation of bound and free non-ulosonic acids

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Sialic acids (Sias) are a subset of Non-ulosonic acids (NulOs) that are widely distributed in nature. The DMB-HPLC method of Hara and colleagues has long been utilized to detect and quantitate the many naturally occurring types of Sias and was combined with mass spectrometry by Klein and colleagues to confirm identification. We have used the method in many studies, including confirmation of the presence of the non-human Sia Neu5Gc in human tissues, and in elucidating how traces of this sugar of dietary origin comes to reside in these tissues. Most recently we used human-like *Cmah* null Neu5Gc-deficient mice to confirm and extend the work of Nohle and Schauer, showing that dietary Neu5Gc from glycoproteins but not free Neu5Gc gives a human-like pattern of tissue incorporation into fetal and adult tissues, as well as into tumors. It is now important to detect and accurately quantitate bound and free Neu5Gc in complex human tissues and food samples. We show that the low-temperature modification of DMB derivatization introduced by Inoue et al. to protect the lability of polysialic acid can also be used to accurately differentiate between free and glycosidically bound Sias. Solvent composition can be manipulated to separate co-eluting peaks in complex mixtures and an internal standard of NeuNPr can better control for variations in derivatization efficiency and/or changes in elution profile. The relative stability of Sia *O*-acetyl groups during the process was also studied. This approach can be used with any samples wherein sensitivity, specificity and quantification of bound and free NulOs are important. We applied these new approaches to the study the distribution of free and glycosidically bound Neu5Gc in common human foods. In addition to confirming and extending prior data in this matter, we report some new findings that have implications for the role of Neu5Gc as a dietary "xeno-autoantigen".

(262) High-throughput *O*-Glycomics

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O-glycosylation is a key biological modification and defects in o-glycosylation have been implicated in a number of human diseases, including congenital muscular dystrophies, cancer and others. *O*-glycomics, or the study of global o-glycosylation typically by mass spectrometric approaches, is a relatively new field. The idea is that the global o-glycan profile can define specific cellular states, such as states of differentiation. Sample preparation and analysis is relatively simple, but data analysis is a time-consuming process. Our group is focused on developing a high-throughput workflow for the analysis and quantification of global o-glycan levels. The method includes selective data acquisition, introduction of standards for absolute quantification and the incorporation of automated data analysis. This technique has initially been applied to quantification of the differences global o-glycosylation in (i) ABO blood groups and (ii) human embryonic stem cell (hESC) differentiation states.

(263) Evolution of haptoglobin glycoforms in liver disease progression

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Haptoglobin is a liver secreted protein with four N-glycosylation sites. Haptoglobin prevents loss of iron by binding to free hemoglobin released by intravascular hemolysis and subsequent kidney damage. We have examined site-specific glycoforms of haptoglobin during the progression of HCV viral infection to hepatocellular carcinoma. Haptoglobin isolated from plasma of patients with premalignant liver disease, hepatocellular carcinoma and healthy controls was characterized by combination of HILIC and nano-reverse phase C18 chromatography coupled with Q-TOF mass spectrometry. Structures of the glycans were determined by specific exoglycosidases and mass spectrometry of the permethylated detached *N*-glycans. We have observed elevation of branched multiply fucosylated glycoforms of haptoglobin in the liver disease and confirmed quantitative differences of several Lewis-type structures by LC-MS SRM. These unusual glycoforms could have important impact on the disease progression. The results show the importance of quantification of site-specific protein glycoforms in the liver disease context.

(264) 2-*O*-sulfated HS domains in syndecan-1 inhibit acetaminophen-induced liverinjury by suppressing hepatocyte apoptosis

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Acetaminophen (APAP) intoxication is the most common drug overdose and the foremost cause of acute liver failure in the US. Although the generation of NAPQI, the toxic metabolite of APAP, is thought to mediate liver injury, other mechanisms have also been suggested to contribute to disease. Because syndecan-1 modulates key processes in tissue injury and syndecan-1 is the predominant heparan sulfate proteoglycan in hepatocytes, we examined the role of syndecan-1 in APAP-induced liver injury. *Sdc1−/−* mice showed significantly elevated levels of liver injury markers (serum ALT and AST) and histological liver damage comaped with Wt mice at 24 h post-APAP. The extent of liver damage was similar in both backgrounds until 12 h post-APAP when liver injury markers started to subside in Wt mice but continued to increase in *Sdc1−/−* mice. However, liver levels of CYP2E1 (enzyme that generates NAPQI) and GSH (detoxifying agent of NAPQI) were similar in APAP-injected Wt and *Sdc1−/−* mice, and isolated Wt and *Sdc1-/−* hepatocytes were similarly susceptible to both APAP and NAPQI cytotoxicity, suggesting that mechanisms other than NAPQI-mediated hepatotoxicity is responsible for the observed difference in liver injury in vivo. Consistent with these data, activated caspase-3, a marker of apoptosis, was markedly increased in *Sdc1−/−* livers comaped with Wt livers starting at 12 h post-APAP. Moreover, a specific inhibitor of caspase-3 (Ac-DVED-CMK) significantly attenuated liver injury in both APAP-injected *Sdc1−/−* and Wt mice. Studies with selectively modified heparan compounds revealed that 2-*O*-sulfated heparan compounds (HS, heparin, *N*-desulfated heparin, *N*- and 2-*O*-sulfated heparosan) inhibit APAP-induced liver injury and caspase-3 activation, but not those without this modification (2-*O*-desulfated heparin, heparosan, *N*-sulfated heparosan). These data indicate that syndecan-1 is a critical endogenous inhibitor of APAP-induced liver injury and suggest that this is accomplished through the inhibition of hepatocyte apoptosis by 2-*O*-sulfated HS domains in syndecan-1.

(265) Development of structurally-defined heparan sulfate/heparin oligosaccharide libraries using 2D chromatography and mass spectrometry-based structural determination

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Heparan sulfate (HS) is a long linear polysaccharide that exists in all animal tissues and functions in diverse biological processes. Although efforts have been made to synthesize structurally diverse HS oligosaccharides, libraries composed of biologically derived saccharides from natural sources would provide a valuable platform for investigating HS structure–function relationships. Developing such a library has long been hindered by lack of techniques for structure determination. Here, we develop and characterize HS oligosaccharide library using two/three dimensional chromatography and structure determination techniques that relies on the combination of chemical derivatization and LC-MS/MS with pulsed post-column addition of sulfolane, plus the newly developed electron excitation dissociation on FT-ICR MS. Various degrees of polymerization (DP) of HS oligosaccharides were generated using single heparin lyase digestion of HS and heparin from porcine intestine mucosa. Specifically, HS oligosaccharides libraries from heparin lyase I, II or III digestion and a heparin oligosaccharide library from lyase I digestion were prepared using a first dimension of gel filtration chromatography. Oligosaccharide composition of each collected fraction were profiled using hydrophilic interaction chromatography mass spectrometry (HILIC-MS) and fractions with the same DP ranging from 4 to 8 were pooled for a second dimension of separation using preparative strong ion exchange chromatography. Fractions corresponding to chromatographic peaks were desalted and profiled using HILIC-MS for compositions and purities. A third dimension of separation using semi-preparative HILIC was used to for impure fractions. Pure oligosaccharide fractions were derivatized using selective de-*N*-sulfation and subsequent d_3-*N*-acetylation. Sulfate reduced fractions were then analyzed using LC-MS/MS with on-line charge state manipulation for more informative tandem MS spectra. A newly developed tandem MS method of electron excitation dissociation in the negative mode of FT-ICR MS was also employed to facilitate the structure determination of the pure fractions for building up the libraries.

(266) Strategy for large-scale release and purification of sialylated *N*-linked glycans by hydrazinolysis and SAX-HPLC

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The goal of this study is to establish a method for large scale release and purification of sialylated *N*-glycans from glycoproteins and subsequent use of the purified sialylated *N*-glycans as

homogenous standards for NMR and MS studies. The most conventional method for release of *N*-glycans is using the enzyme PNGase F. However, large scale release of *N*-glycan by this method is expensive and therefore not practical. Hydrazinolysis would be the best approach for release *N*-glycans in large scale. However, anhydrous hydrazine, which is commonly used for conventional hydrazinolysis, is highly toxic and explosive. Because of that, the use of the procedure in the laboratory level has been strictly limited in small scale (e.g. from a few microgram to a few mg of glycoprotein). We have used hydrazine monohydrate, which is significantly safer reagent than anhydrous hydrazine, as a replacement of anhydrous hydrazine and optimized this method to obtain a higher yield of *N*-glycans in large scale. We have used 100 mg of bovine fetuin as starting material and released the sialylated *N*-glycans and analyzed the products by MALDI-MS and SAXHPLC using post-column fluorescent labeling. We overcame the peeling effect observed in the hydrazine monohydrate-procedure by directly applying the reaction mixture to an anion exchange column chromatography instead of drying/neutralizing hydrazine monohydrate after the reaction. The recovery of sialylated *N*-glycans by our procedure is compatible to that by PNGase F. Our analysis showed that the sialic acids on released *N*-glycans were stable during entire procedures and no peeling product was observed. The released *N*-glycans were further purified according to the number of sialic acids by using SAX-HPLC. The purities of each fraction obtained after entire process were above 90%. We purified sufficient *N*-glycans (1 mg) that can later be used for MS and NMR studies.

(267) Mass spectrometry-based strategies for high-sensitivity mapping of glycoepitopes involved in mediating cell–cell interactions

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Recent advances in mass spectrometry have offered many new tantalizing possibilities for de novo identification of glyco-epitopes. It serves not only to validate mapping by monoclonal antibodies against any cross-reactivity, but also enable unanticipated discovery. We have largely focused our analytical effort on two different fronts. The first relies on glycomic profiling of permethylated glycans, which has the advantage of facile screening for the expression of sulfated and non-sulfated glycotopes from the same sample preparation in two separate LC-MS/MS modes. Critical fragment ions informative of the targeted epitopes including the location of sulfate were identified and several concerted mapping strategies at the MS2 level were developed. A second complimentary approach is based on analysis of glycopeptides, which allows mapping of the terminal glycotopes in its native form and identification of its protein carrier at the same time in favorable cases. We compared the 2 approaches, drew conclusions on their respective strengths and evaluated the sensible amount of cells/tissues needed to gain an informative glycomic impression.

(268) Shotgun glycan glycomics studies of influenza virus pathogenesis

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Influenza virus is a wide-spread disease, which clearly requires novel insights into the mechanisms for influenza virus infection, especially hemagglutinin–host surface interaction. It is known that terminal sialic acids on cell surface glycoconjugates are part of the glycan determinants recognized by influenza viruses. Identification of the endogenous natural ligands for influenza viruses will significantly promote our understanding of the infection and transmission process. In this work, we have applied a novel glycotechnology, termed "Shotgun Glycomics", to this specific question. Glycoconjugates from the pig lung have been isolated, fluorescently tagged and separated using multidimensional HPLC. This tagged glycan library has been printed to generate glycan microarrays, and these arrays have been analyzed with various strains of influenza viruses. The strong binding receptors have also been characterized. The success of this work will lead to identify the natural glycan ligands in lung that are recognized by different avian, swine and human influenza strains and possibly lead to important insights into species specificity and transmission of influenza viruses. The long-term development of these approaches should allow for their extension to human cells and tissues, and those of other species, and have relevance for our understanding of the genesis of influenza pandemics.

(269) An easy way to desulfonate 2 *O*-sulfonate heparin

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Heparin (HP) is highly populated with *O*-sulfonated/*N*-sulfonated groups that appear to mediate its biologic functions. In this project, commercial HP was degraded with pronase in a mixture of 0.1 M Tris/0.5% sodium dodecyl sulfate/10 mM $CaCl_2$, pH 7.73, at 37°C for 24 h. The reaction mixture was then precipitated with 1.2-reaction volumes of 95% (v/v) ethanol at 4°C overnight. Solid HP obtained was dissolved in 5% NaOAc/0.5 mM HOAc, pH 5.0, combined with 20 µg of iduronate 2-sulfatase/IDS and incubated at 37°C for 24 h. Degraded protein was removed with chloroform-amyl alcohol at room temperature for 1 h and centrifuged. HP in the liquid layer was precipitated with 5-reaction volumes of absolute ethanol at 4°C overnight, centrifuged and washed. The precipitate was analyzed using FT-Infrared spectroscopy. The data show the parent HP with 6 S = O sulfonated bands at 1290–1200 cm^{-1} region, one of them being at 1258 cm^{-1}. After digestion with Iduronate 2 *O*-sulfatase, the same sample displayed 5 S = O sulfonates, with no band at 1258 cm^{-1}. Obviously, HP lost its 2 *O*-sulfonates. This method presents an easy way to change HP sulfonated moieties and thus manipulate its biologic functions.

(270) Characterization of new glycosidases

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The study of glycoprotein expression requires an array of techniques and tools to isolate and identify all different glycoforms present in a given sample. Among these, glycosidases of different specificities are useful to elucidate the underlying molecular structure of glycans. PNGaseF is commonly used to remove *N*-glycans. However, its main limitation is that it cannot release *N*-glycans that have an α1-3 fucose on the core GlcNAc residue.This type of modification occurs primarily in plants and insects. PNGase A (an enzyme isolated from Almond) has been shown to release these α1-3 fucose modified *N*-glycans from glycopeptides but not from glycoproteins. Like PNGaseA, there are several other endoglycosidases with defined properties, which makes them useful tools to complement the available analysis tools. We report here on the characterization and activity of new recombinant glycosidases on defined glycan substratums.

(271) Cross-talk Between SCFA-Hexosamine Analog-mediated Metabolic Flux and Intracellular Esterase Activity

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The field of metabolic glycoengineering has come to rely on monosaccharide analogs appended with ester-linked short chain fatty acid (SCFA) groups, primarily acetate or n-butyrate. The SCFA increase the lipophilicity and subsequent membrane permeability of these compounds. Despite improved efficiency of these compounds, the possibility of efflux from cells by P-glycoprotein (Pgp) was raised by the increased lipophility of these compounds comaped with non- SCFA-derivatized monosaccharides. Moreover, the use of SCFA-hexosamine analogs in biological applications has been hindered by poorly understood cytotoxicity that we hypothesized could result from the diversion of esterase activity from necessary cellular functions to analog processing when cells are exposed to these compounds. In this presentation, we report a disconnect between the impact of analog on Pgp in cell free assays (where many butanoylated analogs stimulated Pgp ATPase activity) comaped with whole cell assays where dye efflux assays indicated a buildup, not efflux, of substratum in analog-treated cells. This disparity was resolved by proposing that certain SCFA-hexosamines counter-intuitively increased the uncommitted esterase capacity found within a cell. Interestingly, this increase occurred without an increase in expression levels for a suite of intracellular esterases when analyzed at the mRNA level by qRT-PCR or at the protein level by western blots or flow cytometry. Instead, increased esterase activity was correlated with increased sialylation of *N*-glycans found on human carboxylesterase 1 (CES1), an esterase implicated in "detoxification" of xenobiotics. It is noteworthy that in the past the activities of secreted esterases have been reported to be affected by glycosylation but to our knowledge this is the first example of intracellular (although membrane-associated) esterase activity being modulated by changes to glycan structure.

(272) Extracellular Esterase and Intracellular Processing of SCFA-Hexosamine Analogs: Implications for Metabolic Oligosaccharide Engineering and Drug Delivery

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There are many potential biomedical applications for metabolic glycoengineering ranging from imaging and diagnostics to the treatment of diseases ranging from cancer to viral infections. A pitfall of this technology, which is inefficient analog utilization, has been rectified in part by the introduction of short chain fatty acid (SCFA) groups into analog design to increase cellular uptake. This strategy is based on the premise that outside of a cell, the SCFA moieties ideally will be largely ignored by esterases and the "prodrug" will reach the targeted cell unscathed. Once the analog reaches a target cell, however, the SCFA protecting groups will be removed rapidly allowing the core sugar to intercept the targeted glycosylation pathway. This presentation provides in vitro experimental evidence that esterase processing of SCFA-hexosamine analogs meets these criteria by presenting data that demonstrates that extracellular hydrolysis of these compounds is comparatively slow under normal cell culture conditions while intracellular processing is much quicker. Negligible degradation of butanoylated ManNAc analogs was observed in 10 or 25% fetal bovine serum (FBS) in the absence of cells although slow hydrolysis was observed in 100% FBS. Structure activity relationship analysis of common ManNAc analogs used in metabolic glycoengineering revealed that acetylated compounds were more refractory against extracellular inactivation by FBS than their butanoylated counterparts; for example, Ac_4ManNAc activity was not reduced even after incubation in 100% FBS for 48 hours. By contrast to analog stability in serum-like conditions, both acetylated and butanoylated analogs supported increased sialic acid production in cells within 4 to 8 hours, indicating that intracellular esterase processing was rapid. Finally, in silico docking simulations of analogs to human carboxyl esterase 1 (CES1) were consistent with the experimental results, providing indirect evidence that this, or similar, esterases were primarily responsible for the hydrolysis of ester-linked SCFA groups from hexosamine analogs.

(273) The Urine Glycome as a Source of Biomarkers: GlycanMap® Analysis of Free Oligosaccharides and *O*-Linked Glycans

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Urine is an attractive sample type for biomarker discovery due to its non-invasive nature and the presence of both free and protein-bound glycans. We applied our robust, high-throughput glycan analysis technology, the Glycan *Map* ® platform, to evaluate the

potential of urine for biomarker research. Urinary free oligosaccharides (uFOS) were profiled directly using 20 µl of urine from healthy human donors. Blood-type related oligosaccharides were readily observed, including the previously reported pentasaccharides containing fucosylated glucose at the reducing end (Lundblad *et al.*, 1967): α-GalNAc-(1 → 3)-(α-Fuc-[1 → 2])-β-Gal-(1 → 3)-(α-Fuc-[1 → 4])-Glc, A-Le^b-pentasaccharide for blood type A and α-Gal-(1 → 3)-(α-Fuc-[1 → 2])-β-Gal-(1 → 3)-(α-Fuc-[1 → 4])-Glc, B-Le^b-pentasaccharide for type B. In O-blood type donors, we detected H substance lacking terminal Gal or GalNAc found in A or B substances. Some individuals exhibited high concentrations of the hexasaccharide Hex3HexNAc1dHex2. Interestingly, those individuals who exhibited the hexasaccharide uFOS also showed significantly lower levels of blood type A or B pentasaccharides, suggesting that the presence of the hexasaccharide corresponded to a non-secretor phenotype. Protein-bound, *O*-linked glycans were also evaluated in urine samples. Following concentration by ultrafiltration, Oglycans were chemically released from the proteins and then analyzed using our Glycan*Map*® technology. Several mucin-type *O*-linked glycans could be readily detected and quantitated in urine, demonstrating that urine is a valuable source for glycomics research. In summary, we have evaluated the free oligosaccharide and *O*-linked glycan profiles in human urine. Our method could simultaneously determine both blood type and secretor status and detected several other oligosaccharides in uFOS. This feasibility study demonstrated the potential of our Glycan*Map*® platform to monitor *O*-glycome and glycan metabolites in non-invasive samples, which we believe can be applied to diagnosis and monitoring of diseases including inherited metabolic disorders.

(274) Application of systems biology to construct and simulate glycosylation reaction networks

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Glycosylated proteins play structural and functional roles in diverse biological processes including inflammation, cancer and development. Recent advances in analytical tools now enable the characterization of entire glycomes. In this paper, we present our efforts to develop a mathematical modeling framework that can be applied to analyze such experimental data. This approach uses object-oriented programing and graph theory to construct glycans, enzymes, reactions, pathways and compartments in silico. Such definitions allow the integration of XML (eXtensible Markup Language)-based glycan structures into Systems Biology Markup Language (SBML) files that contain machine-readable computational models. High-quality visualization of glycosylation reaction networks and integration with glycomics-based databases is also enabled. Thus, information cataloged in these databases is used to inform model structure. Quantitative simulation of the glycosylation network is performed by simulating the "Master Pathway" which describes the "maximum-size" glycosylation pathway that includes all possible reactions in a given system. Additional analysis is performed by analyzing subsets of this master model and by collating the findings using hierarchical clustering, principal component and sensitivity analysis. These post-simulation analysis methods identify regulating enzymes and reactions that control system behavior. They also generate experimentally testable hypotheses. All procedures are implemented in a new MATLAB-based toolbox called Glycosylation Network Analysis Toolbox (GNAT). Examples are presented for mathematical models that: 1) simulate N-linked glycosylation initiation and branching, 2) model O-glycosylation networks and 3) determine "feasible" glycosylation reaction networks that can fit experimentally measured mass spectrometry data. Overall, we present a structured modeling approach for the synthesis of glycosylation reaction network models. Beyond the use of statistical approaches, such a framework may allow querying of experimental data using biological or mechanistic hypothesis.

(275) Designer-oligosaccharide microarrays to decipher ligands in mammalian and prokaryotic glucan-recognition systems

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Glucans are homopolymers of D-glucose and are constituents of fungal, bacterial and plant cell walls. Their specific recognition by glucan-binding proteins is important in mediating diverse biological processes in animals, plants and microbes. These include innate and acquired immunity to pathogens, modulation of immune responses, pathogen virulence and plant cell wall biodegradation. This communication will be focused on development and applications of "designer" microarrays of oligosaccharides derived from glucan polysaccharides of fungal, plant and bacterial origins for studies of glucan-recognition systems. The term "designer" microarray refers to a microarray of oligosaccharide probes generated from ligandbearing glycoconjugates to enable the characterization of ligands that these harbor (Palma et al. 2006, 2012). The microarray system is based on the neoglycolipid (NGL)-technology for generating lipid-linked oligosaccharide probes (Feizi and Chai 2004; Liu et al. 2009, 2012). The microarrays comprise 150 glucan-NGL probes, including linear 1,2-, 1,3-, 1,4- and 1,6-linked glucose sequences with α- and β-configurations ranging from 2 to 13 mers; linear sequences with mixed β1,3/β1,4 and α1,4/α1,6 linkages and branched β1,3/β1,6 sequences. The prototype designer microarray has served to assign oligosaccharides recognized by Dectin-1, the major receptor of the mammalian innate immune system for fungal pathogens (Palma et al. 2006, 2012); also by murine antibodies elicited using glucan-based vaccines with potential for anti-fungal therapeutics (Torosantucci et al. 2009; Capodicasa et al. 2011). We have extended this approach to studies of recognition by microbial non-catalytic carbohydrate-binding modules (CBMs) of modular glycoside hydrolases. These play a crucial role in potentiating the efficiency of degradation of

plant cell wall polysaccharides (Fontes and Gilbert 2010). The microarray analyses reveal the different patterns of glucan recognition among these proteins, e.g. glucose linkage specificity and oligosaccharide chain length requirement.

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(276) Synthesis of sialylated poly-LacNAc *N*-linked and *O*-linked glycans

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N-and *O*-linked glycans are biologically important post-translational modifications of glycoproteins on the cell surface, which play a fundamental role in many processes, including cell adhesion, signal transduction and immune response. Extended poly-*N*-acetyllactosamine (pLN) glycans found on glycoproteins as well as glycolipids of some cells have been characterized as specific ligands for different lectins such as selectins and galectins as well as being tumor-associated antigens. Poly-LacNAc chains are able to be further modified by sialylation, fucosylation and *O*-sulfation. Extended sialoside glycans have shown to influence the receptor-binding specificity in studies on influenza-hemagglutinin receptors. Due to the complexity of natural glycans containing extended pLN sialosides, the availability of synthetic structures for biological studies has been limiting. Here, we report the enzymatic synthesis of a library of pLN *N*- and *O*-linked sialosides. We had previously synthesized pLNs on *N*- and *O*-glycans using a new Helicobacter β1-3GlcNAc transferase (Sauerzapfe B. et al. *Glycoconj J.* **2009**, *26*,141) and β4GalT1, for galectin galectin specificity. This series included biantennary and triantennary *N*-glycans with or without corefucose, core 1, 2, 3, 4, 6 *O*-glycans with each arm extended to penta- or hexa-LN. Now, over 100 NeuNAc or NeuNGc sialosides have prepared using either rat ST3Gal-III or human ST6Gal-I to form α2-3 or α2-6 linkages, respectively, on the Gal terminating intermediates. These glycans are suitable for elaborating on a focused microarray for analysis of Siglecs and influenza-hemagglutinin receptor specificity. Supported in part by NIH grants HL107151 and AI058113.

(277) High-Throughput *N*-Linked Glycan Analysis to supports Cell line and Process Development

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Glycosylation is one of the quality attributes critical for the efficacy, safety and stability of therapeutic proteins. In order to produce proteins with proper glycosylation, screening of thousands of clones and process optimization are critical. Conventional glycosylation assays, such as liquid chromatography and mass spectrometry are labor intensive and time consuming. Here, we present applications of Caliper LabChip GXII for high-throughput (HTP) analyses of N-linked glycosylation in 96-well plates. Cell culture samples were purified by HTP small scale purification followed with HTP PNGase-F digestion, isolation of glycan and labeling. We have also studied the comparability between GXII LabChip and HPLC platforms. With GXII LabChip analytical capability, we can assess *N*-linked glycan analysis in the early stage of clone screening, as well as expedite the cell line and process development.

(278) Salt-free guanidination for maximizing characterization of proteins and glycoproteins using matrix-assisted laser desorption/ionization time of flight mass spectrometry

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Full characterization of proteins with respect to the amino acid sequence and glycosylation continues to be a focus of proteomics research using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Maximizing the amino acid sequence coverage using a single digest would benefit the characterization of low-quantity proteins. The characterization of glycosylation is challenged by low-signal intensities of high-mass glycopeptide ions. Guanidination increases the detection of tryptic peptides containing lysine. High-salt loads from this derivatization are problematic even after micro-extraction clean-up which has limited the applications of this method. This study introduces a salt-free guanidination procedure which uses the freebase of the guanidination reagent, *O*-methylisourea (OMIU). Salt-free guanidination of the standard proteins cytochrome c (CYC), bovine serum albumin (BSA) and the glycoprotein horseradish peroxidase (HRP) required no desalting prior to peptide mass fingerprinting. Use of this procedure allowed for higher than 90% amino acid sequence coverage for BSA and HRP. Complementing this procedure with top-down sequencing allowed for full coverage of CYC. For the characterization of HRP glycosylation, the presence of OMIU in the sample/matrix spot resulted an even ion distribution and the enhanced detection of high-mass range ions which significantly improved the characterization of glycosylation. Salt-free guanidination maximizes information gained from a single digest and avoids additional purification steps which are potential avenues for sample losses.

