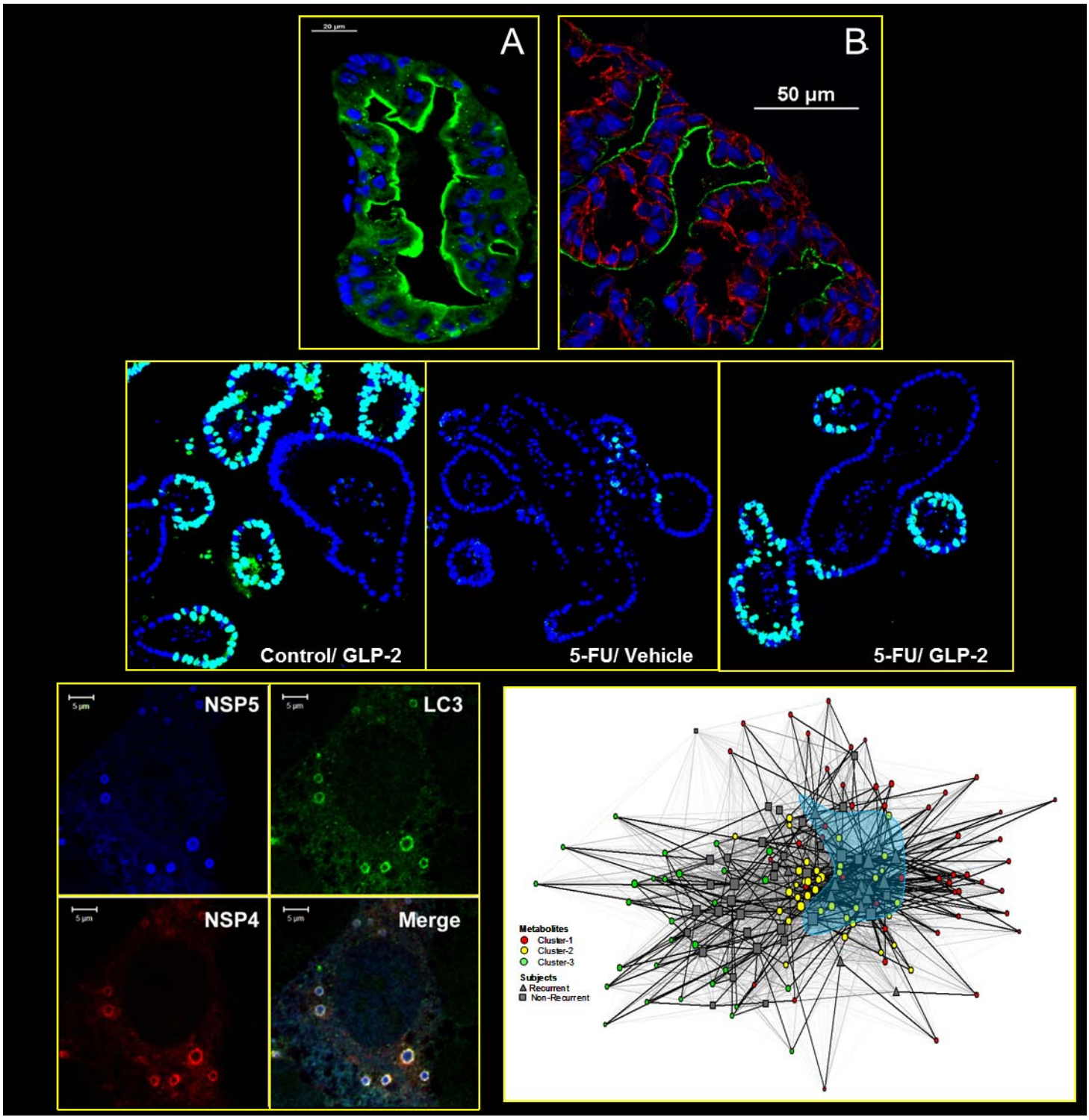
Texas Medical Center Digestive Diseases Center
4th Annual *"Frontiers in Digestive Diseases"* Symposium:
Stem Cells in GI Health and Disease



Saturday, January 12, 2013
Marriot Hotel at the Texas Medical Center
Houston, Texas

Texas Medical Center Digestive Diseases Center
4th Annual *"Frontiers in Digestive Diseases"*: Stem Cells in GI Health & Disease

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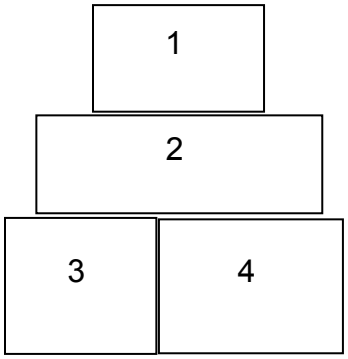
On the cover:



(1) P-10. Kahalil Ettayebi. Villin expression in **(A)** induced human intestinal organoids vs. **(B)** human jejunal intestinal enteroids. Detection of villin expression (green) on brush border membrane of apical absorptive cells; E-cadherin (red); Nuclear staining (blue).

(2) P-29. Xuemei Shi. GLP-2 augments cell proliferation in the mini-gut after 5-fluorouracil treatment. Organoids (cultured from the mouse jejunal crypts) were treated with glucagon-like peptide 2 (GLP-2) and then 5-fluorouracil. Cell proliferation was estimated by BrdU incorporation (in green). The nucleus was counterstained with TOPRO-3 (in blue).