(279) Disrupting the sugar-coat: Inhibition of mucin-type O-glycosylation through metabolic glycan engineering

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Almost all membrane proteins, classified as clusters of differentiation (CD), on mammalian cells are glycoproteins. "Glycocode" envisages that each cell type displays a unique signature of glycan patterns which governs immune response and cell fate. Mucin-type O-glycosylation is initiated by addition of a-*N*-acetyl-D-galactosamine (a-GalNAc) by polypeptide:UDP-GalNAc transferases (ppGalNAcT) to Ser/Thr and orchestrated by a complex, yet hierarchical, biosynthetic machinery. Particularly, mucin-type *O*-glycans are involved in leukocyte homing, extravasation and cell death. Understanding functions of *O*-glycans in cellular physiology is hampered by lack of tools to modulate their biosynthesis. Metabolic glycan engineering (MGE) is a powerful chemical tool for study of glycans that exploits promiscuity of biosynthetic enzymes involved in glycosylation pathways to process non-natural monosaccharides. Herein, we present our results of investigation of effects of a panel of non-natural GalNAc analogs on O-glycosylation using CD43 (leukosialin/sialophorin) as an example. CD43 is a major cell surface antigen in lymphocytes known to act as negative regulator of immune activation. CD43 contains 80 *O*-glycan chains attached to polypeptide and exhibits a rod-like structure. Peracetylated *N*-thioglycolyl-D-galactosamine (Ac_5GalNTGc), but not *N*-glycolyl-D-galactosamine (Ac_5GalNGc), induced drastic hyposialylation and hypoglycosylation of CD43 in Jurkat (human T-lymphoma) cells, in a sulfhydryl and analog structure-dependent manner. Metabolic incorporation of GalNTGc was confirmed by immunoprecipitation and thiol-selective biotinylation of CD43 FLAG-tagged at C terminus. Investigation of global effects in Jurkat cells using lectin staining revealed selective abrogation of MAL-II (NeuAc-a-2 → 3-Gal) epitopes, but not SNA (NeuAc-a-2 → 6-Gal) epitopes, upon treatment with Ac_5GalNTGc. Additionally sialylation of CD45 and CD162 (PSGL-1) were found to be reduced as revealed by neuraminidase-sensitive antibodies. A comparison between Jurkat and K562 (human erythroleukemia) cells revealed similar analog-dependent effects. However, the profile of gene expressions of ppGalNAcT (T1-T14) was found to be distinctly different. Mechanistically, unlike α-*O*-benzyl-GalNAc, a known inhibitor of *O*-glycan biosynthesis that acts as a decoy for glycosyltransferases, Ac_5GalNTGc inhibits elongation of *O*-glycans by direct biosynthetic incorporation. This class of inhibitors provides a novel approach to gain further insight into identities of physiological protein substratums of glycosyltransferases and would be valuable in designing isoform-specific ppGalNAcT inhibitors.

(280) Mass spectrometric profiling of heparan sulfate on tissue histological slides

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Hepran sulfate (HS) is expressed on cell surfaces and in extracellular matrices in a spatially and temporally regulated fashion, constituting the environment for cells to interact, migrate and proliferate. Through binding with a great number of proteins, HS regulates many facets of biological processes from embryonic development to normal physiological functions. Recently, HS has been shown to be involved in pathologic changes and immunological responses including cancer metastasis and inflammation. Past analyses of HS have been focused on cell lines, body fluids and relatively large tissue samples. Therefore, it is important to be able to detect and profile HS at the histological scale to reveal the functions of HS in pathology and to potentially inform diagnosis and prognosis. In this study, we successfully detected HS from bovine brain stem, cortex and cerebellum histological slides by using size exclusion chromatography/mass spectrometry (SEC/MS). Frozen brain sections were cut into 15 μm thickness and with a series of solvent solutions to remove lipids before applying heparin lyases I, II and III on the tissue surface within 5 mm × 5 mm digestion spots. The SEC/MS profiling of HS disaccharides showed differences for spatially separate digestion spots on the same slides and remarkable differences among the three bovine brain tissues analyzed.

(281) Analytical services and trainings at the complex carbohydrates research center

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The Analytical Services Laboratory of the Complex Carbohydrates Research Center (CCRC) at the University of Georgia is a non-profit entity that offers services for the structural characterization of glycoconjugates derived from animal, plant or microbial origin. Our laboratory has many years of analytical experience over a wide array of glycoconjugates. We offer both routine as well as tailor-to-research needs analytical services. The service laboratory is complimented with state-of-the-art instruments such as LTQ-Orbitrap-MS, AB 5800 MALDI-TOF/TOF-MS, and high-field NMRs in addition to HPLC, HPAEC and GC-MS. Here, we present examples of projects in our laboratory that employ a combination of analytical techniques for structural elucidation of glycoproteins and polysaccharides. Briefly described below are some analyses for each class of glycoconjugate. Glycoproteins: release of *N*- and *O*-linked glycans from purified, cells or gel-prepared glycoproteins, mapping N- and O-linked glycosylation sites on peptides, identification of type of *N*-linked glycans, i.e. biantennary, triantennary, tetraantennary, high mannose, hybrid or complex, determination of composition, ring size, anomeric configuration, linkage and sequence of residues in oligosaccharides and determination of points of attachment, composition and linkage of non-carbohydrate constituents such as phosphate and sulfate; polysaccharides: purification to homogeneity by SEC and/or ion exchange chromatography and determination of glycosyl composition, linkage, ring size and anomeric configuration; lipopolysaccharides (LPS): isolation of LPS directly from bacterial cells, release of lipid A from *O*-antigen and characterization of lipid A by MS and *O*-antigen by techniques used for polysaccharides; lipids and glycolipids: determination of fatty acids composition of ceramide, diacylglycerol or triacylglycerol and phospholipids. In addition, we have developed methods for purification and sequencing of glycosaminoglycans through various chromatography, MS and NMR techniques. We also offer annual hands-on training courses for the structural characterization of both glycoproteins and polysaccharides.

(282) α1,6-Fucosyltransferase (Fut8) is implicated in vulnerability to smoke-inducedemphysema in mice and a possible non-invasive predictive marker for humanchronic obstructive pulmonary disease (COPD)

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Deficiency in core fucosylation by the genetic disruption of α-1, 6-fucosyltransferase (*Fut8*) leads to lethal abnormalities and the development of emphysematous lesions in the lung by attenuation of TGF-β1 receptor signaling (Wang et al. 1995). The physiological relevance of core fucosylation in the pathogenesis of COPD was herein investigated using cigaret smoke (CS) exposed heterozygous knockout mice ($Fut8^{+/-}$) (Gao et al. 2012). The $Fut8^{+/-}$ mice exhibited a marked decrease in FUT8 activity and matrix metalloproteinase (MMP)-9 activities were elevated in the lung at an early stage of exposure. Emphysema developed after a 3-month CS exposure, accompanied by the recruitment of large numbers of macrophages to the lung. Moreover, we investigated whether reduced Fut8 levels leads to COPD with increased inflammatory response in humans and is associated with disease progression among symptomatic current or ex-smokers with stable COPD or at risk outpatients (n = 226) (Kamio et al. 2012). Although FUT8 activity significantly increased with age among the at risk patients, this association was not clearly observed in the case of COPD patients. A faster annual decline of FEV_1 was significantly associated with lower FUT8 activity. Patients with lower FUT8 activity experienced exacerbations more frequently. These data suggest that reduced FUT8 activity is associated with the progression of COPD and serum FUT8 activity is a minimally invasive predictive biomarker for progression and exacerbation of COPD.

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(283) Identification of an endogenous *E. coli* glycosidase with activity toward *O*-β-GlcNAc-modified proteins

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Changes in the *O*-β-N-acetylglucosamine (*O*-β-GlcNAc) modification of human proteins have been linked to many health complications, including heart disease, Alzheimer's disease and cancer. The extensive study of the *O*-β-GlcNAc modification is crucial, as it is currently not yet known precisely how it alters the enzyme activity in biological processes. Current methods used to produce *O*-β-GlcNAc-modified proteins only provide low yields, less than milligram quantities. Higher amounts of protein are necessary for the three-dimensional (3D) structure determination of modified proteins, which will aid in the investigation of enzyme mechanisms. We have developed a co-expression system using human *O*-β-GlcNAc transferase (OGT) to produce *O*-β-GlcNAc-modified proteins in vivo. Our preliminary data show that an *E. coli* glycosidase is preventing high yields of *O*-β-GlcNAc-modified proteins with our system. The aim of this project is to isolate and positively identify the enzyme involved in the cleavage of *O*-β-GlcNAc in *E. coli* and use the corresponding knockout strain for the co-expression system.

(284) In vitro inhibition of sialic acid expression

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Sialic acids are essential constituents of glycoconjugates. Due to their position at the outermost of glycan chains, sialic acids are involved in many physiological and pathophysiological cell functions. The bifunctional enzyme UDP-GlcNAc-2-epimerase/ManNAc-kinase (GNE) is the key enzyme of sialic biosynthesis. Divided in an N-terminal domain with 2-epimerase activity and a carboxyterminal part with kinase activity, this enzyme catalyzes the first two steps in the de novo-synthesis of all sialic acids. Inhibition of the GNE would lead to a decreased synthesis of sialic acids and therefore reduce cell surface sialylation. The reduction in sialic acid expression in tumor cells may advance their recognition by the immune system and thus could be a helpful application in cancer therapies. Based on the crystal structure of the ManNAc-kinase domain of GNE (hMNK, PDB ID: 2YHW) 3-*O*- and 6-*O*-modifications of its natural substrate ManNAc have been synthesized and tested as potential inhibitors. There are compounds in both groups that are more potent inhibitors, than all other substrate modifications previously analyzed. Spectrophotometric assays as well as surface plasmon resonance studies and dynamic light scattering with purified hMNK revealed that 3-*O*-modifications of ManNAc are non-competitive but likely specific in their mode of inhibition whereas 6-*O*-modifications competitively inhibit the enzyme's activity. A reduction in cell surface sialylation in various cell lines was attained with peracetylated compounds and demonstrated by flow cytometry experiments with sialic acid-binding lectins.

(285) Ultrahigh-throughput screening campaign to identify compounds enhancing glycosylation of α-dystroglycan

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Proper O-mannosylation of α-dystroglycan (a-DG) is essential for its ligand-binding activity, which is critical for many biological events. Hypoglycosylation of a-DG is associated with human diseases such as muscular dystrophies and cancers. Increasing evidence suggested that enhancing the glycosylation of a-DG may lead to cure these diseases. In order to identify small compounds enhancing the glycosylation of a-DG, we developed a cell-based assay, in which a CHO cell line stably expressing a secreted form of human a-DG with human Ig domain tag is used as the screening cell and negative control. This cell line stably expressing *Large*, a glycosyltransferase, which induces the functional glycosylation of a-DG serves as the positive control cell. The glycosylation of a-DG is monitored by an antibody recognizing the functional glycans on a-DG coupled with AlphaScreen technology. With this system we screened the MLPCN collection of 364,000 compounds at 10 uM. The assay ran well with a Z'(GD-corrected) of 0.61, robust Z'(GD-corrected) 0.59, S/B 63.3, S/N 328 and signal window of 5.1. Total 646 hits were identified from primary screening. After replication of the primary screening hits and dosage experiments, a number of compounds that can significantly enhance the glycosylation of a-DG have been identified, among them a cluster of 5 compounds with IC50 2–6 μM. >50% increase over negative controls. Further study is ongoing. Our results showed the feasibility of Ultrahigh-throughput screening (uHTS) in identifying novel compounds that enhance the glycosylation of a-DG.

(286) Functional glycomic studies of human milk glycans reveals presence of embryonic stem cell biomarkers and virus receptors

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Human milk contains large number of structurally diversified free glycans that are believed to play important roles in infant health. Previous studies of human milk glycans (HMGs) have focused on determining the structures of purified glycans and testing the functions of either limited numbers of defined HMGs or isolated fractions of glycan mixtures. To investigate the biological roles and to identify the functional components of the human milk glycome, we developed "Shotgun Glycomics". We derivatized HMG fractions with a bifunctional fluorescent tag and purified individual fluorescent derivatives by multidimensional HPLC to generate a tagged glycan library (TGL) made up of 127 glycans. The TGL was printed as a shotgun glycan microarray (SGM) and validated by interrogating with defined plant lectins and antibodies. The results not only verified the utility of the HMG microarray, but also provided useful preliminary structural information about the human milk glycome. We discovered that HMGs contained human pluripotent stem cell biomarkers that were identified by Anti-TRA-1 antibodies. The observation that human influenza viruses, as well as Minute Viruses of Mice, specifically bound certain HMGs demonstrated the potential function of HMGs as a part of the infant defense system against pathogens. Finally, the structures of selected, pluripotent stem cell markers and virus receptors among the HMGs were identified via an on-microarray glycan sequencing method that predicts glycan structure utilizing binding data from defined glycan-binding proteins (GBPs) collected before and after exoglycosidase treatments. The human milk SGM provides a valuable resource for studying the functional glycomics of HMGs, which are components of the liquid innate immune system of infants, and once the structures are defined the array will be a natural glycan library for studying the specificities of glycan-binding proteins.

(287) GlycReSoft: A software tool for processing glycomics and glycopeptidomics LC/MS profiling data

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These modifications are biosynthesized in the endoplasmic reticulum and Golgi apparatus by a series of enzymatic transformations that are under complex control. As a result, mature glycans on a given site are heterogeneous mixtures of glycoforms. This gives rise to a spectrum of adhesive properties that strongly influences interactions with binding partners and resultant biological effects. In order to understand the roles glycosylation plays in normal and disease processes, efficient structural analysis tools are necessary. In the field of glycomics, liquid chromatography/mass spectrometry (LC/MS) is used to profile the glycans present in a given sample. This technology enables comparison of glycan compositions and abundances among different biological samples, i.e. normal vs disease, normal vs mutant etc. Manual analysis of the glycan profiling LC/MS data is extremely time-consuming and efficient software tools are needed to eliminate this bottleneck. In this work, we have developed a tool to computationally model LC/MS data to enable efficient profiling of glycans. Using LC/MS data deconvoluted by DeconTools, we built a list of unique neutral masses corresponding to candidate glycan compositions summarized over their various charge states, adducts and range of elution times. Our work aims to provide confident identification of true compounds in complex data sets that are not amenable to manual interpretation. This capability is an essential part of glycomics work flows. We demonstrate this tool, GlycReSoft, using an LC/

MS data set on tissue-derived heparan sulfate oligosaccharides. The software, code and a test data set are publically archived under an open source license.

(288) Evolutionally Conserved Blood Group Glycolipids are Endogenous Antigens forInvariant Natural Killer T Cells

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CD1d-restricted invariant natural killer T cells (iNKT) express evolutionally conserved V 14 $^{+}$ (mouse) or Va24^{+} (human) T-cell receptors (TCR), which are defined by specific recognition of the marine sponge.derived glycosphingolipid a-galactosylceramide. During development of iNKT cells in the thymus, endogenous lipids are necessary for positive selection of randomly generated TCRs from mainstream T-cell precursors. The identities of the self-lipids that select iNKT cells *in vivo* remain elusive. Our glycosphingolipidomics analysis of thymuses of mouse, pig, and human identified blood group glycosphingolipids that are structurally but not genetically related to isoglobotriaosylceramide (iGb3), a previously identified ligand for iNKT cells. We found that some of these iGb3-related glycosphingolipids can activate iNKT cells and stimulate their production of cytokines. These findings indicate a surprising function of evolutionally conserved blood group glycolipids as agonist ligands for iNKT cells and reveal the presence of multiple ligands that might be related to the physiology of iNKT cells.

(289) Formation of an enzyme complex for efficent production of keratan sulfate glycosaminoglycan

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Keratan sulfate (KS) proteoglycan is one of the major components of the corneal extracellular matrix and suggested to have an important role for function of the cornea. Corneal KS glycosaminoglycan (GAG) is highly sulfated by two distinct carbohydrate sulfotransferases, corneal GlcNAc 6-*O* sulfotransferase (CGn6ST, also known as GlcNAc6ST-5/GST4) and KS Gal 6-*O* sulfotransferase (KSG6ST). We prepared a lentiviral vector that produces both sulfotransferases in the infected cells and infected the lentiviral vector to different cultured cell lines, HeLa cells and human corneal epithelial (hCE) cells. We confirmed the sulfotransferases are expressed in the infected cells and also detected production of highly sulfated KS GAG in hCE cells. However, production of highly sulfated KS GAG is minimal in HeLa cells, suggesting that HeLa cells lack required components for efficient production of the carbohydrate. We then analyzed gene expression of β1,3-GlcNAc transferases by RT-PCR and found that HeLa cells lack expression of *B3gnt7*, which encodes β1,3-GlcNAc transferase-7 (β3GnT7). To confirm the requirement of β3GnT7 for highly sulfated KS GAG production in HeLa cells, we overexpressed β3GnT7 in sulfotransferase-expressing HeLa cells and observed enhanced production of highly sulfated KS GAG in the cells. By immunoprecipitation analysis, we found that three tagged enzymes, β3GnT7-HA, CGn6ST-flag and KSG6ST-T7, were co-precipitated by anti-FLAG antibody-conjugated beads, indicating that the three enzymes form a complex to process efficient KS GAG production in the cells.

(290) Complex *N*-glycans are essential for spermatogenesis

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Complex *N*-glycans are generated in the Golgi following the action of MGAT1 (GlcNAcT-I), the enzyme that transfers GlcNAc to initiate hybrid and complex *N*-glycan synthesis. This class of glycans is essential for life. Mouse embryos that lack MGAT1 cannot survive beyond embryonic day 9.5 (E9.5). We have investigated the role of MGAT1 during spermatogenesis using conditional deletion in spermatogonia via a testis-specific *Cre* recombinase transgene driven by the Stra8 promoter (Stra8-*iCre*). The deletion efficiency of MGAT1 floxed alleles was 100%, as shown by the transmission of the deleted allele by heterozygous mice, as well as by lectin histochemistry of conditional mutants. Males, in which the synthesis of complex *N*-glycans was blocked by deletion of *Mgat1* in spermatogonia, did not produce sperm. Sertoli cells, spermatogonia and spermatocytes appeared normal on histological analyses, while most spermatids formed giant multinucleated cells (MNCs) or symplasts associated with increased apoptosis. The phenotype became visible during the first wave of spermatogenesis at 22–26 days post-partum (dpp), when spermatids begin to appear. Both primary and secondary spermatocyte numbers were unchanged at 28 dpp. The mutant mice failed to produce complex *N*-glycans as shown by lectin histochemistry of testis sections using L-PHA and GSA, while high-mannose structures are increased in testes of mutant mice, as revealed by Con A histochemistry. Western blot analyses using a monoclonal antibody against basigin, an *N*-glycoprotein highly expressed in elongated spermatids, combined with PNGase F or Endo H digestions, confirmed that complex *N*-glycans were not detectable in mutant mice and revealed that a small fraction of basigin in wild-type mice remained modified with high-mannose glycans. This high-mannose fraction is consistent with the presence of an inhibitor of MGAT1 that is expressed in spermatocytes. The inhibitor is a Golgi glycoprotein termed GlcNAcT-I inhibitory protein (GnT1IP). A membrane bound form (GnT1IP-L) is the active and specific inhibitor and it is expressed in spermatocytes but not spermatids of adult mice. We are investigating the hypothesis that down-regulation of complex *N*-glycans in spermatocytes is required for their interactions with Sertoli cells and the progression of spermatogenesis. Supported by grant RO1 30645 to P.S.

(291) Functional significance of vascular endothelium-derived ADAMTS9 in angiogenesis and craniofacial development

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The secreted metalloproteinase ADAMTS9 is the most highly conserved member of the ADAMTS family, being similar to the worm proteinase *Gon-1*. ADAMTS9 was identified as a tumor suppressor gene in esophageal and nasopharyngeal cancer and a possible mechanism for this effect was suggested by its ability to inhibit angiogenesis in vitro and in vivo. In contrast to other anti-angiogenic ADAMTS proteins, ADAMTS9 is a constitutive product of the microvascular endothelial cells (mECs), being expressed by embryonic and adult mECs but not large vessel endothelium. *Adamts9*$^{-/-}$ mice die at 7.5 days of gestation, prior to angiogenesis and organogenesis. However, analysis of *Adamts9*$^{+/-}$ mice demonstrated that these mice had spontaneous corneal vascularization and enhanced tumor angiogenesis. ADAMTS9 was implicated in proteolysis of versican, a major component of the embryonic and other provisional extracellular matrices, in vivo during palatogenesis, interdigital web regression and in cardiac and aortic development. More specifically, EC-derived *Adamts9*, working cooperatively with *Adamts20* expressed in the palate mesenchyme, was shown to be crucial for the closure of the mouse palate, as 100% of *Adamts9*$^{+/-}$;*bt/bt* mice developed cleft palate, a phenotype not seen in either mutant allele alone. To study the effect of a complete *Adamts9* deficiency on the cardiovascular system, a floxed *Adamts9* allele (*Adamts9*^{fl}) was developed to allow the conditional deletion of *Adamts9* and bypass the early lethality of *Adamts9*$^{-/-}$ mice. *Adamts9*$^{fl/fl}$ mice were bred with Tie2-Cre mice to allow Adamts9 deletion uniformly in endothelial cells during embryogenesis and adulthood. Tie2-Cre; *Adamts9*$^{fl/fl}$ mice are viable and are apparently without impairment of organogenesis. However, Tie2-Cre; *Adamts9*$^{fl/+}$;*bt/bt* mice developed cleft palate confirming that *Adamts9* expressed by endothelial cells is crucial during palatogenesis. Further analyses are currently performed to better characterize implication of mEC-derived *Adamts9* in angiogenesis and in developmental processes. This work was made possible by NIH-NICHHD awrd RO3HD069747.

(292) Unbiased transcriptome analysis of cleft palate in Adamts9 + Adamts20 deficient mice identifies novel cellular consequences of defective versican proteolysis

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Cleft palate is among the most common human birth defects. It leads to problems in speech development and swallowing and requires surgical correction. Mammalian palatogenesis is a rapid, complex process in which bilateral palatal shelves fuse in the midline of the embryo. It involves highly regulated interactions between epithelial and mesenchymal cells of the palate and remodeling of the extracellular matrix (ECM). Extensive studies of palatogenesis in mice with cleft palate implicated several major signaling pathways, i.e. TGFβ, FGF and EGF signaling in palate morphogenesis. The two most conserved members of the ADAMTS family, ADAMTS9 and ADAMTS20, were previously shown to act locally in palate closure and cooperate in proteolysis of versican (*Vcan*), an abundant, large aggregating proteoglycan found in association with hyaluronan in ECM. 100% of *Adamts9*$^{+/-}$;*Adamts2*$^{-/-}$ *(or bt/bt)* mice developed cleft palate, while such a phenotype was not seen in either single mutant allele alone. The palate mesenchyme of *Adamts9*$^{+/-}$;*bt/bt* mice showed evidence of decreased cell proliferation and reduced versican proteolysis. *bt/bt* mice lacking one *Vcan* allele developed cleft palate with an essentially similar mechanism as *Adamts9*$^{+/-}$;*bt/bt* mice, suggesting that an ADAMTS9- and an ADAMTS20-derived versican fragment was required for promoting mesenchymal proliferation during palatogenesis. In order to identify the signaling pathways implicated in the down-regulation of mesenchymal cell proliferation, Illumina RNA array analysis was performed using mRNA from palates of *Adamts9*$^{+/-}$;*bt/bt* and wild-type embryos at 13.5 days of gestation. Pathway analysis of the data identified among others, several differentially regulated genes in *Adamts9*$^{+/-}$;*bt/bt* vs *bt/+* mouse embryos that are implicated in cell cycle regulation and cell migration. RT–PCR and immunohistochemistry analyses are ongoing to validate and understand the dysregulated mechanisms highlighted by array analysis. This work was made possible by NIH-NICHHD award RO3HD069747

(293) Evidence for an effect of the morphogen retinoic acid through stimulated MMP9 expression on the turnover of collagen type IX by cultured chondrocytes

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In developing the cartilage matrix, three tissue-specific collagens, types II, IX and XI, co-polymerize to form a fibrillar framework that is stabilized by intermolecular cross-links. How the heteropolymeric collagen fibrils can grow laterally and mature into the cross-linked network that typifies the cartilage matrix is a challenge to understand. Yet, such elemental information seems fundamental to the promising field of cartilage tissue engineering. An attractive hypothesis is that type IX collagen is selectively removed by proteinases from the surface of the growing type II collagen fibril allowing the fibrils to grow laterally. We have begun to explore this question, using the Swarm rat chondrosarcoma chondrocyte cell line RCS-LTC, which assembles pN-type II, types IX and XI collagen molecules into nascent heterofibrils and expresses retinoic acid receptors. We investigated type IX collagen proteolysis and matrix metalloproteinase expression in the cell line following treatment with retinoic acid. The results show that the RCS-LTC cells express MMP3 under normal culture conditions. MMP3 has been shown to selectively cleave type IX collagen in the NC2 domain in vitro and would generate α1(IX) chain fragments consistent with our observations. MMP3 is implicated in early limited proteolysis of type IX collagen in these cultures. MMP2 and MMP9 are normally not expressed by the RCS-LTC cell line. This could explain why the α1(IX) fragments are not

further degraded in these cultures. Following the induction of MMP9 by retinoic acid, antibody reactivity to the α1(IX) fragments is lost implicating this enzyme in the further degradation of these fragments. MMP9 activity in stimulated cultures was confirmed by gelatin zymography. Speculation that the NC4 domain, specifically recognized by the antibody, is proteolytically cleaved off with resultant loss of reactivity was further confirmed in vitro using purified MMP3 and MMP9. The data support the hypothesis that MMP3 and MMP9 are important in the turnover of type IX collagen. MMP9, an enzyme that affects cartilage vascularization, may play a role in clearing type IX collagen fragments from the surface of the developing type II collagen fibril in cartilage growth and maintenance.

(294) Homeostasis and degradation of heparan sulfate: involvement in normal and pathological skeletogenesis

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Limb skeletogenesis involves the formation of mesenchymal cell condensations that undergo endochondral ossification. This process is regulated by growth factors that bind to heparan sulfate (HS) chains. Present in the ECM and at the cell surface, the HS chains influence the distribution and bioavailability of these factors. Mutations in HS polymerizing enzymes EXT1 or EXT2 cause hereditary multiple exostoses (HME), a disorder characterized by ectopic cartilaginous outgrowths typically found in long bones. We previously showed that exostosis formation is inversely related to *Ext* expression, accounting in part for variations in disease severity in patients. Up-regulation of heparanase was also suggested to contribute to such variation. However, the exact pathogenic mechanisms of HME remain unclear. To study these mechanisms, we modeled HME in limb bud mesenchymal micromass cultures. Because HME involves HS deficiency, we mimicked this by treating cultures with the HS antagonist Surfen. The treatment provoked a significant increase in chondrogenic differentiation and cartilage nodule formation. Additionally, when we cultured $Ext1^{fl/fl}$ neonatal forelimb elements in medium with Adeno-Cre, we observed ectopic cartilage formation within the perichondrium. We hypothesized that these changes could be due to enhanced activity of HS-binding pro-chondrogenic factors such as bone morphogenetic proteins (BMPs). Indeed, phosphorylatedSmad1/5/8 protein levels as well as BMP ligand and receptor expression were higher in micromass cultures treated with Surfen than controls. Surfen treatment also stimulated the responsiveness of a BMP reporter plasmid to endogenous and exogenous BMPs. Similarly, the nuclear accumulation of pSmad was increased in the perichondrium in *Ext1*-deficient long bones in vivo and in explant culture. We also tested whether this enhanced activity was due to atypical ligand diffusion. Biochemical assays did indicate that Surfen blocked BMP/heparin binding, suggesting that BMP diffusion is enhanced in HS-deficient tissue. Lastly, we investigated heparanase expression and activity in cells and organ cultures with diminished HS function, finding increases in both. Our data reveal that interference with HS function increases cell response to BMP2, stimulating Smad phosphorylation and chondrogenic differentiation. We propose aberrant BMP signaling as a candidate pathway in HME and suggest that this may be further enhanced by increases in heparanase activity.

(295) Developing skeletal muscle activity affects abnormal morphology of lathyritic long bone

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Lysyl oxidase catalyzes the cross-link formation of the extracellular molecules for collagenous and elastic fibers in tissues, and its inhibition by lathyrogens such as β-aminopropionitrile (βAPN) induces a reduction in the cross-linkage and the resultant bone strength. However, aberrant morphogenetic process in lathyritic bone has not been linked convincingly to lower strength of the bone. The present study undertook morphological and histological examinations of the developing leg of βAPN-treated chick embryos to substantiate muscle involvement in the formation of long bones with abnormal bending morphology. Embryos in ovo were treated with βAPN (350 μg/egg) at embryonic day (ED) 4 and the leg skeleton was evaluated after aldehyde fixation and alcian blue and alizarin red double-staining at ED10. An abnormal mid-diaphyseal bending of the tibial bone toward the flexor side was discernible at ED7 and this bending was enhanced after ED9. In the concave side of the bending, the hyperplasia of the fibrolamellar bone was observed. Transposition of extensor muscles to the flexor side was demonstrated histologically at ED8 or later. Therefore, by expecting the immobilization of the developing skeletal muscles, decamethonium bromide (DMBr), a non-competitive blocker of the neuromuscular junction, was administered to βAPN-treated (and untreated control) embryos at ED8. It was shown that the characteristic bending of the tibial long bone in βAPN-treated embryos was alleviated by the effect of DMBr. These findings indicate that skeletal muscle activity elevated at around ED8 is one of causal factors operating on abnormal bending of the lathyritic long bone with reduced mechanical strength.

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(296) The Fibrillin-like fbn-1 Gene Regulates Epithelial Stem Cell and ECM Dynamics in Molts

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The molting cycle involves the periodic removal and deposition of extracellular matrices (ECM). The stem cell-like lateral seam cells contribute to the production of new matrices during the molts, but undergo asymmetric divisions early in every larval stage. In addition, successive transitions between seam cell temporal fates coincide with the molts. However, the molecular mechanisms that coordinate ECM and stem cell dynamics during the molts are not yet understood. Here, we describe FBN-1, a protein that is similar to human fibrillins, which are the major components of ECM

fibers defective in Marfan Syndrome and other inherited disorders of skin and connective tissue. The *fbn-1* gene emerged from a full-genome, RNAi-based screen for larvae unable to fully shed cuticles; *fbn 1(tm290)* mutants also exhibit molting defects. A transcriptional *fbn-1::gfp-pest* fusion gene is transiently but reiteratively expressed in the hypoderm during every molt. In addition, the expression of multiple splice variants of *fbn-1* suggests substantial post-transcriptional gene regulation. To better define the function of *fbn 1*, we characterized the status of the cuticle in *fbn-1* mutants, using cell and molecular biological approaches including TEM. A functional COL 19::GFP fusion protein was improperly deposited and disorganized in cuticles of *fbn-1(lf)* adults, and structural cuticle abnormalities were detected by TEM. Consistent with these findings, rearrangements in the actin cytoskeleton of the hypodermis were not obvious in *fbn-1* mutants undergoing the fourth molt, but were readily detected in wild-type animals stained with rh-phalloidin. Further, the lateral seam cells were detected using standard markers for the cell nuclei and margins. At the L4-to-adult transition, some seam cells failed to fuse or exit the cell cycle in 35% of *fbn-1(-)* animals. The axis of seam cell division was also abnormal in *fbn-1(RNAi)* animals, suggesting de-regulation of the Wnt signaling pathway. Indeed, genetic analyses confirmed that mutations that affect the Wnt or other conserved cell–ECM signaling pathways modify the phenotypes of *fbn-1(-)* larvae. Taken together, our findings indicate that FBN-1 polymers likely serve as both structural and instructive components of matrices remodeled during the molts. We propose that the certain activities of FBN-1 macromolecules orchestrate stem cell and ECM dynamics in larval development.

(297) Imbalance of Vcan Protein Expression Alters Collagen Deposition:Consequence for Heart Development

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The requirement for the proteoglycan versican (Vcan) to early heart formation was clearly demonstrated by the Vcan null insertional mutant mouse. The total absence of the Vcan gene is lethal at a stage prior to the heart's pulmonary/aortic outlet segment growth. However, deletion of exon 7 alone produces viable animals (Vcan $^{(tm1Zim)}$) with reduced levels (50%) of Vcan expression due to the complete lack of two of the 4 alternative splice forms. mice have a spectrum of cardiac outlet defects that include a smaller pulmonary lumen, reduced central AV cushion primordia and alterations in myocardialization of valve leaflets. A comparative proteomic analysis of the Vcan $^{(tm1Zim)}$ mouse hearts, show unexpected changes in the expression of other extracellular matrix-related proteins involved in collagen biology. We further explored these changes in E13.5 pc and neonatal hearts using both biochemical and histological analysis that included collagen I and Hsp47 western blots, sircol quantification, picrosirius red staining patterns and qRTPCR (Vcan V0, V1, V2, col1a2, col3a1, lysyl oxidase, MMP-2, -3 and uPARA). Overall our data measured a significant change in the abundance of both collagen and Hsp47. mouse has a significant reduction in both soluble and insoluble collagen ($P < 0.05$). The null mice have an altered histological pattern of collagen deposition in the forming valve leaflets and walls of the aorta and pulmonary arteries. Changes in mRNA expression indicated that the mechanism for reduced collagen deposition occurred during the later stages of collagen maturation. Our study suggests that a specific splice variants of Vcan play an important role in regulating collagen deposition during aortic and pulmonary artery development that underlie the outlet defects found in the Vcan $^{(tm1Zim)}$ null hearts. NHLBI R01HL66231 (CHM); Janey Briscoe Center for Excellence in Cardiovascular Research (CJLS).

(298) Developmental roles of mucin-type glycosylation in zebrafish

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Mucin-type O-glycosylation is one of the most common post-translational modifications of proteins and is initiated by a family of UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferases (ppGalNAc-Ts). To date, 20 isozyme genes of this family have been isolated from mammals. Analyses of these isozymes indicate that they have partially overlapping, but distinct tissue expression patterns and substratum specificities. We previously cloned mammalian ppGalNAc-T9 and WBSCR17 genes and demonstrated that both are predominantly expressed in the brain. Recently, we found that WBSCR17 had a low but detectable catalytic activity and its knockdown in culture cells induced their morphological changes. To study the developmental roles of ppGalNAc-Ts in vivo, we have been using zebrafish as model organism. Zebrafish genome database searches revealed that zebrafish has most of mammalian ortholog genes. Among the isozymes, we focused on those predominantly expressed in the brain and cloned orthologue genes for ppGalNAc-T9 and WBSCR17 from zebrafish. To analyze their roles in the development, we suppressed their expression using morpholino antisense oligonucleotides and demonstrated that WBSCR17 was involved in the brain development and marker gene expression was disturbed in the hindbrain but not in the forebrain and the midbrain-hindbrain boundary. In addition, we carried out whole mount immunostaining using zn8 antibody and anti-acetylated tubulin antibody, which recognize commissural neurons and reticulospinal neurons, respectively and found that neurogenesis was impaired in the WBSCR17 knockdown embryos. To investigate glycosylation in the hind brain, embryo cryosections were stained with fluorescence-labeled lectins, such as Jacalin and PNA, which mainly recognize mucin-type glycans. The lectin staining showed an altered localization of glycans in the morphant hindbrain, suggesting that WBSCR17 plays important roles in the development of hindbrain through glycosylation.

(299) Breaking barriers: Role of Adamts9 in early embryonic mouse development

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ADAMTS9 is a secreted metalloprotease that is known to cleave ECM proteoglycans versican and aggrecan. In this study, using various mouse lines containing combinations of *Adamts9* alleles (i.e. an allelic series), we investigated the roles of *Adamts9* during early mouse development. In fish, frog, chicken and humans, morphogenesis begins with gastrulation movements of the embryo. In rodents, however, a unique cup shaped structure known as the egg cylinder is formed prior to gastrulation by the distal outgrowth of the implanted embryo. Prior to gastrulation, the egg cylinder is composed of two simple epithelial cell layers, the ectoderm (or the epiblast cells) and the visceral endoderm. These two epithelia are of opposing apical-basal polarity, separated by a basement membrane. We show here that *Adamts9* expression is switched on in both cell layers very rapidly at E 6.5, just prior to gastrulation and is critical for the gastrulation and survival of the mouse embryo. During gastrulation, *Adamts9* expressing cells delaminate from the ectoderm to colonize the newly forming mesoderm. *Adamts9* knockout embryos (−/−), die around E.7.0 without undergoing gastrulation and with a profound disorganization of the embryo and extra-embryonic ectoderm. Second, using a hypomorphic *Adamts9* gene trap allele for (*Adamts9*^{gt}), we investigated the function of *Adamts9* in the post-gastrula mouse embryo. Adamts9 $^{gt/-}$embryos are capable of undergoing gastrulation but die around E 9.0 due to the failure of the embryo to twist, causing a turning and heart looping defect. Mice carrying two alleles of the gene trap alleles (*Adamts9*$^{gt/gt}$) survive even further in gestation until E 14.5 and die with severe intrauterine growth retardation and evidence for an impaired maternal-fetal axis. We are currently investigating how *Adamts9* regulates these crucial developmental processes. This work was made possible by NIH-NICHHD award RO3HD069747

(300) Cellular and transcriptional regulation of collagen fibrils assembly and growth intendons

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A unique feature of tendon fibroblasts is their ability to generate highly organized collagen fibrils that transmit the force generated by muscle contraction. Studies of collagen fibrilogenesis have focused on the complex synthesis, processing and secretion of the structural proteins that make up the collagen fibrils and on regulation of collagen fibril growth by collagen binding proteins. Little, however, is known about the cellular processes and transcriptional activities in tenocytes that regulate collagen fibril assembly, organization and growth. This abstract presents three systems we use to unravel cellular regulators of the collagen matrix. A striking feature of the collagen matrix is the parallel organization of collagen fibrils that is attributed to the direction of force applied to tendons. We show, however, that highly organized collagen fibrils are formed even in tendons that develop in embryos with muscleless limbs or in genetically paralyzed embryos, suggesting that cellular organization or mechanical strains generated by the growth process may be sufficient to direct the organization of the collagen matrix. Scleraxis, a bHLH transcription factor, is a key regulator of tendon differentiation. We find that Scleraxis is also a critical regulator of matrix assembly. Inducible loss of Scx towards the end of embryogenesis results in a highly disorganized collagen matrix and massive deposits of elastin rich microfibrillar structures. Collagen fibril growth occurs in two distinct stages; during embryogenesis fibril diameters are small and uniform. Fibril diameter grows much faster after birth and this phase is characterized by remarkable heterogeneity of collagen fibril diameter that has been attributed to fusion of micofibrils in this stage. Mohawk is an atypical homeobox transcription factor with a distinctive expression in tendons. In Mohawk mutants, we find that embryonic collagen fibrilogenesis is not perturbed but collagen fibrils remain small and uniform even in post-natal stages, suggesting an essential role in the post-natal phase of collagen fibril growth. These mutants highlight a regulatory modularity in the assembly and growth of the collagen matrix and present an experimental opportunity to identify the cellular mediators of assembly and growth of the collagen matrix.

(301) O-glycosylation modulates integrin and FGF signaling by influencing the secretion of basement membrane components

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Extracellular microenvironments play crucial roles in modulating cell interactions during development. Here, we discover that a conserved protein modification (O-glycosylation) influences the extracellular matrix (ECM) composition during mammalian organogenesis, affecting integrin signaling and fibroblast growth factor (FGF)-mediated cell proliferation. Specifically, mice deficient for an enzyme (*Galnt1*) that adds sugars to proteins during early stages of organogenesis resulted in intracellular accumulation of major basement membrane (BM) proteins and endoplasmic reticulum (ER) stress, with resultant effects on FGF signaling, epithelial cell proliferation and organ growth. Exogenous addition of BM components rescued FGF signaling and the growth defects in a β1integrin-dependent manner. Our work demonstrates for the first time that O-glycosylation influences the composition of the ECM during mammalian organ development, influencing specific aspects of the ER stress response, cell signaling, cell proliferation and organ growth. Our work provides insight into the role of this conserved protein modification in both development and disease.

(302) Mammalian GDP-fucose transporters required for notch signaling

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Notch signaling is important during embryonic development and in cell fate decisions during somitogenesis. Notch receptors must

be modified by *O*-fucose glycans on epidermal growth factor-like (EGF) repeats in their extracellular domain for optimal signal induction by canonical Notch ligands to be achieved. Slc35c1 is the only known GDP-fucose transporter. However, mice and humans lacking Slc35c1 do not have an obvious Notch mutant phenotype. In addition, Slc35c1 null fibroblasts transfer *O*-fucose to Notch and exhibit robust Notch signaling. Nevertheless, when we examined Slc35c1 null embryos at embryonic day 18.5, skeletal defects were observed indicating defective Notch signaling during somitogenesis. Slc35c2 is a GDP-fucose transporter related gene that we showed promotes Notch1 O-fucosylation and is required for optimal Notch signaling. We generated *Slc35c2*[−/−] mice by deletion of exon4 of the Slc35c2 gene. Western blot analysis showed that Slc35c2 was not expressed in *Slc35c2*[−/−] testis or *Slc35c2* [−/−] mouse embryonic fibroblasts. No apparent abnormalities were observed in *Slc35c2*[−/−] mice at birth, and both male and female homozygous mutants were fertile. In contrast to *Slc35c1* [−/−] embryos, *Slc35c2*[−/−] embryos had no significant skeletal defects. We therefore crossed *Slc35c1*[+/−] mice with *Slc35c2* [+/−] or *Slc35c2*[−/−] mice to obtain *Slc35c1*[−/−]*Slc35c2* [−/−] double-knockout mice. Double-knockout mice are born, but at a lower ratio than expected. Preliminary results indicate that skeletal defects in *Slc35c1*[−/−]*Slc35c2*[−/−] double-knockout embryos are similar to *Slc35c1*[−/−]*Slc35c2*[+/+] embryos. They have thoracic and lumber skeletal defects in several vertebrae, but exhibit only mild rib and tail defects. In contrast, mice that are unable to add GlcNAc to Ofucose on Notch have severe thoracic, lumbar and rib defects and no tail. Therefore, mice lacking the known and putative GDP-Fuc transporter activities of mammals must nevertheless be adding *O*-fucose to Notch in the secretory pathway, providing strong evidence for the existence of at least one other GDP-Fuc transporter important for the O-fucosylation of Notch. Supported by RO1 95022 to P.S.

(303) Development of the dermal matrix in chick limb bud

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Collagenous and elastic fibers are the essential components of the dermal matrix. The aim of this study was to reveal the temporospacial pattern of development of collagenous and elastic fibers. We focused on tarsometatarsal skin of chick embryo from embryonic day (ED) 4 to 20. Tarsometatarsus is a proximal part of the chicken foot useful for a chronological study of dermal development because it is devoid of feather formation. To elucidate the distribution of collagenous and elastic fibers in the presumptive dermis, we performed immunohistochemistry for fibrillin, elastin and lysyl oxidase (LOX), and laser capture microdissection and qRT-PCR for LOX mRNA expression. Picrosirius polarization and electron microscopy (EM) were used to investigate collagen accumulation and elastic fiber formation. PCR analysis showed that LOX mRNA expression became apparent at embryonic day 13 and increased considerably by ED17 in the presumptive dermis. Immunohistochemical staining for LOX in the dermis was very low at all stages of development. Accumulation of dermal collagen fibers was shown in ED10, and thicker fibers became evident by ED13. Fibrillin microfibrils as an immature form of elastic fibers were seen in ED4. Elastin deposition to microfibrils was observed at ED13. Our findings suggest that the temporal pattern of LOX mRNA expression correlates with collagen fiber accumulation in the tarsometatarsal dermis of the developing chick, whereas LOX expression was relatively constant at the protein level. Fibrillin microfibrils were observed earlier than collagen fiber accumulation. Supported by grants from Sato Fund and Dental Research Center, NUSD.

(304) Cardiac ankyrin repeat protein (CARP/ANKRD1) regulates MMP13 expression

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MMP remodeling of the extracellular matrix is required for cell migration and capillary morphogenesis during wound healing. Overexpression of the transcription co-factor, *Ankrd1*, increases neovascularization, suggesting that *Ankrd1* regulates genes associated with the process of matrix remodeling and angiogenesis. The aim of this study was to identify *Ankrd1*-interacting nuclear factors and their targets. A stress response PCR array showed significantly increased expression of genes that belong to the MMP family in the absence of *Ankrd1*. Deletion of *Ankrd1* results in increased collagenase 3 (*Mmp13*) mRNA expression in *Ankrd1*$^{-/-}$ comaped with *Ankrd1* fibroblasts, while *Ankrd1* overexpression decreased phorbol myristic acetate (PMA)-induced *Mmp13* promoter activity dose-dependently. *Ankrd1*$^{-/-}$ skin fibroblasts exhibited enhanced PMA induction of *Mmp13* mRNA by several fold over *Ankrd1*$^{flox/flox}$ cells and siRNA knockdown of *Ankrd1* in skin fibroblasts also increased *Mmp13* mRNA. Conversely, the transfection of an *Ankrd1*-Flag construct into *Ankrd1*$^{-/-}$ fibroblasts decreased *Mmp13* mRNA. *Ankrd1*$^{-/-}$ mice had higher MMP-13 protein levels in the skin and significantly increased levels of MMP-13 protein and mRNA in day 4 excisional wounds. Therefore, the absence of *Ankrd1* de-represses *Mmp13*. Yeast two-hybrid analysis in microvascular endothelial cell nuclear extracts identified *Ankrd1* association with the transcription factor, nucleolin and the interaction was confirmed by reciprocal pull-down assays. Nucleolin has been shown to repress the AP-1-dependent transactivation of MMP-13. Our data showed that nucleolin knockdown also de-repressed *Mmp13*. Electrophoretic mobility shift assay showed that mutations in the *Mmp13* AP-1 site reduced the binding of transcription factors to the *Mmp13* promoter and abolished *Mmp13* promoter activity. The gel shift pattern comparing *Ankrd1*$^{flox/flox}$ and *Ankrd1*$^{-/-}$ fibroblast nuclear extracts suggested that in the absence of *Ankrd1*, additional transcription factors bind to the AP-1 site in and regulate the *Mmp13* promoter. Understanding the downstream mechanisms through which *Ankrd1*

exerts its pro-angiogenic activities could lead to the development of a therapy that improves the outcome of chronic wounds. Supported by the Department of Veterans Affairs and NIH grants DK65656 (JMD) and GM101947 (KA).

(305) Fibulin-1 as an inhibitor of α-secretase processing of amyloid precursor protein

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Many current pharmacological approaches to combating Alzheimer's disease (AD) seek to block Aβ production through inhibition of the amyloidogenic enzymes known as β- and γ-secretases. An alternative approach is to activate the α-secretase processing of amyloid precursor protein (APP), which is mediated by several members of the disintegrin family of metalloproteases, ADAM9, ADAM10 and ADAM17. Processing of APP by these α-secretases is thought to be beneficial with respect to AD since it limits production of Aβ and generates the neuroprotective soluble APPα (sAPPα) product. Fibulin-1 (Fbln1) is an extracellular matrix protein expressed in the brain by neurons that binds the amino terminus of APP and sAPPα. We tested the possibility that Fbln1 might regulate α-secretase-mediated cleavage of APP. We found increased levels of sAPPα in the conditioned culture medium of Fbln1 null mouse embryo fibroblasts (MEFs) when comaped with wild-type MEFs. We also found that Fbln1 binds to two other membrane anchored α-secretase substratums, heparin binding-epidermal growth factor (HB-EGF) and neuregulin-1 (NRG1). Furthermore, we showed that Fbln1 acts to inhibit the proteolytic release of soluble forms of HB-EGF and NRG1. Based on these findings we conclude that Fbln1 serves as an inhibitor of processing of several α-secretase substratums. With respect to APP, Fbln1 may represent a therapeutic target that if inhibited might lead to augmented α-secretase processing of APP and reduced pathological APP cleavage.

(306) IL-10 promotes a regenerative phenotype by regulation of hyaluronan synthesis via a STAT3-dependent mechanism

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Mid-gestational fetal wounds heal scarlessly with elevated levels of hyaluronan (HA). We previously demonstrated that overexpression of IL-10 recapitulates the fetal HA-rich pericellular matrix (PCM) phenotype in adult fibroblasts (AFbs) and results in scarless post-natal wound healing. We hypothesize IL-10's regenerative effects are mediated by HA-rich ECM deposition via regulation of HA synthases (HAS1–3) through a STAT3-dependent mechanism. Murine AFbs were cultured with IL-10 (200 ng/ml) and IL-10R1 neutralizing antibody (IL-10R1nAB) and assessed for (i) STAT3 phosphorylation (western blot) and nuclear localization (immunohistochemistry; confocal-imaging), (ii) HAS1–3 gene expression (qRT–PCR) and (iii) HA-rich PCM production (ELISA; particle exclusion assay). To determine if IL-10 effects on HA-rich PCM formation are mediated by STAT3; lentiviral shRNA STAT3-silenced AFb cell line was created and treated with IL-10 and assessed for HAS1–3 gene expression, HA synthesis and PCM formation. Statistical analysis were done by *t*-test and ANOVA. IL-10 significantly induces STAT3 phosphorylation (control 0.651 ± 0.133 vs treatment 1.257 ± 0.126; $P = 0.03$) and nuclear localization at 1hr, HAS1 gene expression at 3 h (control 0.95 ± 0.065 vs treatment 1.29 ± 0.05; $P = 0.01$) and HAS2 at 1, 2 and 3 h (1 h: control 0.98 ± 0.04 vs treatment 1.19 ± 0.4; $P = 0.02$, 2 h: control 0.96 ± 0.07 vs treatment 1.35 ± 0.4; $P = 0.007$, 3 h: control 0.88 ± 0.02 vs treatment 1.22 ± 0.06; $P = 0.007$), but not HAS3. Comaped with fetal fibroblasts (FFb), AFb has reduced HA synthesis (1.20 ± 0.02 vs 2.0 ± 0.2 μg/mg; $P = 0.005$) and PCM production (PCM area/cell area 1.84 ± 0.08 vs 2.78 ± 0.14; $P < 0.001$); however, IL-10 treatment increases HA synthesis (AFB 1.20 ± 0.02 vs AFB +IL-10 1.6 ± 0.08 μg/mg; $P = 0.002$) and recapitulates the fetal HA-rich PCM phenotype in AFb (AFb +IL-10 2.74 ± 0.24 vs FFb2.79 ± 0.35; $P =$ NS). IL-10R1 blockade inhibits STAT3 nuclear localization at 1hr and abrogates PCM formation (AFb +IL-10 2.74 ± 0.24 vs AFb +IL-10R1nAB +IL-10 1.53 ± 0.07; $P < 0.001$). In STAT3-silenced AFb, IL-10 effects on HAS1–3 gene expression are attenuated at 0, 1, 2, 3 and 6 h and the effects on HA synthesis (control 1.27 ± 0.23 vs treatment 1.15 ± 0.31; $P =$ NS) and PCM formation (control 1.239 ± 0.0327 vs treatment 1.215 ± 0.0283; $P =$ NS) are abrogated. IL-10 has direct effects on HA-rich PCM formation via STAT3 signaling and regulation of HAS genes. These data suggests IL-10, in addition to its known anti-inflammatory role, may be a key regulator of the ECM; and provides the basis for development of therapeutics aimed at regenerative wound repair in diseases characterized by excessive inflammation and fibrosis.

(307) Engineering fibroblast-remodeled electrospun matrices for full-thickness skinregeneration

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The skin is often severely damaged, resulting in the need for surgical intervention. There are many limitations to current therapies; therefore, a synthetic skin graft would be invaluable for skin regeneration. The focus of the current study is on developing engineered scaffolds with embedded dermal fibroblasts that can be remodeled into the native skin tissue upon implantation. To achieve this goal, we created electrospun scaffolds composed of collagen I and polycaprolactone (PCL), and then introduced pores to allow fibroblast infiltration. Initial experiments performed to optimize the pore size and collagen:PCL concentration determined that a 160-μm pore diameter and the 70:30 ratio of collagen to PCL were ideal parameters based on favorable fibroblast responses. Fibroblasts grown in these scaffolds exhibited proper morphology, rapid proliferation and importantly, the cells secreted and filled the pores with native matrix molecules, including

collagen I and fibronectin. These molecules are important for scaffold remodeling into the human skin. Upon implantation, the scaffolds must support the formation of an epidermal layer; therefore, we evaluated the keratinocyte growth on the scaffolds. H & E staining revealed that keratinocytes proliferated and formed a stratified layer on the scaffold surface. Additionally, fluorescent staining for KI-67 showed a basal layer of proliferating keratinocytes, whereas CK-10 staining demonstrated terminally differentiated keratinocytes at the apical surface. These collective results confirm that fibroblast-embedded scaffolds stimulate the keratinocyte organization into the type of the stratified structure found in endogenous epidermis. Scaffolds also exhibited a low rate of contraction (<19%), which is comparable, and possibly less than, the contraction rates of the current clinical graft products. Finally, preliminary in vivo data suggest that 70:30 collagen:PCL scaffolds regenerate the skin faster than a sham skin wound, and the synthetic components degrade within 3 weeks, an optimal time frame for the scaffold turnover. In conclusion, microporous electrospun scaffolds with embedded fibroblasts are promising new substrata for skin regeneration.

(308) Biosynthetic hybrid fibrin matrices for enhanced wound repair

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The fibrin provisional matrix forms rapidly in response to injury and facilitates wound repair and angiogenesis. In clinical practice, the use of purified fibrin systems is highly attractive as an exogenous hemostat and as a tissue sealant due to the polymer's role in wound repair. However, clinically relevant formulations use high fibrinogen and thrombin concentrations that produce matrices that are inhibitory to cell infiltration and normal remodeling of the matrix. While lower fibrin concentrations facilitate cell infiltration they also result in weak polymers that lack the mechanical integrity necessary for clinical application. We aim to create hybrid synthetic-fibrin assemblies that replicate the properties of clinical formulations but better mimic physiological clots, and thus normal wound repair, through the incorporation of hydrogel microparticles (microgels) into fibrin matrices. Here, we utilize ultralow crosslinked p NIPAm microgels, previously shown to self-assemble and form strong interdigitated networks. To impart fibrin specificity to the microgels, human domain antibodies (DAb) identified through molecular evolution techniques to possess high affinity for fibrin were coupled to the microgels. Using fibrinogen and fibrin ELISAs, we observed binding of DAb-microgels to fibrin but not fibrinogen-coated surfaces, demonstrating the specificity of the microgels for fibrin. Binding of DAb microgels to fibrin surfaces was further confirmed in a label free system utilizing the Fortébio BLITZ. In vitro polymerization assays demonstrate that the incorporation of DAb-microgels in forming fibrin clots does not significantly alter polymerization comaped with controls. However, the analysis of the structure through confocal microscopy demonstrates that the incorporation of DAb-microgels significantly alter the matrix structure comaped with control clots. Fibrin network structure greatly influences wound healing responses; therefore, future studies will characterize cellular responses to microgel-fibrin assemblies including proliferation, migration and angiogenesis.

(309) Manipulating myofibroblast differentiation with growth factors

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Myofibroblasts are highly contractile cells that deposit the abundant extracellular matrix (ECM) and have a crucial role in matrix contraction during tissue repair. Their unregulated activity leads to fibrosis, which impairs tissue structure and function. To study the modulation of myofibroblast activity, we used adipose-derived mesenchymal stem cells (ADSCs), which are used in regenerative medicine. We found that ADSCs treated with TGF-ß1 developed a myofibroblastic phenotype with a 1.5-fold increase in fibronectin mRNA and a dramatic increase in secreted type I collagen. Higher levels of a-smooth muscle actin (SMA), a classic marker of myofibroblasts and of total stress fibers were also observed. These changes correspond with reduced cell migration and activation of the Smad pathway. In contrast, treatment of ADSCs with bFGF, another growth factor involved in tissue repair, has the opposite effect. bFGF-differentiated ADSCs showed a marked down-regulation of fibronectin, type I collagen and a-SMA expression. bFGF-differentiated ADSCs were significantly more migratory and this behavior correlated with an up-regulation of vimentin and of tenascin-C, an anti-adhesive ECM protein. The functional effects of these treatments were measured using a fibrin matrix contraction assay. TGF-ß1-differentiated ADSCs were significantly more active in the matrix contraction assay with a 3-fold higher level of contraction comaped with bFGF-differentiated ADSCs. Interestingly, cells completely changed not only their myofibroblast marker expression but also contractility profiles when switched from culture with one growth factor to the other. These results show that myofibroblast properties can be enhanced and suppressed through the use of growth factors, suggesting that it may be possible to refine the heterogeneity of ADSCs by manipulating the microenvironment. Applying this model to study myofibroblast activity may provide insights into therapeutic strategies that promote repair and reduce the risk of fibrosis.

(310) Collagen biosynthesis balancing act between Ski and Scleraxis

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Interstitial fibrosis afflicts a wide spectrum of organs including heart, liver and lung. Expansion of the extracellular matrix in fibrotic disorders involves activation of myofibroblasts and the inappropriate deposition of fibrillar collagens types I and III. Our previous work supports canonical TGF-β/ Smad signaling in phenoconversion of these indigenous tissue fibroblasts to myofibroblasts and matrix biosynthesis. The current study is focused on interrelationships among transcription factors and transcriptional coactivators as inputs for the initiation of transcription of key

target genes in these cells. We have shown that the transcription factor Scleraxis is expressed in cardiac fibroblasts and myofibroblasts, and directly regulates human collagen Iα2 gene expression. Our lab has previously demonstrated the effects of endogenous inhibitors of TGF-β signaling, e.g. Smad-7 and c-Ski, on cardiac myofibroblasts. Specifically we have shown that Ski can reduce fibrillar collagen synthesis and secretion in cardiac myofibroblasts. Using adenoviral overexpression systems for both Ski and Scleraxis in first and second passage rat cardiac myofibroblasts, we have identified a link between these two regulatory proteins. Scleraxis overexpression was able to significantly decrease Ski expression. Evidence of reciprocal regulation of these two factors was present in that overexpression of Ski was associated with a significant decrease Scleraxis protein expression. These data suggest that Ski and Scleraxis are both important in the regulation of pro-fibrotic signals from TGF-β and the overall balance of the expression levels of these proteins may be influential in the progression of fibrotic disease.

(311) Fibronectin degradation by granzyme b disrupts endothelial cell angiogenic potential and alters vascular endothelia cell growth factor bioavailability

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Immune-derived proteases regulate neovascularization in part through cleavage of the extracellular matrix (ECM) proteins that interact with infiltrating endothelial cells (ECs) on their way to form functional capillaries. Granzyme B (GZMB) is a serine protease that is expressed and released by a variety of immune cells and accumulates in the ECM during chronic inflammatory diseases that are highly associated with dysregulated angiogenesis. Although several ECM proteins have been identified as GZMB substrata, the effect of GZMB-mediated ECM cleavage on EC angiogenic potential has not been determined. Fibronectin (FN) is an important ECM protein that regulates angiogenesis by facilitating EC adhesion and migration and by binding vascular endothelial growth factor (VEGF). We hypothesized that GZMB cleavage of FN will disrupt EC angiogenic behavior due to degradation of FN and alteration of VEGF bioavailability. The GZMB cleavage of plasma FN and cell-derived FN was evaluated by western blot. Human microvascular endothelial cells (HMVEC) adhesion and migration to FN treated with either GZMB or GZMB inhibitor was evaluated in FN-coated culture wells and modified Boyden chamber assay. Changes in VEGF bioavailability due to GZMB-mediated FN cleavage was explored by binding VEGF to FN-coated wells and VEGF release to the supernatant was examined by ELISA. Capillary tube formation assay in collagen gel mixed with FN was performed in order to examine EC capacity to form capillary tube structures in the presence of GZMB. GZMB effectively cleaved human plasma FN and cell-derived FN. GZMB-mediated FN cleavage resulted in a significant reduction in HMVEC adhesion and migration to fibronectin, which was restored by GZMB inhibition. Treatment of FN -bound VEGF with GZMB resulted in a significant release of VEGF to the supernatant that was reduced by using GZMB inhibitor. GZMB treatment reduced EC capacity to form the capillary tube structure in the collagen gel matrix. The GZMB cleavage of FN disrupts EC angiogenic behavior, prevents capillary tube formation and alters VEGF bioavailability. GZMB may contribute to impaired angiogenesis during chronic inflammation, while the inhibition of GZMB activity can prevent FN degradation and may support physiological angiogenesis

(312) Granzyme B contributes to impaired wound healing in apolipoprotein e deficient mice

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Background

Granzyme B (GzmB) is a serine protease that can be released into the extracellular spaces by inflammatory cells during chronic inflammation. Apolipoprotein E (ApoE) is a protein highly expressed in the skin, where it can regulate inflammation through its anti-oxidative and anti-inflammatory properties. Mice deficient in ApoE develop an inflammatory skin phenotype when fed a high fat diet featuring increased levels of GzmB capable of degrading several components of the extracellular matrix (ECM) including the proteoglycan, decorin. GzmB-mediated decorin degradation in the skin of ApoE knockout (KO) mice can result in collagen disorganization and skin frailty. We hypothesized that ApoE KO mice will demonstrate increased inflammation in response to injury, featuring increased GzmB-mediated degradation of the ECM and impaired wound healing.

Methods

C57BL/6 wild type, ApoE KO and ApoE/ GzmB double knockout (DKO) mice were fed a high fat diet for 30 weeks and given a single 1 cm diameter full thickness skin wound on their mid backs. Wound tissue was harvested at 16 days post-wounding and analyzed histologically.

Results

Wound healing was delayed in ApoE KO mice compared to wild type controls. After 16 days of healing, only 40% of wounds were fully closed in ApoE KO mice compared to 80% of wounds closed in the GzmB/ ApoE DKO mice. In addition to improved healing, GzmB/ ApoE DKO mice also displayed increased decorin levels and collagen organization compared to ApoE KO mice.

Conclusions

Wound healing in ApoE KO mice is altered compared to wild type controls, featuring delayed healing, increased inflammation and altered collagen/ dermal structure. GzmB contributes to ECM degradation and impaired wound healing in aged ApoE KO mice.

(313) Matrix Metalloproteinase-9 Inhibition Attenuates ADAMTS2 and TOLLIP Expression Post-Myocardial Infarction in Mice

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In the post-myocardial infarction (MI) left ventricle, matrix metalloproteinase-9 (MMP-9) regulates cardiac remodeling,including the inflammatory response and extracellular matrix production to form the infarct scar. Targeted deletion of theMMP-9 gene attenuates post-MI remodeling, but whether inhibiting MMP-9 after MI is also effective has not beenevaluated. Accordingly, we treated mice with a novel, triple-helical transition state analog MMP-9 inhibitor given at 3hours post-MI. Adult male C57BL/6 mice (3–6-month old, n = 3/group) were subjected to left coronary artery ligation.Three hours post-surgery, osmotic pumps containing saline or MMP-9 inhibitor (0.03 μg/day) were inserted subcutaneously and the mice were sacrificed at day 7 post-MI. The 7-day survival rate after MI for saline-treated mice was 42% and for MMP-9 inhibitor-treated mice was 50% ($P = 0.64$). The infarct areas were evaluated by 1% 2,3,5-triphenyltetrazolium chloride metabolic staining, and the LV function was assessed by echocardiography. No change ininfarct areas was observed between saline-treated (57 ± 6%) and MMP-9 inhibitor-treated (58 ± 3%) mice ($P = 0.55$) indicatingthat both groups received a similar initial injury. The decrease in ejection fraction at day 7 post-MI was also similarbetween saline-treated (10 ± 3%) and MMP-9 inhibitor-treated (7 ± 2%; P = not significant) mice. We measured 168inflammatory and ECM genes by quantitative RT-PCR gene arrays. At day 7 post-MI, the mRNA levels of *ADAMTS2* and *TOLLIP* genes were increased from (0.046 ± 0.001 to 0.113 ± 0.008) and (23.5 ± 5.4 to 62.1 ± 9.7) 2^{-}x 1000 units in saline-treated mice respectively (both $P < 0.05$). In the MMP-9 inhibitor-treated group, neither gene was elevated post-MI. *ADAMTS2* may regulate infarct wall remodeling by influencing collagen deposition, while *TOLLIP* is a pro-inflammatorymediator. Together, these results highlight roles for MMP-9 in removal of necrotic tissue and in stimulating theinflammatory response.

(314) Recombinant Human Collagens and Associated Reagents

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Among the largest and most abundant macromolecules in mammals, type I collagen interacts with a variety of molecules and is synthesized as a pro form, which undergoes multiple post-translational modifications such as proteolytic processing of the N- and C-propeptides by procollagen N- and C-proteinases (PNP and PCP). While the mature type I collagen from several species can be purchased, the pro form has not been commercially available from any species until now. Here, we present recombinant human collagens encoded by COL1A1, COL2A1 and COL3A1 genes and preliminary analysis of their properties. Expressed by mammalian cells, the three purified collagens contain both the pro form and the C-propeptide. In addition, COL1A1 and COL2A1 contain the pN fragment. Both the pro form and the C-propeptide form disulfide-linked oligomers and contain N-linked carbohydrates. The pro form can be used in assays for the proteolytic activity of PNP and PCP and for PCP enhancer 1 (PCPE1), which increases PCP activity. The recombinant collagens can also be used in studies targeted at their binding partners. We provide such an example, as in the case of pigment epithelium-derived factor (PEDF). Recombinant human COL1A1 and COL2A1 bind PEDF similarly to each other and to the native bovine type I collagen. In addition, the recombinant collagens have been used as immunogens to generate monoclonal and polyclonal antibodies with distinct specificity. In summary, the availability of recombinant human collagens and associated reagents should facilitate future studies of collagens focusing on their biosynthesis, structure, function and regulation.

(315) Engineered antibody for reducing localized fibrotic scarring

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Localized fibrotic changes continue to be a significant clinical problem. Although these changes may be triggered by various events such as trauma or surgical procedures, all them are developed through fundamentally similar pathways that, in the end, lead to excessive formation of fibrotic deposits of which the main component is collagen fibrils. Because current treatments of localized fibrosis are not fully effective, novel approaches have yet to be identified and explored. Recently, we defined a new target to limit excessive fibrosis, namely the formation of collagen fibrils. Initially, we described a monoclonal antibody of the IgA class able to inhibit formation of collagen fibrils by blocking critical collagen-collagen interaction. The utility of that antibody was demonstrated in vitro and in an animal model. As the next step toward developing clinically-relevant inhibitors of localized fibrosis, here, we present genetically engineered versions of the blocking IgA antibody: (i) a chimeric mouse-human IgG antibody variant and (ii) an scFv variant. The IgG construct was produced in CHO cells, while the scFV variant was expressed in bacteria and yeast systems. The inhibitory potential of these antibody constructs was evaluated *in vit*ro and in cell-based systems. The results indicate that both constructs decrease the rate of cleavage of the C-terminal propeptide of procollagen I, most likely, by blocking C proteinase cleavage sites. Assays of the inhibitory potential of the IgG form demonstrated a significant reduction in collagen-rich deposits in cell layers formed by normal dermal fibroblasts, keloid-derived fibroblasts, and cells isolated from normal tendon, a fibrotic joint capsule and fibrotic deposits associated with Dupuytren's contracture. Our results indicate the great

potential of recombinant inhibitors to reduce localized deposition of fibrotic tissue. Further tests in clinically-relevant animal models will establish the utility of the proposed inhibitors for future applications in patients. Supported by NIH R21AR061118.

(316) Chondroitin sulfate-mediated stabilization of hyaluronan-rich matrices

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Hyaluronan (HA) provides structural organization to many extracellular matrices (ECMs). HA is stabilized in the ECM through a trans-esterification process where the heavy chain (HC) proteins of inter-α-trypsin inhibitor (IαI) become covalently bound to HA and serve to cross-link HA through HC–HC interactions. IαI is a complex between two HCs, designated HC1 and HC2, covalently bound to the chondroitin sulfate (CS) chain that decorates bikunin. This study demonstrated that the bikunin CS chain structure controls the formation of HA–HC. Analysis of the CS chain covalently attached to Ser-10 of bikunin in plasma and urine revealed differences in the linkage region sulfation patterns. Bikunincontaining fractions in the biological fluids were separated by anion exchange chromatography, where fractions were analyzed for their reactivity with the 4-sulfated CS linkage region antibody (2B6). The fractions in urine that were 2B6 positive contained 10% un-sulfated, 30% 4-sulfated, 30% 6-sulfated and 30% di-sulfated CS disaccharides. In contrast, the 2B6 negative fraction contained 70% un- and 30% 4-sulfated CS disaccharides. Complexes of bikunin in plasma that were 2B6 positive were able to promote the trans-esterification of HCs to HA in vitro in the presence of TSG-6 while 2B6 negative fractions had a significantly reduced capacity to transfer HCs to HA. Thus, the bikunin CS chain structure is a key determinant of the stabilization of HA-rich matrices. These data also suggest that the transfer of HCs to HA is mediated by CS chains with high levels of sulfation and we hypothesize that the sulfated regions mediate binding of co-factors required for the trans-esterification process. Analysis of proteoglycan-enriched synovial fluid from pooled samples from individuals with rheumatoid arthritis indicated low levels of 2B6 reactivity, suggesting a limited ability to form HA–HC. These data have implications for the ability of HA to be stabilized during inflammatory processes which is essential to stabilize the matrix and prevent loss of matrix components.

(317) Fibronectin is essential for survival but is dispensable for proliferation of hepatocytes in acute liver injury

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The extracellular matrix (ECM) is a major component of tissue microenvironment. An ECM glycoprotein fibronectin is prominently expressed during adult tissue repair. We have recently identified fibronectin-independent mechanism of collagen fibrillogenesis in adult liver remodeling (Moriya et al. 2011). However, the extent of fibronectin dependence on hepatocyte response to acute liver damage remains to be defined. Because the identification of hepatic survival factors is critical for successful therapeutic intervention in liver failure, this relationship has been investigated using a fibronectin-deficient mouse model of acute liver injury. Here, we show that the lack of fibronectin induces significantly increased hepatocyte apoptosis, which is accompanied by a significant down-regulation of the antiapoptotic protein, Bcl-xL. Furthermore, fibronectin deficiency leads to a significantly elevated the production of the hepatocyte growth factor in hepatic stellate cells post-injury, which, in turn, results in an earlier onset and the acceleration of hepatocyte regeneration. Primary hepatocytes on fibronectin are protected from reactive oxygen species-induced cellular damage, retaining the expression of Bcl-xL, whereas those on type I collagen are not. This retained expression of Bcl-xL is inhibited by the PI3K inhibitor LY294002. Thus, we provide evidence that fibronectin-mediated matrix survival signals for hepatocytes are transduced through the PI3K/Bcl-xL-signaling axis in response to injury. This work defines fibronectin as a novel antiapoptotic factor for hepatocytes after acute liver injury, but demonstrates that fibronectin is not essential for subsequent hepatocyte proliferation. Supported by NIH grant DK074538.

Reference

Moriya K, Bae E, Honda K, Sakai K, Sakaguchi T, Tsujimoto I, Kamisoyama H, Keene DR, Sasaki T, Sakai T. 2011. A fibronectin-independent mechanism of collagen fibrillogenesis in adult liver remodeling. *Gastroenterology* 140:1653–1663.

(318) Mast cells produce novel shorter forms of perlecan that contain functional endorepellin: A role in angiogenesis and wound healing

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Mast cells are derived from hematopoietic progenitors that are known to migrate to and reside within connective and mucosal tissues where they differentiate and respond to various stimuli by releasing pro-inflammatory mediators including histamine, growth factors and proteases. This study demonstrated that primary human mast cells as well as the rat and human mast cell lines, RBL-2H3 and HMC-1 produce the heparan sulfate proteoglycan, perlecan with a Mr of 640 kDa as well as smaller molecular weight species

of 300 and 130 kDa, respectively. Utilizing domain-specific antibodies coupled with N-terminal sequencing, it was confirmed that both forms contained the C-terminal module of the protein core known as endorepellin, which were generated by mast cell-derived proteases. Domain-specific quantitative PCR experiments demonstrated that transcripts corresponding to the various domains including endorepellin were present suggesting that these cells were capable of producing the complete protein core. Fractions from mast cell cultures that were enriched for these fragments were shown to bind endothelial cells via the α2β1 integrin and stimulate the migration of cells in "scratch assays", both activities of which were inhibited by incubation with either anti-endorepellin or anti-perlecan antibodies. This study shows for the first time that mast cells secrete and process the extracellular proteoglycan perlecan into fragments containing the endorepellin C-terminal region that regulate angiogenesis and matrix turnover, which are both key events in wound healing.

(319) Characterization of the outer wall structure of the hydrocarbon-producing alga *Botryococcus braunii*

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Hydrocarbons from abundant microalgae offer an intriguing, renewable alternative to fossil fuels. *Botryococcus braunii* is a colonial green alga whose cells associate through a complex extracellular matrix and produce prodigious amounts of liquid hydrocarbons that can be readily converted into conventional combustion-engine fuels. The colonies are surrounded by an outer wall ("retaining wall") that holds in the hydrocarbons that the algae produce to stay afloat on the water surface and to maximize the received sunlight. The existence of this wall has been known for almost 40 years, but its structure remains elusive. The retaining wall is extremely resistant to degradation and represents a significant obstacle to extracting the oil from the algae. It also hinders the direct exploration of how to increase oil production by biotechnological means. Therefore, it is imperative that we understand the wall structure, with the aim to disrupt its synthesis or to break it down efficiently. During growth, the colonies shed pieces of the retaining wall. These cup-shaped pieces are termed "shells". We have isolated these shells and performed glycosyl composition and linkage analysis on them. We also partially hydrolyzed the shells, isolated the resulting fragments and acquired 1D and 2D NMR spectra. We found that the shells are almost entirely made up of carbohydrate, having a composition of 42% arabinose, 39% galactose and 9% 6-deoxyaltrose, in addition to minor amounts of glucose and mannose. The shells consist mostly of a complex arabinogalactan whose structural details are still under investigation, but also features a not yet fully identified polysaccharide-like large acidic polymer. The results of this study will ultimately lead us to higher oil yields, more efficient extraction methods and to make this and other algae economically viable renewable energy sources.

(320) A systems-level view of the extracellular matrix: From definition to domains

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Advances in high-throughput omic technologies are starting to provide unprecedented insights into how components of biological systems are organized and interact. In humans, dysregulation of the extracellular matrix (ECM) is associated with a number of complex and progressive diseases including atherosclerosis, arthritis, fibrosis and cancer. However, until recently little has been known about the underlying physical interaction network and how its architecture contributes to health and disease. In a recent survey of the ECM, we identified 357 proteins that represent core components of a high-quality ECM interactome, together with an additional 524 genes that mediate related functional roles. Using publicly available interaction data sets such as MatrixDB we defined a high-quality interaction network and derived a set of predicted functional modules and used additional metadata (e.g. functional descriptions, gene expression, MeSH disease terms and phylogenetic conservation patterns) to demonstrate biological relevance and predict disease associations. Phylogenetic profiles of ECM proteins highlight large groups of proteins restricted and/or expanded in metazoans, deuterostomes or mammals, suggestive of taxon-specific tissue innovations. Here, we further investigate the evolution of this complex system. It has been suggested that vertebrate complexity has predominantly arisen through the duplication and subsequent modification of retained, pre-exising ECM genes. We report that while approximately two-thirds of the ECM genes found in vertebrates appear to be vertebrate specific most domains in ECM proteins are of pre-deuterostome origin. Many of these domains participate in novel domain arrangements in vertebrates suggesting that the sampling of new domain combinations has been an important mechanism leading to the neo-functionalization of retained, preexisting ECM genes during vertebrate evolution. This is in contrast to the introduction of comparatively few recent domains.

(321) Insights on the unique glycosylation of the S-layer and surface glycoproteins of *Methanosarcina mazei*

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Little is known about glycosylation in Archaea, the least understood kingdom of life, and it may differ substantially from that of other kingdoms, including plants and mammals. Epi-fluorescence microscopy of ConA fluorescein with single cell cultures of the methanogenic archaeon *Methanosarcina mazei* suggests that surface glycosylation may influence cell morphology. Only aggregating cells interact with a labeled lectin. We have previously

identified MM1976 as the major S-layer protein of *M. mazei* and used MALDI-TOF MS to determine that it has three major glycoforms with MWs 73.2, 74.5 and 75.9 kDa, indicating glycan contributions of 1.6, 2.8 and 4.3 kDa, whereas SDS–PAGE migration of the major glycoform corresponded to 100 kDa vs protein standards. CAD MS/MS experiments on MM1976 glycopeptides determined glycan mass contributions ≤2.8 kDa. Glycopeptide enrichment and MS/MS analyses have enabled assignment of glycopeptides to 13 different surface glycoproteins. For some of these glycopeptides, MS/MS de novo sequencing indicated glycan masses ≤5544 Da. A 1305-Da glycan with composition Hex5dHex2HexNAc appears to be widely present on the *M. mazei* glycoproteins, along with longer and shorter analogs. The hotECD MS/MS structural determinations of MM1976 and MM0002 glycopeptides allowed definition of *N*-linked glycosylation at the classic sequon NXT ($X \neq P$) as the attachment mode for the 1305- and 1467-Da glycans. ECD MS/MS spectra obtained under standard conditions showed O-linked glycosylation of a single Hex residue at Thr and Tyr. To our knowledge, Tyr glycosylation, although reported on bacteria, has not been described for Archaea. Unique glycopeptides were assigned to specific glycoproteins. Although the present stage of this study did not aim to elucidate the full structures of the observed glycans, these glycans appear to contain a unique glycan core; experiments now underway should fully detail the glycans. Support: NIH P41 RR010888/GM104603, S10 RR020946, S10 RR025082, F31 AI061886 and DOE DEFC-02-02ER63421.

(322) Apolipoproteins E and AV are required for heparan sulfate-dependent clearance of triglyceride-rich lipoproteins

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Recent genetic experiments in mice have confirmed that the heparan sulfate proteoglycan, syndecan-1, acts as a receptor for triglyceride-rich lipoprotein (TRL) clearance in the liver (Stanford et al. 2009). Binding depends on the structure of the heparan sulfate chains based on the accumulation of plasma TRLs in mice bearing hepatocyte-specific mutations in the heparan sulfate modifying enzymes *N*-deacetylase *N*-sulfotransferase-1 (*Ndst1*, MacArthur et al. 2007) and uronyl 2-O-sulfotransferase (*Hs2st*, Stanford et al. 2010. J. Biol. Chem. 285:286). To identify the major apolipoproteins on the surface of TRLs that mediate binding to the heparan sulfate, we measured hepatocyte binding of TRLs isolated from mice lacking the major apolipoproteins, ApoB100, ApoB48 or ApoE in the presence or the absence of heparin lyases, bacterial enzymes that selectively digest heparan sulfate chains. Particles derived from $ApoE^{-/-}$ mice did not bind, whereas no significant difference was noted in binding of TRLs containing only ApoB100 or ApoB48 comaped with TRLs from wild-type mice. Reconstitution of ApoE-deficient TRLs with recombinant human ApoE3 restored heparan sulfate-dependent binding. Antibodies to ApoE blocked binding of human VLDL to the ectodomains of heparan sulfate proteoglycans shed from hepatocytes, whereas ApoB antibodies had no effect. Genetic studies of compound mutant mice with hepatic inactivation of *Ndst1* on an ApoE-deficient background resulted in elevated fasting plasma triglyceride levels in comparison with their single mutant littermates, suggesting another ligand in TRL binding. Interestingly, antibodies to ApoAV prevented binding of TRLs to heparan sulfate. Western blot analysis of TRLs from Ndst1-deficient mice exhibited the accumulation of ApoAV comaped with wildtype controls. Taken together, these data demonstrate that ApoE and ApoAV are required for heparan sulfate-dependent clearance of triglyceride-rich lipoproteins.

(323) Reduced ICAM-1 expression in N-glycosylation-deficient cells is associated with impaired inflammatory and immune responses

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Congenital Disorders of Glycosylation (CDGs) are caused by genetic defects in N-linked glycosylation. Patients exhibit highly variable motor and intellectual disabilities, seizures, developmental delay, hypoglycemia, clotting and digestion abnormalities. PMM2-CDG (CDG-Ia) is the most common CDG and is caused by mutations in *PMM2*, encoding phosphomannomutase-2, which converts mannose-6-phosphate to mannose-1-phosphate. Nearly, 20% of patients die in infancy often due to recurrent infection. Immunodeficiency was proposed as one of the underlying reasons, but this is unproven. We previously showed that intercellular adhesion molecule-1 (ICAM-1) was universally reduced in fibroblasts from CDG patients. Since ICAM-1 deficiency drastically mitigates innate immune responses, we speculated that reduced ICAM-1 on vascular endothelial surface in CDG patients may lead to an attenuated localized inflammatory or pathogen-stimulated response and subsequently to recurrent infection. To test the hypothesis, we first tested inflammatory responses in human endothelial cells (HUVECs) challenged by TNF-α together with tunicamycin (Tm) treatment. TNF-α-induced increase in ICAM-1 was greatly diminished by Tm. TNF-α increased monocytic adhesion to HUVECs, which was completely abolished after Tm treatment. CDG-Ib is caused by mutations in *MPI*, encoding phosphomannose isomerase, which converts fructose-6-phosphate to mannose-6-phosphate. MPI-deficient mice exhibited marked decrease in neutrophil extravasation in response to acute peritonitis. More surprisingly, we found a drastic decrease neutrophil number and proportion in the peripheral blood of these mutant mice as well, suggesting that reduced ICAM-1 may be also associated with the impaired neutrophil mobilization from the bone marrow into the circulation. We also found that neutrophil Mac-1 (CD11b), which is the adhesion receptor for ICAM-1, was also reduced dramatically in MPI-deficient mice both unchallenged and during acute inflammation. This may further attenuate adhesion between leukocytes and vascular endothelium in the process of leukocyte transmigration during innate immune response. The work was supported by the Rocket Fund and NIH R01DK22615.

(324) Biglycan and Decorin Dependent Fetal Membrane Signaling

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The leading cause of newborn mortality in the US is preterm birth, 40% of which is caused by preterm premature rupture of fetal membranes (PPROM). Patients with Ehlers-Danlos Syndrome are at increased risk of PPROM. One subtype of Ehlers-Danlos Syndrome is caused by the abnormal secretion of the proteoglycans biglycan and decorin. Biglycan/ decorin double-knockout mice are a model of preterm birth. Biglycan and decorin are associated with the TGF-β-Smad signaling pathway. Downstream targets of this pathway are collagens and tissue inhibitors of metalloproteinases, which block matrix metalloproteinases (MMPs). MMPs are linked to the pathogenesis of PPROM. TGF-β, Smad-2 and -3 expression was tested in biglycan-and decorin-knockout and wild-type fetal membranes at E12 and E18 using Western blotting. Fetal membrane MMP-8 and MMP-9 expression was tested at E18 in the same genotypes using ELISA assays. At E12, TGF-β, Smad-3 and phospho-Smad-3 were increased in the absence of decorin compared to wild-type, while at E18, Smad-3 and phospho-Smad-3 as well as Smad-2 and phosphoSmad-2 were decreased and MMP-8 and MMP-9 were increased. The absence of biglycan, on the other hand, did not lead to changes in signaling at E12, while at E18, TGF-β, Smad-2 and phospho-Smad-2 were decreased and MMP expression was unchanged. These results suggest that decorin downregulates the TGF-β-Smad pathway at E12, possibly leading to remodeling of the fetal membranes associated with increased fetal growth during early gestation, while by E18 it upregulates the signaling pathway, leading to a decrease in MMP expression and thus stabilization of fetal membrane integrity. Biglycan, on the other hand, plays a role in fetal membrane stabilization via TGF-β-Smad signaling at E18 but not earlier in gestation. Taken together, these results suggest differential roles for biglycan and decorin in signaling pathways that stabilize the fetal membranes during distinct phases of gestation.

(325) Lubricin, the Basis of Faulty Biolubrication and Immunological Consequences

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Biolubrication is key for sustaining the mobility of joints. The consequence of faulty biolubrication is pronounced in pathological conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA), where degradation of the joint is exacerbated by defect of the lubricating superficial layer on the cartilage. We have identified how a lubricating glycoprotein (lubricin) in the synovial fluid associates with joint surfaces using proteomic techniques and mass spectrometric identification of lubricin-associated protein macrocomplexes in synovial fluid. Our data suggest that part of lubricin becomes covalently linked to proteins in the extracellular matrix (ECM) of joint tissue. This explains how lubricin can provide efficient lubrication even under high-stress conditions in a healthy joint. The association of lubricin to joint surfaces allows oligosaccharides attached to lubricin to generate a friction free joint surface. In pathological conditions such as RA, the association of lubricin to joint surfaces is lost. Instead, lubricin will interact with synovial neutrophils accumulated in the synovial fluid due to the inflammation. We hypothesize that lubricin glycosylation, while necessary for the biolubrication, is responsible for inappropriate activation of synovial neutrophils present in high amounts in RA synovial fluid. This is partly due to binding to the subpopulation of L-selectin positive neutrophils in the synovial fluid with sialylated and sulfated oligosaccharides present on lubricin. The data suggest that the mechanisms for localization of surface active biolubricating molecules to synovial surfaces provide insight into transformation from a healthy state to pathological state.

(326) In vivo reduction in Smad2 concomitant with proteoglycan accumulation results in high penetrance of bicuspid aortic valves

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Bicuspid aortic valve (BAV) is the most prevalent cardiovascular malformation resulting in two rather than three mature cusps and occurs in at least 1–2% of the population. However, only NOTCH-1 loss of function has been linked to human BAV disease, and there are limited genetically modified mouse models that display BAV. We reported that loss of proteolytic cleavage of versican, a proteoglycan abundant in cardiac outlet cushions, results in myxomatous valves in ADAMTS5 deficient mice. Recently we discovered that a failure to cleave versican was concomitant with a reduction in cell–cell condensation, phosphorylated Smad2 and fibrous ECM organization in Adamts5 − / − valves. To test the hypothesis that that versican cleavage via ADAMTS5 is required, in part, to elicit Smad2 phosphorylation we further reduced Smad2 in Adamts5 − / − mice through intergenetic cross. The resulting Adamts5 − / − ; Smad2 + / − mice developed a more dramatic valve phenotype than Adamts5 − / − ;Smad2 + /+ mice and displayed a high penetrance of BAV (7/10). Interestingly, the pulmonary valve was also bicuspid in 6/10 (5/10 displayed both bicuspid PV and BAV). All Adamts5 − / − ;Smad2 + / − semilunar valves displayed wide hinge regions at the juncture of the annulus concomitant with loss of fibrous ECM. Use of the ADAMTS5 floxed model demonstrated that endothelial (Tie2-Cre) cell expression of ADAMTS5 is critical for normal valve maturation and to prevent endothelial fusion during valve formation. Collectively, these data suggest that the dramatic changes in proteoglycan turnover, during remodeling of the truncal cushions, elicit changes in cell behavior and signaling required for normal semilunar valve formation. Further studies of the Adamts5 − / − ;Smad2 + / − mice may elucidate a novel

etiology of BAV pathogenesis and lead to new pharmacological treatments for valve disease.

(327) Acid hydrolysis of maltooligosaccharides, cellooligosaccharides and glycogen

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During our investigation of cellulose from cotton fibers we have found small amounts of sugar alcohols and sugars other than glucose. As a result the question of the presence of these carbohydrates in α-glucans arose For this reason we investigated the acid hydrolysis maltooligosaccharides, starch and human liver glycogen from normal individuals and a Pompe disease patient. The hydrolysis products of maltooligosaccharides include *s*-inositol, iditol, sorbitol, galactose and mannose in addition to glucose. Maltooligosaccharides are produced by partial hydrolysis of starch. In a number of investigations of starch from many different plant sources, there is no mention of minor constituents other than glucose. Hydrolysis products of maltooligosaccharides and glycogen included gentiobiose, a ß-1,6 glucan, as well as isomaltose however, as expected, no gentiobiose was observed in the hydrolysis products of the phosphorylase limit dextrins of either normal or Pompe liver glycogen. The fact that the release of the minor monosaccharide and sugar alcohol components do not increase with hydrolysis time suggests that these components are not artifacts of glucose hydrolysis. In other control experiments, incubation of glucose or mixtures of sugars in either 2 N TFA or 6N HCl at 100°C for up to 3 hours does not result in any interconversion of sugars or sugar alcohols. The same absence of reports of carbohydrates other than glucose in glycogen exists.The possibilities of common steps in their biosynthetic pathways and common functions is intriguing from the standpoint of our understanding of basic biochemistry of glucan polymers. It is known that glycogen is synthesized on a protein primer. There appears to be an association between starch and protein and in other work we have found an association between cellulose and protein. We hypothesize that the minor carbohydrate components of these glucans may be involved in the complex structure of these polysaccharides.

(328) Interactions of DC-SIGN with 2α-Mannobiose and Lewis-x

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Dendritic Cell-Specific Intracellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) is a C-type lectin highly expressed on the surface of antigen presenting dendritic cells. DC-SIGN is believed to mediate the interaction between dendritic cells and T-cells through interactions with the Intracellular adhesion molecule-3 (ICAM-3). Thus, DC-SIGN serves as an important factor in the initiation of cell-mediated immune response and phagocytosis. DCSIGN also facilitates dendritic cell migration from blood to peripheral tissues through interactions with ICAM-2 during tethering and rolling of dendritic cells along endothelial linings. It has also been shown that DC-SIGN mediates interactions between dendritic cells and a variety of epithelia, myeloid cells and endothelia by binding to fucosylated Lewis^x (Le^x) and high-mannose residues on the carcinoembryonic antigen-related cell-adhesion molecule 1 (CEACAM1). Full-length DC-SIGN is a type II membrane protein that consists of a cytoplasmic tail, a trans-membrane helix, and an extracellular portion consisting of an α-helical tetramerization stalk plus a C-terminal C-type carbohydrate-recognition domain (CRD). Titration of the monomeric DC-SIGN CRD alone with each of 2α-Mannobiose and Le^x, monitored by 2D NMR, revealed significant perturbations of DC-SIGN cross-peak positions in each case. Saturation transfer difference (STD) and transferred NOE (trNOE) NMR experiments can provide additional information about ligand-protein interactions. However, these require larger protein constructs for sufficient transfer of magnetization to occur. Here, the extracellular tetrameric portion of DC-SIGN was used to investigate the binding of Le^x through STD and trNOE experiments, providing constraints for the orientation and conformation of Le^x within the binding site.

(329) Periductal induction of vascular addressin on high endothelial venule-like vessels in autoimmune pancreatitis

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Autoimmune pancreatitis (AIP) is a chronic, relapsing inflammatory disorder affecting the head of the pancreas and other organs such as salivary glands. Yoshida et al. characterized AIP in 1995, but the turning point did not come until Hamano and researchers at Shinshu University discovered elevated levels of serum IgG4 in patients with AIP in 2001. Since then, the IgG4-related disease has been catching the world's attention as a kind of systemic inflammatory disorder based on autoimmunity. However, the etiopathogenesis of AIP is not fully understood. On the other hand, some glycoproteins such as peripheral lymph node addressin and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) are reportedly expressed on high endothelial venule (HEV)-like vessels induced in various chronic inflammatory diseases (CID) and play a role in lymphocyte recruitment in inflamed tissues. AIP is also a kind of CID; histologically characterized by dense lymphoplasmacytic infiltration with marked storiform fibrosis, and obliterative phlebitis, which are observed around large- and medium-sized interlobular ducts. Such lymphocyte recruitment is thought to be elicited by dysregulation of mechanisms governing physiological lymphocyte homing. Here, we undertook immunohistochemical analysis of AIP using a battery of vascular addressin-related antibodies. We observed that the number of periductal MECA-79$^+$ HEV-like vessels was increased in AIP relative to that seen in

non-AIP chronic pancreatitis, while the number of MAdCAM-1$^+$ HEV-like vessels did not differ between the two conditions, suggesting that AIP is characterized by periductal induction of MECA-79$^+$ HEV-like vessels. We also found that the MECA-79 antigen is expressed on exocrine duct-forming epithelial cells not only in pancreas but also in salivary glands. Given that MECA-79 antigen is distributed on duct-forming epithelium in organs susceptible to AIP, our findings suggest that MECA-79$^+$ 6-sulfo sialyl Lewis X-related carbohydrate antigens on these epithelial cells could be associated with AIP pathogenesis.

(330) Fibronectin induces Crk protein recruitment of caspase-8 to the cell migration machinery

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Key regulators of programmed cell death, caspases also have recently identified roles regulating diverse non-apoptotic events including necrosis resistance and cellular migration. With respect to the latter, caspase-8 has been shown to have pro-migratory roles associated with recruitment of procaspase-8 to an NP-40 insoluble cellular compartment at the leading edge of migrating cells. However, it is not known how signals from the microenvironment control which roles are played by caspase-8, or how they are regulated. Here, we find that interaction with Crk-L, but not Crk, is critical for this recruitment and is dependent upon Src kinase activity induced by cell adhesion to fibronectin. Pharmacological and genetic blockade of Src or Crk-L inhibits caspase-8 recruitment to the pro-migratory complexes. The results suggest a mechanism by which the local microenvironment can toggle caspase-8 from proapoptotic to non-apoptotic functions.

(331) Human milk Glycosaminoglycans inhibit HIV-1 primary isolates in vitro infection.

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Background: In previous in vitro studies of human milk glycosaminoglycans (GAGs) effect on cell lines infection by HIV-1 reference strains, we found ≥80% inhibition of macrophage-tropic virus (R5) infection but not of lymphotropic virus (X4) infection. Here, we tested GAGs inhibition of in vitro infection of peripheral blood mononuclear cells (PBMCs) by HIV-1 primary isolates. GAGs were extracted from 2 L of pooled human milk purified by ion exchange chromatography and characterized by mass spectrometry. Six HIV-1 primary isolates from antiretroviral-naïve patients were used. To determine HIV-1 tropism, V3 loop of HIV-1 gp120 was sequenced to infer viral co-receptor utilization: CCR5 (R5) or CXCR4 (X4) and interpreted by Geno2Pheno algorithm. For inhibition assays, PHA-stimulated PBMCs were used. Serial dilutions of GAGs were incubated for 2h with each isolate before cell infection. After 7 days, HIV-1 P24 antigen was quantified by ELISA in culture supernatants. Significant inhibition of viral infectivity was defined as ≥80% reduction in P24 concentration with respect to controls. For comparison, we tested all isolates with non-human GAGs (shark cartilage chondroitin sulfate). Four isolates showed R5 tropism and two were X4. GAGs significantly inhibited infection by R5 isolates (87–100%) at a concentration of 33 μg/mL and only one R5 was inhibited (92%) with a higher concentration, 167 μg/mL. One X4 isolate was inhibited (96%) with 33 μg/mL and the other isolate was not inhibited. Chondroitin sulfate had no inhibitory effect. Human milk GAGs significantly inhibited in vitro infection of PBMCs by HIV-1 primary isolates from antiretroviral naïve patients. R5 and X4 HIV-1 primary isolates appeared to have different susceptibility to GAGs inhibition, which was dose-dependent.

(332) Platelet functions require correct protein O-glycosylation

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Platelets express a variety of membrane and secreted glycoproteins, but the importance of glycosylation to platelet functions is poorly understood. To explore the importance of O-glycosylation, we generated mice with a targeted deletion of Cosmc in murine endothelial/hematopoietic cells (EHC) (EHC Cosmc-/y). X-linked Cosmc encodes an essential chaperone that regulates protein O-glycosylation. This targeted mutation resulted in lethal perinatal hemorrhage in the majority of mice, and the surviving mice displayed severely prolonged tail-bleeding times and macrothrombocytopenia. EHC Cosmc-/y platelets exhibited a marked decrease in GPIb-IX-V function and agonist-mediated integrin αIIbβ3 activation, associated with loss of interactions with the von Willebrand factor and fibrinogen, respectively. Both GPIbα and αIIb, as well as the platelet collagen receptor GPVI, were partially proteolyzed in EHC Cosmc-/y platelets. These results demonstrate that extended *O*-glycans are required for normal expression and functions of these essential platelet glycoproteins and that variations in O-glycosylation may contribute to altered hemostasis.

(333) Overlapping basic sequences in the polysialyltransferases, ST8SiaII and ST8SiaIV, are required for the polysialylation of NCAM, Neuropilin-2 and SynCAM 1

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Polysialic acid (polySia) is critical for modulating cell–cell adhesion, is required for proper brain development and is involved in synaptic plasticity, neuronal regeneration, memory formation and tumor growth and invasiveness. Despite our understanding of the biological roles of polySia, the precise mechanism by which the polysialyltransferases (polySTs), ST8SiaIV (PST) and ST8SiaII (STX), synthesize polySia on substrata remains poorly understood. Previous studies from our laboratory have identified a polybasic

region (PBR) prior to the conserved catalytic sialylmotifs of the polySTs that is necessary for substratum polysialylation but not overall enzyme activity. Additional studies demonstrated that the PBR is likely involved in substratum recognition. In this study, we identify specific basic residues within the polyST PBRs that are important for the polysialylation of neural cell adhesion molecule (NCAM), neuropilin-2 (NRP-2) and synaptic cell adhesion molecule 1 (SynCAM 1). Replacing individual basic residues in the PST and STX PBR regions with alanines revealed that the requirements for polysialylation differed for these substratums. We find that PST Arg-82 is critical for the polysialylation of all three substratums. Efficient NCAM polysialylation also required Arg-93, while NRP-2 polysialylation required Lys-99 and SynCAM 1 polysialylation required both Arg-87 and Lys-99. A larger set of STX PBR residues reduced NCAM polysialylation when mutated to alanine, including Arg-97 (analogous to Arg-82 in PST), Lys-98, Lys-102 and Lys-108 (analogous to Arg-93 in PST). In contrast, STX Lys-102, Lys-114 and Lys-118 were required for NRP-2 polysialylation, and SynCAM 1 polysialylation required all the STX residues identified for the other substratums. Taken together, these results indicate that different combinations of basic residues within the PST and STX PBRs are critical for the polysialylation of distinct substratums and suggest that overlapping basic surfaces in the polySTs are used for the recognition of each substratum.

(334) Bacterial mucus-binding proteins: Structural and functional insights into their role in the adhesion to host glycans

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The mucus layer is a defensive barrier that covers the epithelium of the gastrointestinal (GI) tract. The main structural components of mucus are highly glycosylated mucin proteins carrying mainly *O*-linked glycan chains characterized by a high level of structural complexity and diversity. Protein–carbohydrate interactions are believed to play an important role in the adhesion of resident gut bacteria to the mucus layer. However, the nature of the ligands and the specificity of the interaction remain to be elucidated. Our research focuses on lactobacilli mucus-binding proteins (MUB) whose presence on the bacterial cell surface contributes to bacterial attachment to the protective mucus layer. The 353 kDa MUB from the *L. reuteri* strain ATCC 53608 consists of two types of modular mucus-binding repeats (Mubs) of 200 amino acids,Mub1 and Mub2, present in six and eight copies, respectively. We previously determined the first crystal structure of a type 2 Mub repeat at 1.8 Å, displaying high structural similarity to the repeat-unit of the *Peptostreptococcus magnus* Protein L (PpL), an immunoglobulin (Ig)-binding surface protein. Our recent small angle X-ray scattering (SAXS) experiments revealed an elongated boomerang-like solution structure of single and multiple Mub repeat proteins. These findings suggest a fiber-like appearance of MUB on the bacterial cell surface, mediating interaction to the host, as also observed for the purified native MUB by atomic force microscopy (AFM). Mub repeat proteins showed binding to different mucins, glycosylated mucin peptides and a number of mammalian Igs in vitro. Current work focuses on the characterization of the glycan moieties involved in the interaction between mucin glycoproteins and MUB using a range of biophysical methods.

(335) The role of galectin-3 in macrophages during Listeria monocytogenes infection: Critical role of galectin-3 in macrophage caspase-1 activation and intracellular Listeria survival

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Galectin-3 is a β-galactoside-binding animal lectin expressed in various immune cell types including macrophages. It has been shown to regulate multiple macrophage functions such as phagocytosis, LPS-mediated inflammation and alternative macrophage activation. Recently, galectin-3 was suggested as a marker for vacuole lysis by invasive pathogens and is co-distributed with inflammasome proteins. We have investigated the role of galectin-3 in macrophage antibacterial response during Listeria monocytogenes infection and found that galectin-3 is essential for efficient caspase-1 activation and the ensuing IL-1β processing and release when mouse bone marrow-derived macrophages (BMM) were infected with Listeria or stimulated with LPS and ATP. In addition, we showed that Listeria infection-induced autophagy activation was enhanced in gal3 −/− BMM. Autophagy serves as a defense mechanism against infection by targeting intracellular pathogens for degradation. Indeed, the enhanced autophagy activation correlated with lower bacteria burden, which was restored when autophagy was inhibited by 3-methyladenine. Immunofluorescence staining of Listeria-infected macrophages revealed a higher incidence of LC3-coated bacteria in gal3 −/− BMM comaped with that in gal3 +/+ cells, suggesting that galectin-3 may protect bacteria from LC3 targeting and autophagic degradation. Together these results demonstrated previously unrecognized roles of galectin-3 in promoting caspase-1 activation but dampening autophagy activity in Listeria-infected macrophages.

(336) Syndecan-1 attenuates lung injury during influenza infection by limiting apoptosis

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Syndecan-1 is a cell surface heparan sulfate proteoglycan that has multiple roles in healthy and pathogenic conditions. In the lungs, syndecan-1 has been shown to regulate lung repair and the inflammatory response after various injuries. Here, we investigated the role of syndecan-1 in influenza infection. Wild-type (WT) and syndecan-1 deficient ($Sdc1^{-/-}$) mice were intranasally infected

with 250–1000 PFU A/PR/8/34 influenza A virus (PR8), and lung injury and survival was evaluated. Primary cultures of WT and *Sdc1*$^{-/-}$ airway epithelium cultured at an air-liquid interface (ALI cultures) were also infected with PR8 virus (MOI 0.01). WT comaped with *Sdc1*$^{-/-}$ mice had better survival 12 days after high-dose PR8 infection (75 vs 20%, respectively; $P < 0.005$). Low-dose PR8 infection also induced greater weight loss in *Sdc1*$^{-/-}$ mice vs WT mice starting at day 4 after infection ($P < 0.001$). Moreover, *Sdc1*$^{-/-}$ mice had significantly worse lung injury comaped with WT mice as defined by increased IgM levels, PMN infiltration and RBCs in the bronchoalveolar lavage. Interestingly, measuring apoptosis by caspase-3 activity in lung homogenates revealed more apoptosis in infected lung tissue of *Sdc1*$^{-/-}$ mice in comparison with WT mice. Treatment of *Sdc1*$^{-/-}$ mice with zVad, a pancaspase inhibitor, trended toward improvement comaped with DMSO-treated *Sdc1*$^{-/-}$ mice as indicated by a reduced cellular infiltrate in the airspaces ($P = 0.09$). Because the influenza virus primarily infects the airway epithelium to manifest disease, we infected WT and *Sdc1*$^{-/-}$ ALI cultures with PR8 virus and found apoptosis was increased by 2-fold ($P < 0.005$) in the *Sdc1*$^{-/-}$ cells comaped with WT cells as measured by a caspase-3 activity assay. The lack of syndecan-1 profoundly aggravates the course of influenza virus infection in mice. Our studies show that syndecan-1 regulates apoptosis in lung epithelium to limit the lung injury after influenza infection.

(337) Identification and characterization of hyaluronan in human milk and potential functions in innate host defense of newborns

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Hyaluronan (HA), especially in its lower molecular weight form, is increasingly being recognized as a regulator of innate host defense mechanism, acting primarily through Toll-like receptors (TLRs) 4 and 2. We have recently demonstrated that very specific-sized HA fragments induce microbial defense pathways in intestinal epithelium in vitro and in vivo, specifically the expression of the antimicrobial peptide, human β defensin 2 (HβD2). We reasoned that this mechanism could promote healthy microflora development in intestinal tissue that was not yet colonized with bacteria and hypothesized that HA is present in human milk and serves as part of the maternal protection mechanism. Multiple human milk samples were collected from 45 donors at regular time points over the first year after delivery and analyzed for HA content and HA size, using the HA-specific enzyme-linked sorbant assay to determine quantity and carbohydrate gel electrophoresis to evaluate size distribution. Additionally, HT29 human intestinal epithelial cells were treated with HA purified from milk and HβD2 expression was measured to ascertain whether milk HA had the ability to induce defense mechanisms. HA is present in human milk and is found at the highest levels (up to 2 μg/ml) in the first 3 months after delivery. Milk HA is present in a wide size range from 750kDa to 20 kDa. Treatment of HT29 cells with HA purified from milk increases HβD2 protein expression and digestion of milk HA preparations with specific hyaluronidase abrogates the effect. Human milk contains HA, and concentrations are highest in the first months after onset of lactation, decreasing to a steady-state level thereafter. Milk HA is present in a polydispersed size range and 10–15% is present in the specific size range important for inducing innate defense.

(338) Influenza A Interactions with the Host Mucus Matrix Sialome

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Influenza A viruses infect the digestive tract of birds and the airways of mammals, including humans. These viruses bear two major glycoproteins on their envelope: hemagglutinin (HA) and neuraminidase (NA). In order to infect the host, the virus HA binds to sialic acids (Sias), which are found on the terminal position of glycosylated proteins and lipids on host cells. In contrast, NA cleaves Sias, and its activity is required to release newly budding viruses from the infected cells. Both digestive and respiratory tracts are covered with a thick mucus matrix, which is heavily sialylated and thus present potential decoy epitopes for Influenza binding. It has been speculated but not rigorously proven that the mucus matrix may inhibit Influenza A infection. We developed a fluorescent probe for internal labeling of Influenza A virions, such that the virus envelope and infectivity are both unaffected by the labeling reagent. Using this method we were able to track individual virions using fluorescent microscopy and analyze virus interaction with the mucus matrix. We show that Influenza A virus directly binds to sialylated mucins on host target tissues and in vitro and that the mucus matrix can protect underlying cells from infection. In addition, the enzymatic activity of virus NA is required for the release of influenza virions from the mucus "decoy" Sias to allow infection. We thus propose that NA activity is required for virus penetration through the protective mucus during initial infection process. This novel role for influenza NA has direct implications on screening for anti-influenza drugs. Anti-influenza drugs, e.g. oseltamivir (Tamiflu) and zanamivir (Relenza), target the virus NA. New screening strategies for putative NA inhibitors should be developed in a more naturalistic molecular context that include the mucus component. Furthermore, host-mucus composition should be considered as an important host factor for influenza species specificity and may be an important factor in events of multiple virus infections and the subsequent risk of viral re-assortment.

(339) Adenovirus infection induces changes in glycosylation of human-infected cells

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Cell surface glycosylation has been clearly involved in the entry mechanism of viruses like rotavirus and influenza (Guerrero 2000;

Kirkeby 2009). Nonetheless, little is known on how virus infection induces glycosylation changes that could play a role in the infectious process. It has been described that infection with HIV-1, CMV, HZV and HTLV-1 viruses modifies glycosylation profiles of infected cells particularly at the expense of changes in terminal glycosylation modifications, specifically fucosylation and sialylation (Nyström 2009). These changes have been associated with alterations in the expression of genes coding for fucosyltransferase (FUT) or donor substratum biosynthetic enzymes (Giodanengo 2004). The aim of this work was to determine if Adenovirus serotype 5 (Ad5), a common pulmonary human pathogen, induces glycosylation changes in infected cells. Lectin flow cytometry of Ad5-infected A549 cells (lung cell line) was determined at 8 and 16 h post-infection (p.i.). Lectins *Sambucus nigrans* (SNA; α2,6 sialic acid), MAL II (*Maackia amurensis*; α2,3 sialic acid), AAL (*Aleuria aurantia*; *core* α1,6 fucose and α1,2/3 terminal fucose), LCA (*Lens culinaris*; *core* α1,6 fucose) and *Ulex europaeus* (UEA; terminal α1,2 fucose) were selected to identify changes in fucosylation and sialylation of control and Ad5-infected cells. We identified that adenovirus infection was capable of inducing significant changes in fucosylation of A549 cells at 16 h p.i. that could be relevant in changing the cell signaling response (Liu 2011). We also show the association of these changes to regulation in the expression of FUT genes.

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(340) Specific-sized hyaluronan frgments promote innate defense of the intestinal epithelium

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Hyaluronan (HA) is a glycosaminoglycan polymer found in the extracellular matrix of many mammalian tissues. Recent findings have suggested a role for small, fragmented HA polymers in initiating innate defense responses in immune cells, endothelium, and epidermis through interaction with innate molecular pattern recognition receptors such as TLR4. Despite these advances, little is known regarding the effect of fragmented HA at the intestinal epithelium, where numerous pattern recognition receptors act as sentinels of an innate defense response that maintains epithelial barrier integrity in the presence of abundant and diverse microbial challenges. As part of healthy mucosal defense, intestinal epithelial cells secrete the 7kD antimicrobial protein human b-defensin 2 (HbD2) into the mucus lining of the intestine. We have observed that highly purified HA fragments promote expression of intracellular HbD2 in intestinal epithelial cells. Treatment of HT-29 colonic epithelial cells with HA fragment preparations resulted in time- and dose-dependent up-regulated expression of HbD2 protein in a fragment size-specific manner, with 35 kDa HA fragment preparations emerging as the most potent inducers of intracellular HbD2. Furthermore, oral administration of HA fragments promotes the expression of an HbD2 ortholog in the colonic epithelium of both wild-type and CD44-deficient mice, but not in TLR4-deficeint mice. Importantly, HA fragments promote functional enhancements to the defense of the intestinal epithelium. For example, pretreatment of HT-29 cells with 35kDa HA fragments promotes resistance to intracellular infection by the pathogen *Salmonella typhimurium*. Future investigations will be focused on characterizing the functional effects of HA fragments in the inhibition of intracellular infection in intestinal epithelium, as well as evaluating the therapeutic applications of fragmented HA in diseases in which epithelial integrity is compromised.

(341) Helicobacter pylori cholesteryl α-glucosides are critical for bacterial growthand activation of invariant NKT cells

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Helicobacter pylori (*H. pylori*) uniquely contains cholesteryl α-glucosides (αCGs) as the major components of its cell wall. Cholesteryl-αglucoside (αCGL) is synthesized by cholesterol α-glycosyltransferase (αCgT), and αCGL is a precursor for cholesteryl-acyl-α-glucoside and cholesteryl-phosphatidyl-α-glucoside (αCPG). We reported that mucin-type glycoproteins expressing α1,4-*N*-acetylglucosamine residues suppress bacterial growth by inhibition of αCGs synthesis (***Science*** 2004, ***BBRC*** 2006). To determine roles of αCGs, we analyzed amino acid substitutions in αCgT of *H. pylori* from 24 Japanese clinical isolates. Recombinant α CgT showed diverse activity from 15 to 174% comaped with the αCgT in the wild-type *H. pylori* 26695. Analysis of the mutated αCgT activity and histological features revealed that the enzyme activity is associated with the progression of gastric atrophy. We generated αCgT deficient *H. pylori* (αCgT^{Δ}) and αCgT^{low}, which expressed higher and lower αCgT activity then wild-type, respectively. αCgT^{Δ} exhibited delayed growth and morphologic abnormality caused by loss of all of αCGs. These results suggested that αCGs are essential for *H. pylori* growth. We investigated functions of αCGs in immune response. Phagocytosis and the activation of dendritic cell were observed at the same degrees in the wild-type *H. pylori* and all

mutants, regardless of αCgT activity. However, response by invariant NKT (iNKT) cells was correlated with the activity of αCgT. In synthetic compounds of αCGs, monoacylated αCPG was the most potent antigen by isoelectrofocusing and in vivo assays. *H. pylori* was more retrieved from the stomach of iNKT deficient ($J\alpha18^{-/-}$) mice than wild-type mice in *H. pylori* infection assay. Furthermore, the expression level of cytokines in infected $J\alpha18^{-/-}$ mice was significantly decreased than infected wild-type mice. Our findings suggested that recognition of αCGs by iNKT cells is necessary for bacterial clearance and stimulation of iNKT cell response (Submitted). Supported by NIH grants PO1CA71432, CA33000, KAKENHI 24790388, Toyobo Biotechnology Foundation Fellowship and Ube Industries Research Foundation.

(342) Trans-sialidases of Trypanosma congolense

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Trypanosoma congolense is a parasite causing Nagana, the trypanosomiasis in African ruminants, a socio-economic threat throughout sub-Sahara Africa. Trypanosomes express trans-sialidases (TS) catalyzing the transfer of sialic acids to another glycan, forming α2,3linkages to galactose or N-acetylgalactosamine. Several lines of evidence showed that TS play important roles in pathogenesis and survival of the parasite in the insect vector and possibly also in the mammalian host. However, the role of TS in protection against the host's and vector's defence systems has remained unclear. In order to test the hypothesis that TS and/or TS-like gene products play a role in the infection cycle of *T. congolense*, we have cloned 14 TS-like genes found in the genome of these parasites. Eleven of these (TS1 variants) are very similar with over 95% amino acid identity, whereas the other three are more distantly related with 45–60% sequence identity. We have expressed the corresponding recombinant proteins and characterized their enzymatic properties. All proteins have trans-sialidases with more or less sialidase activities, but differ significantly in their substratum specificities and kinetics. To address whether TS-like genes are expressed by the bloodstream form of *T. congolense*, we have investigated the expression of TS-like mRNAs in a natural infection cycle. For this, teneral tsetse flies were allowed to feed on a rat infected with *T. congolense* and then these flies were fed on a healthy goat. Blood samples collected daily from the goats were analyzed by RT-PCR for the presence of mRNA encoding TS-like genes. In addition, the presence of parasites was investigated by PCR of the ITS1 region of *T. congolense* from genomic DNA extracted from thee blood samples. Interestingly, mRNAs for several TS-like genes were found in the blood of infected goats as soon as parasites were detected by PCR.

(343) Sialic acid catabolism by *Gardnerella vaginalis* in bacterial vaginosis

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Bacterial vaginosis (BV) is an imbalance of the vaginal flora associated with pregnancy complications and other adverse health outcomes. In BV, lactobacilli of the normal flora are replaced with Gram-negative anaerobes and *Actinobacteria*, including *Gardnerella vaginalis*, a bacterium found to correlate with the clinical Amsel criteria for BV. How BV bacteria lead to the symptoms and complications of BV has not been resolved. One of the diagnostic features of BV is the presence of sialidase enzyme activity in vaginal fluids. We have established that sialylated glycoproteins found in mucus, such as mucins and immunoglobulin A, are substrata for BV sialidases. Sialidases present in clinical specimens are capable of cleaving 2,3- and 2,6-linked sialic acids from *N*- and *O*-glycans. However, the fate of free host sialic acids after hydrolysis is unknown. Here, we investigate the sequence of events in mucus sialoglycan hydrolysis and catabolism by *G. vaginalis*. This study demonstrates that *G. vaginalis* can release, take up, catabolize and deplete sialic acid residues from a variety of mucosal sialoglycoprotein substrata, including human vaginal mucus. Furthermore, analysis of human vaginal specimens showed that women with BV have lower levels of total sialic acids than normal healthy controls, providing evidence that foraging and depletion of host sialoglycans occurs in the human vagina during BV.

(344) Roles of sialidase in bacterial vaginosis: *Gardnerella vaginalis* in a new murine model

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Bacterial vaginosis (BV) is an imbalance of the vaginal flora characterized by a shift from a lactobacillus-dominant environment to a polymicrobial mixture of other anaerobes including *Actinobacteria* and Gram-negative bacilli. BV is associated with increased risk of sexually transmitted infection and adverse pregnancy outcomes, including preterm birth. Sialidase activity in vaginal fluids is one of the key diagnostic features of BV, but little is known about the role of sialidase during host-microbe interactions in the vaginal niche. Lack of a validated animal model with clinical features of BV has been a major impediment in this field. Here, we show that *Gardnerella vaginalis*, one of the most frequently isolated bacterial species in BV, is sufficient to cause several BV-like features in a murine vaginal inoculation model, including a robust exfoliation response, the presence of "clue-like" cells and sialidase activity in vaginal washes, depletion of sialic acids from the vaginal mucosa and ascending infection of the uterine horns. Additional experiments revealed that *G. vaginalis* sialidase activity reduces the overall viscosity of mucus and promotes bacterial interactions with galactose residues underlying sialic acids on vaginal epithelial cells. These results suggest that while BV is a *polymicrobial* condition in the clinical setting, infection with *G. vaginalis* alone is sufficient to cause BV-like phenotypes in an animal model. There has been much debate in the

literature concerning the contribution of *G. vaginalis* to the etiology and pathology of BV, since it is also present in a significant proportion of healthy women. These results conclusively demonstrate the capacity of sialidase-positive *G. vaginalis* to contribute to the clinical features of BV. Future studies will use this animal model to investigate important questions regarding BV etiology, pathogenesis and associated complications.

(345) Silent structural features of *Trichomanas vaginalis* CPI-GC

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Human trichomoniasis is the most common sexually transmitted infection (STI) caused by *Trichomonas vaginalis* (TV). The disease is linked to premature delivery, low birth weight, infertility and vaginitis and has been reported to heighten HIV infection as well. The molecular mechanism underlying the pathobiochemistry is poorly understood. We have shown that a predominant cell surface glycoconjugate, lipophosphoglycan (LPG) is involved in adherence of TV to host cells and mediates host inflammatory responses to infection. We have partially characterized a biologically active part of LPG, the CPI-GC (ceramide phospho-inositol glycan core) which is required in triggering immune inflammatory pathways in human vaginal and cervical epithelial cells. CPI-GC binds to galectins (Gal) -1 and -3. In this study, (i) we compared the monosaccharide compositions of CPI-GCs from several isolates, (ii) compared binding of CPI-GCs to gal-1, -3, Tomato and Ricin lectins using Biolayer Interferometry and (iii) further structural characterization of CPI-GC by NMR and enzymatic methods. Both the monosaccharide composition and lectin-binding properties vary between the strains. The varying degree of binding to lectins suggests the degree and amounts of n-LacNAc repeats as well as branching in glycan. NMR results showed that CPI-GC (from our UR-1 strain) contains terminal non-reducing β-Gal, β-GalNAc. However, -3-α-Gal-1-3-GlcNAc-1- and -3-α-Gal-1-4-β-GlcNAc-1-disaccahride repeat units are the predominant structural features. The presence of α-Gal and β-GlcNAc was further confirmed by specific enzyme treatments. The presence of terminal GalNAc and ß-Gal suggests that the chains are either terminated at the non-reducing end of the sugars or that the chains have some GalNAc and ß-Gal side chains. Further, structural analyses are needed to understand the nature and binding of this complex glycan. Supported by NIH-NIAD grant RO1 AI079085.

(346) Overexpression of UDP-GlcNAc:polypeptide *O*-α-*N*-acetyl-Dglucosaminyltransferase-2 (TcOGNT2) inhibits metacyclogenesis of Trypanosomacruzi

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Biosynthesis of *O*-glycans in all life-cycle stages of *T. cruzi* begins via addition of αGlcNAc to threonine in a reaction catalyzed by a Golgi UDPGlcNAc:polypeptide *O*-α-*N*-acetyl-D-glucosaminyltransferase (ppaGlcNAcT). Previously, we identified three genes in *T. cruzi* (*TcOGNT-1*,*-2* and *-L*) encoding putative ppaGlcNAcTs and, after heterologous expression in *Leishmania tarentolae*, TcOGNT2 was shown to be an authentic ppaGlcNAcT. Here, we report that *TcOGNT2* is transcribed into a mature mRNA in epimastigotes and monospecific anti-TcOGNT2 detects the endogenous protein by western blot (WB) and immunofluorescence (IMF). However, TcOGNT2 was not detected after differentiation into metacyclics. Overexpression of catalytically active (with "DSH"motif) or inactive myc-TcOGNT2 in epimastigotes showed 10-fold higher levels of normal or mutant protein. Microsomes from cells overexpressing active TcOGNT2 exhibited 1.5x higher ppaGlcNAcT activity and 5-fold higher UDP-[^{3}H]GlcNAc hydrolysis activity than mock transfectants. Although sub-cellular fractionation and WB indicated a microsomal association, IMF revealed both Golgi and extra-Golgi localization. Despite no difference in growth, parasites overexpressing catalytically active or inactive TcOGNT2 were strongly inhibited in metacyclogenesis both in vitro and in vivo. Little effect on glycosylation was observed in the overexpressors based on WB of epimastigote mucins and cysteine proteinase, flow cytometry using fluorescent ConA or WGA (except a 15% reduction in GS-IB4 binding) and MALDI-TOF-MS of *O*- and *N*-glycans. The minimal changes in glycosylation suggest that overall Golgi protein burden may be a factor that regulates the ability of epimastigotes to differentiate into metacyclic trypomastigotes. Supported by Fogarty-NIH, CNPq, FAPERJ.

(347) Roles of active and inactive forms of the group A *Streptococcus hyaluronidase* (HylA) in bacterial interactions with host innate immunity

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Streptococcus pyogenes (group A *Streptococcus*; GAS) is a leading Gram-positive bacterial pathogen associated with a wide spectrum of human disease including pharyngitis, rheumatic fever and toxic shock syndrome. GAS expresses a surface hyaluronic acid (HA) capsule, identical in structure to host HA, which is a major virulence factor promoting survival in the host. Despite this fact, many GAS strains also express hyaluronidase enzymes (including the secreted HylA), while others express enzymatically inactive forms of this enzyme, often correlated with their M protein serotype. In this study, we analyzed the roles of active and inactive HylA, using a representative M1 GAS strain (5448) and M4 GAS strain (4063_05) and recombinant forms of the enzyme prepared from each strain. Using a spectrophotometric assay to measure release of dissacharide units from the HA polymer, M4 GAS and recombinant M4 HylA showed hylauronidase activity, whereas M1 GAS and recombinant M1 HylA did not. Isogenic mutant strains lacking the *hylA* gene (Δ*hylA*) were constructed in both the M1 and M4 background by allelic replacement, and expression vectors for the M4 (active) and M1 (inactive) versions

of HylA were produced for complementation analyses (pM4HylA and pM1HylA). The wild-type (WT) M4 GAS strain possessed negligible capsule comaped with the WT M1 strain. Introduction of pM4HylA but not pM1HylA into the M1Δ*hylA* strain led to a marked reduction (90%) in HA expression on the bacterial surface. In human blood survival assays, both the M1Δ*hylA* and M4Δ*hylA* were cleared more quickly than their respective parent strains. Furthermore complementation of the M1Δ*hylA* mutant with either the active pM4HylA or the inactive pM1HylA plasmids promoted an increase in bloodstream survival comaped with untransformed mutant. These results indicate that the active M4 HylA can degrade the GAS HA capsule, but that HylA contributes to bacterial survival in human blood through a mechanism independent of its HA activity. The intersecting roles of GAS HA mimicry and of the active and inactive HylA enzymes in GAS immune invasion, epithelial cell interactions, and animal virulence are currently under investigation.

(348) Role of bacterial surface factors in adherence of enteric bacteria to plantpolysaccharide

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It is generally recognized that bacterial attachment to plant or animal surfaces constitutes the first step in colonisation. This mechanism is mediated by interactions between adherence factors that are expressed on the bacterial cell surface, such as fimbriae or flagella, and host cell surface glycans. For example, *E. coli* type 1 fimbriae are well characterised structures for their interaction with mammalian epithelial cells and are known to recognise specific glycans present on host cell surfaces, such as α-D-mannose. *In planta,* recent papers have demonstrated the implication of curli fibers and flagella from Enteropathogenic *E coli* and *Salmonella* species respectively. However the molecular recognition pattern implicated in adhesion of human pathogen bacteria to plant tissues has yet to be described. In this work plant glycan arrays have been used to investigate specific interactions with bacterial adherence factors. The work provides an improved understanding about the molecular mechanisms underpinning foodborne outbreak associated pathogens with plants.

Late-breaking Abstracts

(LB01) X-ray diffraction and microscopy study of non-enzymatic decomposition ofcollagen fibers by a Biglycan antibody and a plausible mechanism for rheumatoid arthritis

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Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory and destructive joint disorder that affects tens of millions of people worldwide. Normal healthy joints maintain a balance between the synthesis of extracellular matrix (ECM) molecules and the proteolytic degradation of damaged ones. In the case of RA, this balance is shifted towards matrix destruction due to increased production of cleavage enzymes and the presence of (autoimmune) immunoglobulins resulting from an inflammation induced immune response. Herein, we demonstrate that a polyclonal antibody against the proteoglycan biglycan (BG) causes tissue destruction that may be analogous to that of RA affected tissues. The effect of the antibody is more potent than harsh chemical and/or enzymatic treatments designed to mimic arthritis-like fibril de-polymerization. In RA cases, the immune response to inflammation processes causes synovial fibroblasts, monocytes and macrophages to produce cytokines and secrete matrix remodeling enzymes, whereas B cells are stimulated to produce immunoglobulins. The specific antigen that causes the RA immune response has not yet been identified, although possible candidates have been proposed, including collagen types I and II, and proteoglycans (PGs) such as biglycan. We speculate that the initiation of RA-associated tissue destruction in vivo may involve a similar non-enzymatic decomposition of collagen fibrils via the immunoglobulins themselves that we observe here ex vivo.

(LB02) Novel anti-HIV-1 activity produced by conjugating unsulfated dextran with poly-L-lysine and characterization of the unique antiviral mechanism

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To understand significance of higher-order structure of proteoglycans (PGs) and create a bioactive compound, we synthesized unique pseudoproteoglycans (pseudoPGs) that simulate PG macromolecular structures with combinations of different sizes and different types of glycans and core polymers. A conjugate of poly-L-lysine (PLL) with unsulfated dextran (Dex) was found to have remarkable anti-HIV-1 activity against both the macrophage-tropic R5 virus Ba-L and T-cell line tropic X4 virus IIIB strains, although neither PLL nor dextran has such activity (Nakamura et al. 2012). Conjugation with dextran was found to produce an antiviral effect showing the chain-lengths dependency in three kinds of assay systems including a human CD4 + T-cell line. The pseudoPG synthesized using 10 kDa PLL and 10 kDa dextran showed EC_{50} 4–40 times lower than that of sulfated dextran or heparin against Ba-L and EC_{50} equal to that against IIIB virus, indicating that PLL-Dex was more effective against R5 virus than sulfated polysaccharides. PLL-Dex suppressed a clinically isolated R5 virus from primary peripheral blood mononuclear cells. PLL-Dex significantly inhibited virus adsorption to cells and subsequent virus entry into the cells (Nakamura et al. 2012). On membrane analysis using separated HIV-1 proteins demonstrated that PLL-Dex bound to viral core proteins but not to envelope glycoproteins including GP160. Flow cytometry using budding MOLT-4-IIIB cells supported that Dex-PLL bound at a site different from that of heparin. When lysates of MOLT-4 were separated by SDS-PAGE and transferred to a PVDF membrane, several protein bands were observed to bind to PLL-Dex, suggesting that PLL-Dex binds to cellular proteins. These results suggest that PLL-Dex has unique multiple preventive mechanisms against HIV-1. Although the antiHIV-1

mechanisms of PLL-Dex have to be elucidated, we propose the use of the neoglycoconjugate, pseudoPG, to develop a novel anti-HIV-1 treatment.

Reference

Nakamura K, *et al* (2012) Novel anti-HIV-1 activity produced by conjugating unsulfated dextran with polyL-lysine*Antiviral Res*2012, 9489–97.

(LB03) Microfibril-associated Protein 4 (MFAP4) as a biomarker of COPD

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To enhance accuracy in evaluating prognosis and target therapy, there is a need for biomarkers in COPD. Yet, there are no reliable biomarkers that can differentiate between phenotypes of COPD. MFAP4 is a glycoprotein, co-localized with elastin and microfibrils in elastic fibers. We hypothesized that circulating MFAP4 reflects elastin degradation and thereby emphysema in COPD patients. Plasma levels of MFAP4 (pMFAP4) were determined by ELISA in 74 Danish COPD patients from the multicentre ECLIPSE (Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-points) study. Associations between log-transformed pMFAP4 levels and clinical outcomes were assessed by multiple regression analysis. Age, gender and current smoking were included as covariates. Levels of pMFAP4 associated significantly with GOLD stages and were significantly higher in GOLD IV than in II. LogpMFAP4 was positively correlated with percentage of low-attenuation area on HRCT (LAA%; $\beta = 0.007$, $P < 0.005$). There was moreover a significant negative association between plasma log-pMFAP4 and FEV1/FVC index ($\beta = -0.007$, $P < 0.05$) and reversibility ($\beta = -0.6$, $P < 0.05$). There was a significant interaction between gender and BMI and Fat Free Mass Index (FFMI), respectively, showing a significant negative association between MFAP4 and BMI ($\beta = -0.03$, $P < 0.01$) and FFMI ($\beta = -0.08$, $P < 0.005$) among women. MFAP4 levels in plasma were associated with severity of COPD and were positively correlated with HRCT, and negatively correlated with FEV1/FVC, and reversibility.

(LB04) Trypanosoma cruzi surface calreticulin interactswith Thrombospondin-1 to enhance infection

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Trypanosoma cruzi causes Chagas heart disease, which is a neglected tropical disease that produces severe pathology and mortality. The mechanisms by which the parasite invades cells are not well elucidated. We recently reported that *T. cruzi* up-regulates the expression of thrombospondin-1 (TSP-1) in human coronary artery smooth muscle (HCASM) cells to enhance the process of cellular invasion. Furthermore, we show that labeled TSP-1 interacts specifically with the surface of *T. cruzi* trypomastigotes. We used TSP-1 to pull down interacting parasite surface proteins that were identified by mass spectrometry. Full length TSP-1 and the N-terminal domain of TSP-1 (NTSP) interact with *T. cruzi* surface calreticulin (TcCRT) and other surface proteins. Pre-exposure of recombinant NTSP or TSP-1 to *T. cruzi* significantly enhances cellular infection of wild-type mouse embryo fibroblasts (MEF) comaped with the C-terminal domain of TSP-1, E3T3C1. In addition, blocking TcCRT with specific monovalent Fab antibody fraction significantly inhibits the enhancement of cellular infection mediated by the TcCRT-TSP-1 interaction. Taken together, our findings indicate that TSP-1 interacts with TcCRT on the surface of *T. cruzi* through the NTSP domain and that this interaction enhances cellular infection. Thus, surface TcCRT is a novel virulent factor that enhances the pathogenesis of *T. cruzi* infection through TSP-1, which is up-regulated by the parasite. Supported by NIH grants: SC2AI083925**;** 5 U54 RR026140-03; AI080580; GM05999; G12RR003032; 5T32 AI007281-23.

(LB05) Keratinocyte-derived Laminin-332 Promotes Melanin Synthesis via Regulating the Uptake of Tyrosine

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Melanocytes, which produce the pigment melanin, are known to be closely regulated by neighboring keratinocytes. However, how keratinocytes regulate melanin production is unclear. Here, we report that melanin production in B16F10 mouse melanoma cells was markedly increased on a keratinocyte-derived extracellular matrix (ECM) comaped with a B16F10-derived ECM. siRNA-mediated reduction in keratinocyte-derived laminin-332 expression decreased melanin synthesis in melanoma cells, and laminin-332, but not fibronectin, enhanced melanin content and α-melanocyte stimulating hormone (α-MSH)-regulated melanin production in melanoma cells. Similar effects were observed in human melanocytes. Interestingly, however, laminin-332 did not affect the expression or activity of tyrosinase. Instead, laminin-332 promoted tyrosine uptake into melanoma cells via ERK activation. Taken together, these data strongly suggest that keratinocyte-derived laminin-332 contributes to melanin production by regulating tyrosine uptake.

(LB06) Fer tyrosine kinase down-regulates α-dystroglycan glycosylation in C2C12 myotube cells

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In skeletal muscle, dystrophin-glycoprotein complex, which consists mainly of α/β-dystroglycan (α/β-DG) and dystrophin, serves as a linker between cytoskeleton and the extracellular matrix. *O*-mannosylated glycans attached to α-DG plays a key role in interaction with laminin since their defects cause severe congenital muscular dystrophies (CMDs). While seven causative genes, including *like-acetylglucosaminyltransferase* (*LARGE*) and β*3-Nacetylglucosaminyltransferase 1* (*B3GNT1*), are found to be associated with α-DG glycosylation so far, little is known about the upstream mechanism that controls expressions of laminin-binding glycans. Recently, our laboratory has identified, by siRNA screening, Fer tyrosine kinase as a crucial regulatory factor for the laminin-binding glycan synthesis in prostate cancer cell lines (Yoneyama et al. 2012). In this study, we verified Fer activity in muscle cells using established differentiation model of mouse myoblast C2C12 cell. We found by RT-PCR that C2C12 myoblast cell expresses *Fert2*, a mouse homolog of human *Fer*, as well as α-DG, LARGE and β3GnT1. C2C12 myoblast transfected with Fer-cDNA down-regulated the expressions of β3GnT1. As reported previously (Suzuki et al. 2003), C2C12 myoblast cells grew along the direction of the underlying fibers and form an aligned pattern similar to those in native skeletal muscle tissues when cells were cultured in differentiation medium (DMEM containing 2% horse serum). These differentiated myotube cells showed increased generation of glycosylated forms of α-DG detected by IIH6 antibody. Interestingly, we found Fer overexpression in C2C12 myotube cells suppressed laminin-binding glycan synthesis and they grew into rather rounded chunks. Conversely, Fert2 siRNA up-regulated the laminin-binding glycan expression and myotube formation. These results indicate that Fer suppresses expression of the glycosyltranseferases associated with α-DG glycosylation and thus down-regulates synthesis of laminin-binding glycans. This gives a strong suggestion of physiological significance of Fer in the muscle cells. Up-regulation of α-DG glycosylation, which can be mediated by blocking of Fer kinase signaling, will be potentially a valuable strategy for treatment of CMDs. Supported in part by NCI PO1CA71932 grant.

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(LB07) Transmembrane domain plays a specific role in regulating the functions of syndecan

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Syndecans, cell surface adhesion receptors initiate intracellular events through receptor clustering, which is mediated by the transmembrane domain. However, the exact role of the transmembrane domain is not fully understood. Here, we investigated the role of the transmembrane domain in syndecan function. Syndecan-2 mutants that transmembrane domain was replaced with that of syndecan-4 were defective in syndecan-2-mediated attachment and migration of rat embryonic fibroblasts cells on fibronectin, suggesting the specific role of syndecan transmembrane domain. Compared with wild type, syndecan-2 mutant showed reduced SDS-resistant dimer formation and reduced intermolecular interaction between syndecan-2 molecules, which was critical for oligomer formation of syndecan-2. Consistently, altered oligomerization tendency in syndecan-2 mutant resulted in weaker interaction of syndecan-2 on fibronectin and subsequent focal adhesion formation. Taken together, these data suggest that each transmembrane domain of syndecan plays a specific role in regulating syndecan functions.

(LB08) A proximal region within endorepellin binds Ig domains 3–5 of VEGFR2 and blocks proangiogenic signaling by VEGFA in endothelial cells

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Endorepellin, a processed fragment of the basement membrane proteoglycan perlecan, possesses anti-angiogenic activity by antagonizing endothelial cells. Endorepellin contains three laminin G-like (LG) domains and binds simultaneously to vascular endothelial growth factor receptor 2 (VEGFR2) and α2β1 integrin, resulting in dual receptor antagonism. Treatment of endothelial cells with endorepellin inhibited transcription of VEGFA, the natural ligand for VEGFR2, attenuating the pro-survival and migratory activities of VEGFR2 through blockade of this positive feedback loop. In this study, we investigated the specific binding site of endorepellin within the ectodomain of VEGFR2. Full-length endorepellin was not capable of displacing VEGFA binding from VEGFR2 and LG3 domains alone did not bind VEGFR2. This suggested different binding mechanisms of the extracellular immunoglobulin (Ig) domains of VEGFR2. Therefore, we hypothesized endorepellin would bind through its proximal LG1/2 domains to VEGFR2 in a different region than VEGFA. Indeed, we found that LG1/2 did not bind Ig1–3, but did bind with high affinity to Ig3–5, distal to the known VEGFA binding site, i.e. Ig2–3. These results support a role for endorepellin as an allosteric inhibitor of VEGFR2. Moreover, we found that LG1/2 blocked the rapid VEGFA activation of VEGFR2 at Tyr1175 in endothelial cells. We also present evidence for distinct signaling roles for LG1/2 vs LG3 including the inhibition of activation and cytoplasmic-nuclear transfer of NFAT1 by LG1/2 in endothelial cells. In contrast, LG1/2 did not result in actin cytoskeletal disassembly in endothelial cells whereas LG3 alone did induce cytoskeletal collapse. These studies provide a mechanistic understanding of how the different LG domains of endorepellin signal in endothelial cells while serving as a template for protein design of receptor tyrosine kinase antagonists.

(LB09) Right Ventricle Outflow Tract Reconstruction using Extracellular Matrix Scaffolds in GFP Chimera Rats

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The current prevalence and severity of congenital heart defects requiring functional replacement of cardiac tissue pose a serious clinical challenge. Biologic-based scaffolds are an attractive approach to cardiac repair because they avoid sensitization associated with homograft materials and theoretically have growth potential. Both urinary bladder matrix (UBM) and cardiac ECM (C-ECM) have been investigated as scaffolds for cardiac repair with modest success, but have not been compared directly. In other tissue locations, bone marrow-derived cells have been shown to participate in the remodeling process, but this has not been investigated for UBM in the cardiac location and has never been studied for C-ECM. The objectives of the present study are to compare the effectiveness of UBM and C-ECM for cardiac repair and determine the role of bone marrow-derived cells in the remodeling response. A chimera rat model was generated in which >95% of all bone marrow cells express GFP. Reconstructed hearts at 8 weeks post-implantation showed minimal ventricular pressure changes. After 16 weeks, reconstructed hearts showed thinning of the reconstructed RVOT when comaped with native cardiac wall, but no stenosis, dilation or fibrosis was observed. Immunofluorescent staining confirmed a large number of GFP expressing cells in both patches and along the interface with native tissue. A more complete penetration and uniform distribution of cells was observed in the UBM patches after 4 and 8 weeks. Small areas of cells in both scaffolds stained positive for α-actinin and Cardiac Troponin T, indicating the formation of immature muscle tissue in the remodeling area. To date, cells within UBM patches appear to express cardiac-specific markers more uniformly than cells within C-ECM patches. Co-localization studies for cardiac, endothelial and macrophage markers with GFP are underway to further elucidate the role that bone marrow-derived cells play in remodeling of these scaffolds.

(LB10) Looking beyond MGP in warfarin-induced vascular calcification

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Calcification of the vessel wall correlates with cardiovascular disease. It is an active and complex process which involves osteoblast-like transformation in vascular smooth muscle. Accumulating experimental evidence implicates a complicated regulatory network in calcification induced by various stimuli. In this study, the roles of γ-carboxylated MGP (matrix gla protein), of the nine major signaling pathways implicated by previous studies in osteoblast-like transformation in vascular smooth muscle (VSMC) and of the inflammatory cytokines were studied in the model of warfarin-induced calcification *in vitro*.A minimal role for Notch, ERK, C/ EBP, JNK, NFκB, and pRb signaling pathways, and for inflammatory cytokines including TNFa, is implicated by the lack of significant warfarin-induced changes in their activities and/ or expression. In contrast, warfarin activates β-catenin, BMP and PKA pathways. Inhibitory analysis reveals that activation of the PKA and TGF/ BMP pathways is not causal, while inhibition of β-catenin with shRNA or bioflavonoid quercetin prevents warfarin-induced calcification supporting a critical role in osteoblast-like transformation and calcification in VSMC. Unexpectedly, quercetin prevents calcification in VSMC underexpressing MGP suggesting that warfarin-induced osteoblast-like transformation in VSMC is at least partially mediated by β-catenin signaling independently of MGP. Finally, in rat model of warfarin-induced elastocalcinosis, quercetin completely prevents vascular calcification suggesting a novel therapeutic strategy for clinic.

(LB11) A Crystal Structure of Human β-1,4-Galactosyltransferase 7

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Proteoglycans (PGs) are a major component of the extracellular matrix, providing structural support to cells. Many membrane-embedded PGs are involved in signaling cascades that affect cell proliferation/differentiation and mediate adhesion of leukocytes during inflammatory responses. All proteins destined to be PGs must undergo the common tetrasaccharide (GluUAb1,3Galb1,3Galb1,4Xylb1-*O*-Ser) assembly, where β-1,4-galactosyltransferase 7 (β 4GalT7) catalyzes the second reaction step, the formation of a glycosidic bond between galactose and xylose via b1,4-linkage, in the presence of MnCl. Gene knockout studies have shown that β4Gal-T7 is essential for embryogenesis, and point mutations in β4GalT7 are associated with Ehlers-Danlos syndrome, a painful condition characterized by joint hypermobility, frequent dislocations and poor wound healing. We used, previously expressed in our laboratory, galectin-fused human β4GalT7 that includes the stem and the catalytic domain, encompassing residue 51 to 327, for crystallization trials. Since this recombinant protein could not be crystallized, we cloned and expressed galectin-fused human β4GalT7 with the N-terminal deletion of up to residue 80 (D81) in *Escherichia coli*. The purified β4GalT7D81, which is catalytically active protein, was crystallized. The crystal diffracted up to 2.7 Å resolution. The crystal structure of human β4GalT7 was solved by molecular replacement using *Drosophila* β4GalT7 crystal structure as a model structure. The structure has been refined and revealed unexpected features that were not observed in the *Drosophila* β4GalT7 structure. More details will be presented. Funded by NCI Contract HHSN261200800001E

(LB12) Fer tyrosine kinase regulates breast and prostate cancer cell motility through α-dystroglycan glycosylation

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Laminin-binding (LB) glycans of α-dystroglycan (α-DG), which is expressed at the epithelial cell-basement membrane (BM) interface, play an essential role in epithelium development and tissue organization. LB glycans on α-DG expressed on cancer cells suppress tumor progression by attenuating tumor cell migration signal from the BM. However, mechanisms controlling LB glycan expression are not known yet. Here, we performed siRNA library screening and identified Fer tyrosine kinase, a non-receptor type tyrosine kinase, as a key regulator of LB glycan expression on breast and prostate cancer cells (Yoneyama et al. 2012). Human Kinase siRNAs targeting 704 genes were prepared at the Functional Genomics Facility of Sanford-Burnham Medical Research Institute. MDA-MB231 breast cancer cells and DU145 prostate cancer cells were used for screening. Next, to investigate function of Fer tyrosine kinase on regulation of LB glycans expression, we performed RT-PCR, FACS, immunoblot analyses of LB glycan expression, and migration and invasion assay by using Fer kinase down-regulated or overexpressed breast and prostate cancer cell lines. Fer overexpression decreased LB glycan expression, while siRNA-mediated knockdown of Fer kinase increased glycan expression on both breast and prostate cancer cell lines. Fer expressed more aggressive prostate cancer cell line and human prostate cancer tissues. Loss of Fer kinase function via siRNA increased transcription levels of glycosyltransferases, including protein *O*-mannnosyltransferase (POMT1), β3-*N*-acetylglucosaminyltransferase 1 (β3GnT1), and likeacetylglucosaminyltransferase (LARGE), which are required to synthesize LB glycans. Consistently, inhibition of Fer expression in breast and prostate cancer cells increases LB glycan thereby decreases cell migration and invasion in the presence of laminin fragment. These results indicate that the Fer pathway negatively controls expression of genes required to synthesize LB glycans, thus impairing BM attachment and increasing tumor cell motility. The results also suggest that Fer kinase is an excellent target for down-regulation of both breast and prostate cancer progression. At present, we are going to screen potential Fer substratums that plays any role in regulating LB glycans synthesis (Lock et al. 1998). Supported in part by NCI PO1CA71932 grant.

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(LB13) Cell Surface Glycosylation and Glycosaminoglycan Composition Profiles inImmature and Mature Intervertebral Discs

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Back pain is a predominant cause of disability, and the major cause is intervertebral disc (IVD) degeneration. Proteoglycan (PG) content of the IVD is decreased during degeneration, and glycosaminoglycan (GAG) composition is altered. In this study, the cell surface glycosylation and GAG composition profiles of immature and mature ovine IVD tissue were profiled in an effort to understand potential signals involved in cell-extracellular matrix crosstalk during maturation and degeneration. IVD tissue from L3-L4 and L4-L5 lumbar segments were harvested from 3 and 11-month-old animals. One portion of the tissue was fixed for histochemical profiling with a panel of fluorescently-labelled lectins. The remaining portion was treated with chondroitinase and the released unsaturated disaccharide constituents of the GAGs present were analysed by HPLC.

The glycosylation expression differed between annulus fibrosus (AF) and nucleus pulposus (NP) tissue by lectin histochemistry and certain lectins could distinguish AF and NP tissue. With maturity, the expression profiles of cell surface glycosylation changed, notably an increase in α(2 → 6)-linked sialic acid and terminal GalNAc expression. HPLC analysis revealed that the quantity and ratio of sulfated GAG components differed from NP to AF tissue and that there was a change in sulfation pattern upon disc maturity. Sulfation is critical for maintaining water content and specific patterns of sulfation are influential in cellular signaling and differentiation events. A better understanding of sulfation and glycosylation profile changes would help to modulate the IVD environment and promote therapeutic regenerative approaches.

(LB14) Scleraxis is a general regulator of extracellular matrix gene expression incardiac myofibroblasts

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The transcription factor scleraxis is expressed in a wide variety of collagen- and extracellular matrix (ECM)-rich tissues including tendons and cardiac valves. We have previously demonstrated that scleraxis is expressed by cardiac fibroblasts and myofibroblasts and is sufficient to induce expression of the collagen Iα2 A dominant negative form of scleraxis blocks fibroblast collagen I production and attenuates pro-fibrotic responses to TGF-β1. We also showed that scleraxis expression is greatly increased in the collagen-rich scar that forms after myocardial infarction. We hypothesize that scleraxis is a general mediator of ECM formation in the heart and plays a central role in driving cardiac fibrosis. To test this hypothesis, we overexpressed scleraxis in primary rat cardiac myofibroblasts by adenoviral gene delivery and examined the expression of several ECM components and regulators. Scleraxis overexpression resulted in decreases in the expression of several ECM proteoglycans including versican, decorin and syndecan-2,

all of which were inhibited by 60–80%, but did not affect expression of syndecan-1. Scleraxis also induced the up-regulation of thrombospondin-4 but not thrombospondin-2. Matrix metalloproteinases MMP9 and MMP11 were down-regulated by more than 50%, and the tissue inhibitor of matrix metalloproteinases TIMP3 was down-regulated by 35%, but there was no effect on the fibrillar collagen-processing enzyme lysyl oxidase. The loss of decorin, thrombospondin-4 and TIMP3 have all been strongly associated with increased fibrosis in a variety of tissues, thus these results agree with our hypothesis that scleraxis is a key player in the development of fibrosis in the heart. The lack of the effect of scleraxis on lysyl oxidase and thrombospondin-4 indicates that scleraxis is not indiscriminate in target gene regulation. The effects of scleraxis on versican, syndecan-2 and MMPs 9 and 11 further suggest that scleraxis may not only promote fibrosis, but may contribute to overall ECM remodeling as well. Collectively, these data support the hypothesis that scleraxis mediates cardiac ECM remodeling and fibrosis.

(LB15) Glycosaminoglycans as specific regulators of signaling pathways driving stem cell differentiation

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Glycosaminoglycans (GAGs) are essential cofactors for many signaling molecules regulating stem cell (SC) expansion and differentiation and remain an under-appreciated factor in the design of SC expansion strategies or methods to direct differentiation. Several issues need to be addressed before human SCs can fulfill their potential for therapy and understanding of disease. Foremost among these is the development of defined culture systems for the high-efficiency differentiation of SCs to the target cell type. These systems must be scalable, fully-defined and xeno-free. Our work, and that of others, has improved the understanding of specific GAG epitopes in mouse ES cells, demonstrating that selected GAG saccharides can influence-specific signaling pathways during neural and mesodermal differentiation. Importantly, the sulfation pattern and size of saccharides required for neural differentiation were distinct from those driving the formation of alternative cell fates (e.g. hematopoiesis). This suggests that GAG saccharides could be used in addition to, or possibly in place of, protein additives in differentiation protocols for the generation of therapeutically relevant cell types from SCs. Recent improvements in the chemoenzymatic production of xeno-free structurally-defined GAG oligosaccharides allow the use of these compounds in the determination of specific sulfation pattern requirements for the control of cell signaling. We are combining this work with studies into the design of artificial cell environments where we have optimized 3D scaffolds, generated by electrospinning or by the formation of hydrogels, for the culture of stem cells. By permeating these scaffolds with defined GAG oligosaccharides, we can control the mechanical environment of the cells (via the scaffold architecture) as well as their biological signaling environment (using the oligosaccharides). Early results demonstrate that this allows us to control SC expansion and differentiation in a 3D setting, allowing the generation of differentiated cell types for use in drug discovery/testing or in therapeutics.

(LB16) Molecular mechanisms controlling trafficking of adipose stromal cells

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Mesenchymal stromal cells (MSC) have emerged as an attractive candidate for cell-based tissue repair due to their trophic, vascularization and immunosuppression functions. White adipose tissue (WAT) contains large numbers of MSC, known as adipose stromal cells (ASCs), that serve as adipocyte progenitors and pericytes/adventitial cells. Our laboratory has uncovered the phenomenon of ASC mobilization in obesity and cancer. We also demonstrated the recruitment of ASC by tumors and the positive effect of ASC engraftment on tumor growth. The mechanisms that govern ASC trafficking in disease remain unclear. Our recently reported data suggest that CXCL1 secreted by human tumor cells and signaling via receptors CXCR1 or CXCR2 could be implicated in migration of human ASC. In our validation studies, multiplex analysis of mouse plasma identified CXCL1 as a chemokine specifically elevated in circulation of obese animals bearing tumors. We show that CXCL1 is up-regulated by malignant mouse cells in obesity and can induce migration of mouse ASC in ex vivo assays. Importantly, CXCR1 (but not CXCR2) is strongly expressed by ASC in WAT of tumor-bearing animals. These results indicate that CXCL1 secreted by tumors in obesity may regulate ASC migration through CXCR1. As a candidate pathway upstream of CXCL1, we identified IL-22R expressed by tumor cells and IL-22 secreted in WAT of obese tumor-bearing animals. In another project, we analyzed the secreted protein acidic and rich in cysteine (SPARC), which had surfaced as a matricellular factor inducing ASC migration. We provide evidence that SPARC activity is mediated by α5/β1 integrin signaling through focal adhesion kinase (FAK) and paxillin. In summary, our studies establish candidate mechanisms that govern ASC trafficking.

(LB17) Renal Progenitors On Extracellular Matrix As A Potential Tool for Kidney Regeneration

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Congenital abnormalities of the kidney and urinary tract (CAKUT) still remains a significant contributing factor for chronic kidney disease and end organ failure. Current treatments are directed towards slowing progression before dialysis and/or transplantation become necessary. The scarcity of allografts, cost and side effects have prompted efforts at investigating new tools for kidney regeneration. Tissue engineering that combines a natural or biodegradable scaffold with cells and growth factors could be a new approach to discover an alternative tool for replacing significantly impaired or non-functional kidney tissue. Our laboratory has previously reported that human amniotic fluid (AF) presents a new

source of renal progenitor cells. A subpopulation of renal progenitor cells expressing CD24 and OB cadherin were isolated from AF. Using a detergent-enzymatic method, we produced mouse decellularized the whole kidney extracellular matrix (ECM). Mouse kidney ECM was seeded with renal progenitor cells, implanted into the kidney of nude mice and harvested at different time points to look for structure and phenotypic expression. After surgery, implanted ECM in situ showed invasion of vessels (angiogenesis) and renal tubular-like structures. In addition to seeded scaffolds configuring into 3D renal structures, cells positive for markers associated with several essential renal cell types, such as mesangial, podocytes and tubular cells were detected. These results suggest that renal progenitor cells from AF seeded into renal ECM could represent a unique investigational approach for kidney regeneration that may be used to augment or replace damaged or compromised kidney tissue from either congenital or chronic kidney disease.

(LB18) A novel source of cultured podocytes for in vitro studies

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Background: The podocyte is the pivotal cell maintaining normal structure and function of the glomerulus. Podocytes present a very limited ability to replicate and loss of podocytes is associated with progression of kidney disease. Various aspects of podocyte biology have been studied using in vitro systems. The current state-of-the-art cultured podocyte is conditionally immortalized, which replicates only some features of this highly specialized cell. Herein, we describe a novel cell population of human amniotic fluid kidney progenitor cells (AKPC) that can be cultured and differentiated toward mature and functional podocytes without immortalization. AKPC were differentiated into podocytes and comaped with human immortalized podocyte cells. Differentiation efficiency was assessed by FACS analysis. Morphology was evaluated by light and electronic microscopy. Microarray, quantitative PCR and western blotting analysis were performed to confirm expression of mature podocyte and GBM markers. Contractile ability was assessed by Angiotensin II stimulation and FFA calcium intake assay. AKPC were seeded onto the mouse kidney extracellular matrix (ECM) and their structure was evaluated by SEM. AKPC can be fully differentiated into mature podocytes, acquiring an arborized morphology, formation of foot processes and rearrangement of F-actin fibers. Expression of podocyte markers including WT1, nephrin, synaptopodin was confirmed. AKPC showed the ability to vigorously contract upon stimulation. AKPC showed superior traits when comaped with a human immortalized podocyte, in particular in the higher expression of podocyte genes confirmed by microarray, in the secretion of collagen IV α 3,4,5, the major constituent of the GBM and the ability to attach, replicate, differentiate and develop mature foot processes when seeded onto decellularized ECM. In conclusion, for the first time we showed in a comprehensive and broad manner that an extra-renal cell population can be differentiated into fully mature and functional podocytes in vitro and established a culture system that will allow a better understanding of podocyte biology than conventional immortalized cell lines.

(LB19) Cartilage-specific Deletion of the Site-1 Protease in Mice Results in the IntracellularEntrapment of both Type IIB Procollagen and Adamts3

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Introduction: Site-1 protease is an enzyme required to activate latent endoplasmic reticulum (ER) membrane-bound transcription factors such as the sterol regulatory element binding protein (SREBP) involved in lipid homeostasis or ATF6 involved in ER stress response. Cartilage-specific ablation of Site-1 protease in mice results in chondrodysplasia with a complete lack of endochondral bone development and lack of proper lipid synthesis. Site-1 protease ablation immediately disrupts type IIB collagen (Col IIB) processing and secretion causing a buildup of Col IIB in the ER and a deficiency of Col IIB in the ECM. The floxed Site-1 protease gene was inactivated in mice using Col2-Cre or the inducible Col2-CreER(T) technology. Genome wide expression analysis was used to examine differences between knockout and wild-type chondrocytes. Tissue was examined by immunohistochemistry using antibodies to Col II (C-propeptide, N-propeptide and triple helical domain) and Adamts3, the enzyme required to cleave the N-propeptide from the P_N-procollagen IIB. Comparison of knockout and wild-type mice demonstrated that the down regulation of lipid enzymes was not the cause of the cartilage defect; nor is the ER stress response affected in the chondrocytes. However, Col IIB entrapment in cells is synchronous with intracellular retention of Adamts3. Examination of Adamts3 revealed a cleavage site for Site-1 protease. Surprisingly, in wild-type tissue, Adamts3 is primarily located in the ECM whereas the Col IIB N-propeptide is intracellular and thus studies are underway to examine the role of N-propeptide cleavage in the procollagen and to examine the hypothesis that lack of processing of Adamts3 by Site-1 protease causes an inhibition of Col IIB maturation and secretion. These studies suggest that Site-1 protease has additional substratums specific to cartilage that involve collagen synthesis and secretion and are not related to the previously discovered role in lipid metabolism.

(LB20) Lectin-Like Ubiquitin Ligases are the Final Arbitrators in Glycoprotein Quality Control

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Alpha-1 Antitrypsin (A1AT) is a glycoprotein made and secreted by the liver to neutralize serum proteases. A1AT deficiency is the most common cause of childhood liver failure, in adults it is responsible for two percent of all cases of emphysema and is one of the three most common lethal genetic diseases in Caucasians. The term A1AT deficiency is a misleading term; in most cases mutant glycoprotein A1AT-Z accumulates

within the endoplasmic reticulum in the liver of A1AT deficiency patients causing liver scarring, inflammation and carcinoma. The accumulation also results in reduced secretion into the blood and that causes the pulmonary sequelae. Endoplasmic reticulum-associated degradation (ERAD) is known to be responsible for clearing most mutant A1AT-Z, and we wanted to know if there were additional steps or factors involved. We examined the degradation of A1AT-Z in liver cell lines and identified the novel lectin-like E3 ubiquitin ligases FBG1 and FBG2 that participate in the clearance of A1AT-Z. Furthermore, we show that soluble A1ATZ is degraded by the ubiquitin proteasome system and by autophagy while the insoluble form of A1AT-Z is predominately degraded by autophagy. FBG1, FBG2 and A1AT-Z are all found in the liver, in the same cellular compartment, and they directly interact as shown by immunoprecipitation. Overexpression of FBG1 and FBG2 decreases the half-life of A1AT-Z from 13 hours to 4 hours and 10 hours respectively. Based on our data, we believe that FBG1 and 2 may function in a novel step in ERAD to re-ubiquitinate glycoproteins that have been de-ubiquitinated prior to their degradation.

(LB21) A Novel Mode Of Action To Stimulate Collagen Production In Aged Skin Cells

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Collagen is the major component of the skin's dermis; it accounts for 80% of the skin's dry weight. Collagen provides both tensile strength and elasticity to the skin: properties that are critical for supporting the skin architecture. However, there is a significant decrease in collagen levels in aged skin, resulting in loss of structural support of the skin and appearance of wrinkles. Collagen in the skin is produced by fibroblasts and its synthesis and maturation is a complex multistep process. Collagen 1 is a helical trimeric protein made up of three α 1 chains of collagen 1. The monomeric strands are synthesized and then assembled into the triple helix, while undergoing post-translational modifications such as hydroxylation on various amino acid residues. PLOD-2 (procollagen-lysine, 2-oxoglutarate 5dioxygenase 2 [lysine hydroxylase-2]) is a critical enzyme in the collagen maturation process; it catalyzes the hydroxylation of lysine residues on the nascent procollagen strands. The resultant hydroxylysyl groups aid in the formation of the triple helix and serve as attachment sites for cross-linking in the extracellular matrix. Thus, this modification is critical for the stability of procollagen and the intermolecular cross-linking of collagen fibrils. We have demonstrated that there is a reduction in PLOD-2 enzyme expression in older human dermal fibroblasts and we have established that a reduction in PLOD-2 leads to a decrease in collagen production. We have demonstrated that a novel cosmetically acceptable active, a tyrosine derivative, N-acetyl tyrosinamide, A-F33, that stimulates PLOD-2 expression in older skin cells and increases collagen production. Thus, inclusion of A-F33 in cosmetic products could provide improvements in the appearance of aging skin.

(LB22) Modulation of miRs: A Novel Approach to Targeting Skin Aging

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Skin aging is accompanied by significant changes in the expression of proteins, particularly those that constitute the extracellular matrix of the dermis, and such changes can contribute to the appearance of aging signs such as lines, wrinkles and loss of firmness. Intrinsic aging and extrinsic factors such as UV can lead to aberrant expression of proteins such as collagen and elastin in dermal fibroblasts. microRNAs (miRs) are a class of short non-coding RNA molecules that are post-transcriptional regulators of gene expression. miRs bind complementarily to protein coding mRNAs and typically result in gene silencing. A single miR can regulate more than one protein-coding mRNA and reciprocally, a single mRNA can be regulated by several miRs. To date, over 1000 miRs have been identified and collectively, they can potentially target/regulate more than 60% of human genes. It is very likely that the expression of skin matrix proteins such as collagen and elastin, may be regulated by activity of miRs. However, identification of specific miRs that regulate extracellular matrix proteins in skin cells is a largely uncharted research area. We have identified specific members of the miR-29 family, miR-29a and miR-29b, as negative regulators of key extracellular matrix proteins, collagen, elastin and fibrillin in skin cells. We have demonstrated that miR-29a and miR-29b increase with age, which can contribute to a decline in collagen, elastin and fibrillin expression. Down regulation of these microRNAs can allow for increased expression of their target skin matrix proteins in aging skin cells. Hence, topical compositions containing compounds that can down-regulate miR-29 maybe be used to deliver anti-aging benefits to skin. In sum, this work describes a novel approach to increasing key extracellular matrix proteins, which improve characteristics of aging skin such as wrinkles and firmness.

(LB23) Overexpression of ST3Gal-IV induces activation of cell signaling pathways and alteration in gastric cancer cell line phenotype

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The overexpression of Sialyl-Lewis antigens is a common feature in cancer. The presence of these glycan structures has been implicated in biological features of cancer cells and is potential cancer biomarkers (Reis et al. 2010). We have previously demonstrated, in stable transfected gastric cell lines, that ST3Gal-III and ST3Gal-IV are involved in the production of SLe^a and SLe^x antigens in gastric carcinoma cells (Carvalho et al. 2010). In the present study, we assessed the biological behavior of these cells regarding cell proliferation, aggregation and invasion in classical in vitro assays and in the in vivo chicken chorioallantoic membrane (CAM) model. The in vitro characterization of the cell lines showed that cells expressing ST3Gal-IV are more invasive

although no differences in cell proliferation were observed. In addition, the ST3Gal-IV expressing cells showed a significant increase in cell invasion in the in vivo CAM model, while no differences were observed in angiogenesis and tumor growth. Constitutive activation of receptor tyrosine kinases was observed. We further identified proteins carriers of SLe^a and SLe^x by studying the cell proteome and secretome. We observed differences in Sialyl-Lewis expression in the transfected cells, and proteins carrying the Sialyl-Lewis were identified. The cell secretome showed, by western blot, that the transfected cells are secreting proteins with $SLe^a$and SLe^x antigens and the proteins identified are being further evaluated as possible new biomarkers. Our results demonstrate that these sialylated glycans contribute for the invasive phenotype of gastric cancer cells, playing a role in the process of cancer cell invasion.

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(LB24) Structure and Expression of Glycosaminoglycans in Cancerous Tissue

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Glycosaminoglycans (GAGs) are linear, highly negatively charged polysaccharide chains that serve a wide variety of functions in the body, including those regulating cell growth, development, vascularization and cell adhesion . all aspects central to the growth and metastasis of cancer. Here, GAG chains were isolated and analyzed from cancerous and normal tissues taken from patients with different forms of cancer. The properties of the isolated GAGs were compared across the different tissue types. Cancerous tissue was found to produce more GAG content, longer GAG chains and changes in the sulfation pattern. Changes were also found to exist between the tissues from patients with lethal cancer and those with cancer that was successfully treated. Lethal, metastatic cancer was found to have lower levels of sulfation and higher levels of unsulfated disaccharides. While based off of a limited sample size, these data suggest that there may be innate variation in the body's construction and composition of GAG chains which facilitate the metastasis of tumors in some patients.

(LB25) Amino acids determining regiospecificity of the *E. coli* K1 and K92 capsule polysialyltransferases

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The enteropathogenic *E. coli* K92 synthesizes a unique capsule consisting of polysialic acid with alternating α2,8- and α2,9-linkages. The demonstration that a single enzyme, the polysialyltransferase (polyST), is responsible for synthesis of these alternating regioisomeric linkages (Shen *et al.*, 1999), raises the question of whether the enzyme catalyzes these reactions with one or two active sites (May *et al.*, 2012; Steenbergen & Vimr, 2003). The availability of a close homolog, the K1-polyST, which catalyzes only the α2,8-linkage, has facilitated comparative studies investigating the basis of this dual regiospecificity (Steenbergen & Vimr, 2003). In our approach, we have utilized a high-throughput polyST screening methodology (Keys *et al.*, 2012) to explore the relevant sequence space between the *E. coli* K1- and *E. coli* K92-polySTs. The linkage specificity of the selected mutants was subsequently confirmed using a polySia permethylation linkage analysis technique (Galuska *et al.*, in press). With these newly established methods, we have identified key residues involved in determining regiospecificity in *E. coli* K1- and *E. coli* K92-polySTs.

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(LB26) Large Scale Glycan Array Analysis of Commercial Lectins and Antibodies: 86 lectins and 15 antibodies

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Th[e] majority of glycan analysis in the clinic and laboratory relies upon glycan binding proteins. A detailed understanding of the specificities of these reagents is critical to the fields of glycobiology and glycomics. Recent work by the Consortium for Functional Glycomics (CFG) and others have started to provide such profiles, but our current data set is inadequate and incomplete. The CFG has the most comprehensive glycan array available consisting of 612 glycan epitopes ranging from *O*-linked and *N*-linked glycans to glycolipid structures. Herein, we report the results of a comprehensive glycan array analysis of the commercially available lectins and a handful of antibodies. The effect of lectin source on glycan binding profile is examined and lectins are classified into major binding groups. This work provides the community with the most comprehensive binding profiles of these well known and used proteins.

(LB27) Cross-platform comparison of carbohydrate microarrays: Six groups and five lectins

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To date, the vast majority of data gathered on glycans is done via analysis with either anti-glycan antibodies or lectins.These reagents have been used to elucidate the role of glycans in biological systems from stem cell differentiation to tumormetastasis. A detailed understanding of the specificities of these reagents is critical to the fields of glycobiology and glycomics.Glycan microarrays are increasingly used to determine the binding specificity of carbohydrate binding proteins (CBPs). Glycanmicroarray platforms run the gamut from large diverse arrays of linker bound glycans (Consortium for Functional Glycomics, Huflejt) or neoglycoproteins (GIldersleeve) to smaller more specialized arrays such as glycodendrimer arrays (Pieters), *N*-linked glycan arrays (Reichardt) and more focused neoglycoprotein presentations (Joshi). It has been known for some time that the manner in whicha glycan is presented to a CBP can alter the apparent binding affinity and specificity of the interaction. The interpretation of datagathered from these reagents relies on our knowledge of their specificities, thus it is critical that we systematically profile them inmultiple formats for direct comparison. Herein, we report the results of a multiplatform comparison between arrays from six differentgroups using five well known lectins; Con A, WGA, SNA, MAL-I and HPA. Issues of data analysis, including thresholding and rankingare discussed. This work has important implications for the implementation of microarrays in the characterization of carbohydratebinding proteins.

(LB28) Tuning docking algorithms for carbohydrates: VIVA-CARB and MD simulations

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We report the creation and screening of a virtual library of 3D structures for the known human glycome (Glibrary-3D), comprising approximately 7000 N and O-linked glycans, populating approximately 500,000 stable conformations.

In addition, we introduce the technique of Computational Carbohydrate Grafting as an improvement over automated docking for the prediction of the 3D structures of protein . glycan complexes.

Lastly, we demonstrate that screening the virtual glycan library with Computational Carbohydrate Grafting leads to correct predictions of substrate specificities for carbohydrate . binding proteins. This is illustrated for carbohydrate-binding proteins and enzymes.

(LB29) Modulating the effector function of rMAbs by host cell glycoengineering

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Glycosylation has attracted major interest from biopharmaceutical industries as a means to control the safety and efficacy of biopharmaceuticals. The N-glycan attached to the conserved glycosylation site at Asn 297 in the Fc region of recombinant monoclonal IgG's (rMAbs) for example, plays a multifunctional role in the physiochemical and biological properties of the IgG molecule (1). These N-glycans sequestered within the interstitial space enclosed by the CH2 domains maintain the thermodynamic stability and quaternary structure of the two heavy chains for the correct binding of the IgG molecule to the Fcγ receptors as part of the antibody cell-mediated cytotoxicity (ADCC) and C1q of complement. The absence or addition of specific sugar moieties to the N-glycan has been shown to modulate the immunoeffector function of the molecule. The absence of core fucose on the IgG N-glycan can greatly increase antibody dependent cellular cytoxicity through an enhanced IgG -FcγIIIa receptor interaction while the addition of α2,6 linked sialic acid enhances the anti-inflammatory activity of the molecule (2,3).

The selection of the mammalian Chinese Hamster Ovarian (CHO) cell line as the primary producer of rMAbs is largely influenced by the host cells ability to generate therapeutically acceptable glycoprofiles. Building upon the recent availability of the CHO genome, we have taken a glycomics based approach to implement a number of cell based glycoengineering strategies to alter the composition of the IgG N-glycan structure and improve various critical characteristics of selected rMAbs including half-life and therapeutic efficacy.

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(LB30) Mesangial cell matrix deposition in high glucose as a model of glomerulosclerosis

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Diabetic nephropathy is among the most devastating long-term consequences of diabetes mellitus and is now the leading cause of end-stage renal disease (ESRD) in the United States. The effects of the disease are predominantly focused in the glomerulus and are characterized by increased levels of extracellular matrix (ECM) proteins that disrupt the function of the glomerular filtration apparatus. Mesangial cells, which support the glomerular capillary network, are the primary matrix-producing cells of the glomerulus. Fibronectin (FN), a major ECM constituent, is found in the normal glomerulus and at significantly elevated levels in diabetic

nephropathy. High-glucose conditions have been shown to increase mesangial cell expression of various ECM proteins, but the effects of high glucose on mesangial cell-mediated FN matrix assembly are as yet undetermined. To analyze matrix assembly, we are using a temperature-inducible cell line that differentiates into mesangial cells when cultured at 37 degrees. Levels of secreted FN are increased during differentiation comaped with the immortalized parental cell line grown at 33 degrees. Preliminary assessment of deoxycholate detergent-insoluble FN matrix deposition by these cells indicates that assembly increases with cell differentiation. This increase in matrix assembly was also observed by indirect immunofluorescence staining for FN fibrils. Experiments are underway to determine whether high glucose affects FN expression or matrix assembly. Non-enzymatic glycation of matrix proteins represents another mode of glucose-mediated ECM disruption. Glycated FN is found at elevated levels in diabetes and glycation of FN can affect its binding activities. Analysis of a protein fragment containing the cell-binding domain (III) of FN that had been exposed to a high-glucose solution suggested an increase in carboxymethyllysine content. The binding properties of glycated fragments will be assessed. We propose that defects in mesangial cell matrix assembly represent a final common pathway in glomerulosclerosis. Further insight into the mechanisms of increased matrix assembly in diabetic nephropathy may pave the way for the development of a new generation of therapeutics, which target this process in hopes of reversing the progression towards ESRD.

(LB31) Nitrogen mustard exposure of the cornea induces erk activation of adam17 within minutes

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Nitrogen mustard (NM) exposure induces microblisters in the cornea, separating the epithelial and stromal layers. This is, in part, due to ADAM17 cleavage of the hemidesmosomal component, collagen XVII. We hypothesized that NM exposure activates ERK, which serves to activate ADAM17 via phosphorylation. To test this, pull down assays were used to test whether ERK and ADAM17 had a direct interaction, and whether the MEK/ ERK inhibitor PD98059 was able to reduce the interaction. 100 nmoles of NM were applied to air lifted organ cultures of rabbit corneas. For some corneas, NM was immediately washed off with either medium or medium plus inhibitor (i.e. 0 min exposure). For others, NM remained on the cornea for 5 or 10 min, then was washed off with medium or medium plus PD98059. Incubation after exposure was in medium (either + or -inhibitor) for 10 min for all cultures. Immunofluorescence analysis showed that unexposed corneas had little activated ERK (i.e., pERK) and no activated (i.e., phosphorylated) ADAM17. In contrast, all NM-exposed corneas showed signal along the basement membrane zone for both pERK and phosphorylated ADAM17. The NM-exposed corneas washed with medium containing PD98059 showed a decrease in pERK expression at all timepoints, but phosphorylated ADAM17 was only able to be attenuated if NM exposures were for 0 or 5 min. A 10 min NM exposure activated ADAM17 whether or not inhibitor was added. Westerns of immunoprecipitated ERK were probed with ERK, pERK and ADAM17 antibodies. These showed both pERK and phosphorylated ADAM17 were abundant after NM exposure. Addition of PD98059 after NM exposure reduced the quantity of ADAM17 found in the pull down assay for early timepoints–The ERK inhibitor PD98059 was only able to decrease the activation of ADAM17 when it was applied to corneas within about 5 min of exposure to NM.

(LB32) Heparan Sulfate 3-O-sulfation: A Rare Modification in Search of a Function

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Heparan sulfate binds many growth factors, chemokines, morphogens, extracellular matrix proteins, enzymes and enzyme inhibitors via relatively short sulfated saccharide sequences. One relatively rare modification to the chains, the addition of sulfate to carbon-3 of glucosamine residues, is poorly understood in terms of its biosynthesis and function. A family of seven glucosaminyl 3-O-sulfotransferases (Hs3st) exists in vertebrates. Although the enzymes have been cloned and partially characterized, little is known about the context in which these modifications occur and even less is known about the endogenous ligands that bind to heparan sulfate chains that contain 3-O-sulfate groups. Heparan sulfate devoid of 3-O-sulfation was collected from the conditioned medium of CHO cells and was modified in vitro using recombinant Hs3st1 or Hs3st2. Conditions were optimized and a low degree of modification was achieved (ca. 1 3-O-sulfate group/20 disaccharides). Native and 3-O-sulfated heparan sulfate was immobilized and heparin-binding proteins were isolated from serum by chromatography. Gel electrophoresis and silver staining of the eluates revealed a complex mixture of proteins with high similarity of protein content between the three matrices. Proteomic analysis of the eluates revealed an abundance of heparin-binding proteins, a number of which were eluted specifically from an Hs3st-modified matrix. Current efforts are underway to fractionate protein from other fluid and tissue sources and validate these candidate proteins.

(LB33) Comparison of Docking Software to Predict Saccharide Binding to Proteins Combination with In silico Protein Engineering

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We have more and more information now showing that protein-carbohydrate interactions are involved in many biological processes including cell signaling, immune response and /or cell–cell adhesion. In order to understand and to be able to influence such processes, it is important not only to analyze binding components, but also to be able to predict geometry of protein-carbohydrate complexes and their binding energy. Computational methods may help substantially within this respect. They are mainly based on the description of two kind of interactions. These are polar interactions among the hydroxyl groups of a carbohydrate and polar functional groups of a protein and/or stacking interactions between aromatic amino acids and the carbohydrate's cyclic moieties, similar to base-base stacking interactions observed in nucleic acids. The situation is often complicated by the fact that the polar interactions may often be mediated or enlarged by bridging water molecules or by ions. We will show in this paper, how different docking software may behave differently when predicting binding mode and binding affinity in protein-carbohydrate complexes. As model proteins, lectins from pathogens are chosen. The CV-IIL lectin from *Chromobacterium violaceum*, the RSL lectin from *Ralstonia solanacearum*, and their mutants will be used. Our testing includes AutoDock, ICM Dock, DOCK and X-Score docking software. This work was funded by the European Community's 7th Framework Programme under the European Regional Development Fund (CZ.1.05/1.1.00/02.0068) and Capacities specific programme (286154) and the Czech Science Foundation (GD301/09/H004). The authors would like to thank the Czech National Supercomputing Centre for providing computational resources.

(LB34) Novel pH-dependent lectin from bioluminescent bacterium Photorhabdus luminescens

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Lectins are capable of specific and reversible binding to carbohydrate moieties and are able to agglutinate cells. Carbohydrate-mediated recognition plays an important role in the ability of pathogenic bacteria to adhere to the surface of the host cell in the first step of their invasion and infectivity. *Photorhabdus luminescens* is a Gram-negative bacterium that is mutualistically associated with entomopathogenic nematodes from the genera Heterorhabditis. These nematodes infect the insect in larvae stage and release the bacteria into insect blood. Afterwards, killed insect serves as a food source suitable for nematode growth and development. The protein PLU from P. luminescens has a strong sequence homology with the recently characterized lectin BC2L-C-nt from the human opportunistic pathogen *Burkholderia cenocepacia*. So far, PLU is the only one supposed homologous of BC2L-C-nt lectin. BC2L-C-nt has unique β sandwich trimeric structure and is the first lectin with TNF-fold [1]. It was also shown, that BC2L-C-nt is bound to the bacterial cell surface and elicits a pro-inflammatory response in cultured respiratory epithelial cell cultures. The gene coding the protein PLU was cloned and both the protein expression conditions and optimal purification were successfully found. For detecting lectin activity was used surface plasmon resonance and hemagglutination. Preliminary information about a quaternary structure of the protein was obtained using analytical ultracentrifugation. The results show interesting pH-dependent binding properties of the protein, which is not always true in the lectin world. The research was further focused on the interaction of insect hemocytes with the lectin. Fluorescence microscopy shows that PLU binds to hemocytes leading to agglutination of the insect cells. The work was supported by Czech Science Foundation (GA303/09/1168, GD301/09/H004) and CEITEC -Central European Institute of Technology with research infrastructure supported by the project Z.1.05/1.1.00/02.0068 from European Regional Development Fund.

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(LB35) Analysis of Toxoplasma gondii genome for Glycosyltransferases

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Toxoplasma.gondii, which can infect many warm-blooded animals including human, is a parasitic protozoa that can cause life threatening disease in immune compromised individual. The lifelong persistence of this parasite is dependent on its ability to establish encysted states within the host. The cyst is characterized by the presence of a heavily glycosylated cyst wall detectable by their reactivity with a broad spectrum of lectins including SNA and JAC that indicate the presence of sialic acid. Despite the central role in the pathogenesis of T. gondii, almost nothing is known about the composition and functions of these glycans associated with the cyst and cyst wall. At this time, little is known about the parasite glycome or the encoded capacity for glycosylation. By conducting a systematic survey of potential glycosyltransferase(s) in silico, it will be possible to establish the glycan profile in the Toxoplasma genome. Sequencing of this parasite genome of about 63 Mb in size with 14 chromosomes has recently been completed. The availability of this sequence information from multiple isolates in a specifically designed database, toxodb, which also include transcriptomic and proteomic data sets, has made this parasite an ideal candidate for in-depth bioinformatics interrogation. In addition, the availability of sequenced genomes of several related parasites housed at www.eupathdb.org provides an ideal resource for expanding these studies to other parasites including Plasmodium species, the agent of malaria. In silico identification of potentially unique enzymatic activities validated by experimental results could open doors toward the discovery of novel drugs to treat these often deadly infections.

(LB36) An Integrated Strategy for N-Glycan Sample Preparation and Analysis Suitable forAll Stages of Therapeutic Protein Discovery, Characterization, Manufacture andQuality Release

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The GlykoPrep. N-Glycan Sample Preparation (GlykoPrep) Modules have been expanded to allow the conversion of most methods and fluorescent dye combinations for a number of analytical platforms for fast, automatable sample preparation. Waters has expanded its analytical capabilities for increased sensitivity, speed and resolution of glycans: the Waters UPLC®-HILIC/ FLR/ Q-Tof. Mass Spectrophotometer (MS) system enables high-resolution glycan separation using the ACQUITY UPLC® BEH Glycan 1.7 ìm column (Glycan Separation Technology, GST Column), and produces sensitive FLR and MS chromatographic signals in a single analysis without splitting the flow. The combination of improved options for sample preparation with selected instruments and consumables reduces time to results for new screening applications such as discovery, clone selection and cell culture optimization that have hardly been contemplated. In addition, such applications as bioreactor monitoring and control (PAT assays) are now within reach, as a small number of samples can return results within a single shift. Standard procedures have benefitted as well, due to highly robust and reproducible UPLC technology, and the improved sensitivity and resolution of the Waters SYNAPT® G2-S MS, which has the capability of detecting glycans, even minor ones, with high mass accuracy and sensitivity.

In this poster we outline an integrated strategy for N-Glycan sample preparation and analysis that yields reliable results with different fluorescent labels and methods, which can be related across diverse applications, from screening to in-depth characterization to quality release and stability testing, taking into account the associated requirements for throughput and time to results vs. data quality. Specifically, we compare results of N-glycan analysis of human IgG (hIgG) and Enbrel® (an Fc-fusion protein) obtained when using various GlykoPrep modules coupled with Waters analytical instruments and protocols.

(LB37) N-Glycan Profiling Using Orthogonal Methods: UPLC® and CE

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The demand for screening applications, with their requirements for high-throughput and short time to results, has significantly reduced the time allotted for analysis. We have explored the trade-off between faster analysis time and the ability to resolve (or estimate) individual species of interest. Two general approaches in the analysis of N-Glycans involve ultra-performance liquid chromatography (UPLC)-and capillary electrophoresis (CE)-based separation of fluorescently labeled N-Glycans. We present an overview of these methods and discuss the advantages, disadvantages and complementarity of each for high-throughput applications. Furthermore, we highlight recent advances in these technologies that increase throughput for such biotherapeutic applications as screening, cell-culture optimization and process scale-up.

(LB38) Glycoprofiling of plant-derived glycoproteins

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Genetically modified plants for production of "humanized" glycotherapeutics present an attractive expression system due to their low cost and ease of production. However, plants still show signs of inconsistency between plant and mammalian N-linked glycosylation by incorporating high mannose, truncated complex, paucimannosidic-type glycans as well as structures containing non-human residues. Notably, Lewisa (Lea) type antigens and known immunogenic epitopes such as ß (1,2)-xylose and a (1,3)-core fucose are present on plant glycoproteins. Protein glycosylation plays a significant role in plant bioproduction as subtle changes to glycan structure can greatly impact safety, efficacy and biological activity of biotherapeutics. The links between the complex nature of the glycans and their associated functions have raised serious concerns within the drug regulatory authorities and require more explicit guidelines on carbohydrate analysis. Glycans are complex, heterogeneous and are assembled in a non template driven biosynthetic pathway which makes glycan characterization a difficult task. As there is no universal method capable of characterising the complete intact glycoprotein structure, it is essential to apply several orthogonal methods to measure individual parameters such as glycosylation site analysis, oligosaccharide sequence and monosaccharide content of a therapeutic glycoprotein [1]. Here we present two HPLC (HILIC/ Reversed Phase) based high-throughput glycoanalytical approaches that can characterize various glycoforms and their oligosaccharide sequence on plant based bio therapeutics.