(3) P-30. Zhongcheng Shi. Colocalization of SOX9 and IGFBP-4 in the intestinal epithelium is visualized by immunofluorescent staining. This supports our in vitro data that igfbp4 is directly regulated by SOX9 (and that SOX9 suppresses cellular proliferation through IGFBP-4).

(4) P-34. Petri Urvil. Bipartite network showing metabolite cluster associations in patients with Clostridium difficile infection. The red metabolite cluster is associated with patients susceptible to disease recurrence and represent potential diagnostic biomarkers.

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A G E N D A
Saturday, January 12, 2013
The Marriot Medical Center Hotel
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7:45am	***Coffee and Continental Breakfast***
Session I. Theme:	**GI Stem Cells**
8:15- 8:45am Digestive	***Welcome.*** Moderator: Mary K. Estes, PhD, Director, Texas Medical Center Diseases Center; Professor, Baylor College of Medicine (BCM).
8:45 – 9:25am	***"Intestinal stem cells during regeneration and homeostasis"*** Calvin Kuo, MD, PhD, Associate Professor, Medicine, Stanford University School of Medicine
9:25 – 10:05am	***"Human intestinal organoids from pluripotent stem cells"*** Noah Shroyer, PhD, Assistant Professor, Pediatrics, Cincinnati Children's Hospital Medical Center.
10:05 – 10:35am	***"Function & mechanism of LGR4/5 in GI stem cells and carcinogenesis"*** Qingyun (Jim) Liu, PhD, Professor, Texas Therapeutics Institute, UTHealth
10:35 – 11:00am	***Morning Coffee Break***
Session II. Theme:	**Liver Stem Cells & Therapeutics** Moderator: Tor Savidge, PhD, Associate Professor, Pathology, BCM
11:00 – 11:40am	***"Developmental morphogens, progenitors and adult liver repair"*** Anna Mae Diehl, MD, Professor, Medicine; Chief, Division of Gastroenterology, Duke University Medical School
11:40 – 12:10pm	***"Exploring human cell therapy for metabolic liver disease"*** Karl-Dimiter Bissig, MD, PhD, Assistant Professor, Center for Cell and Gene Therapy, Stem Cells and Regenerative Medicine, BCM
12:10 – 1:45pm	***Lunch & Poster Viewing/Judging***
1:45 – 2:15pm	***"Transplantation of human stem cells for translational ID studies"*** Gustavo Valbuena, MD, PhD, Assistant Professor, Pathology, University of Texas Medical Branch, Galveston TX.
2:15pm	**Discussion and Closing Remarks**

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L I S T O F A B S T R A C T S

Texas Medical Center Digestive Diseases Center
4th Annual *"Frontiers in Digestive Diseases"*: Stem Cells in GI Health & Disease

21	Dorottya Nagy-Szakal, MD Postdoctoral Associate Pediatric – Gastroenterology, BCM	DNA methylation and microbiome separation of ulcerative colitis in treatment naïve children
22	Numan Oezguen, PhD Instructor, Pathology & Immunology BCM	S-nitrosothiols: novel regulators of gut-microbial crosstalk
23	Luis Ojeda, PhD Postdoc Fellow Center for Cell and Gene Therapy, BCM	Generation of human iPSC by RNA reprogramming and novel 3D culture and differentiation of iPS cells into hepatocyte like cells
24	Narayan Sastri Palla, MS Postdoctoral Associate Molecular Virology and Microbiology, BCM	Biochemical and biophysical evidence of calcium binding to coiled coil domain of rotavirus NSP4
25	Shujuan Pan, PhD Assistant Professor Pathology, BCM	Roles of ERManI in liver disease associated with alpha1-antitrypsin deficiency
26	Karina Pokusaeva, PhD Postdoctoral Associate Pathology and Immunology, BCM	Production of gamma-aminobutyric acid by intestinal microbe *bifidobacterium* dentium and other commensal bacteria
27	Muralidhar Premkumar, MBBS ☆ Assistant Professor Neonatology, BCM	Loss of enterocyte derived argininosuccinate lyase results in increased incidence of necrotizing enterocolitis
28	Sasirekha Ramani, PhD Postdoctoral Associate Molecular Virology and Microbiology, BCM	The VP8* of neonatal rotavirus strain G10P[11] binds to type II precursor glycans
29	Xuemei Shi,MD Research Associate Pediatrics-Nutrition, BCM	GLP-2 receptor is required for the growth of intestinal crypts ex vivo
30	Zhongcheng Shi, MD Postdoctoral Associate Immunology and Pediatrics, BCM	SOX9 directly activates IGFBP-4 and suppresses proliferation in Apcmin/+ adenomas and colorectal cancer cells
31	Shumei Song, MD, PhD ☆ Assistant Professor Gastrointestinal Medical Oncology MDACC	Loss of TGF-β adaptor β2SP activates notch signaling and SOX9 in esophageal adenocarcinoma
32	Jennifer K. Spinler, PhD Instructor, Microbial Genetics & Genomics Texas Children's Microbiome Center, BCM	Pangenomic analysis of Lactobacillus reuteri highlights the evolution of a human-specific ecotype
33	Bryan Tackett Graduate Student Texas Children's Liver Center, BCM	P2Y2 purinergic receptor signaling plays a key role in diet-Induced obesity in mice
34	Petri Urvil Laboratory Director Pathology, BCM	A multiomics approach to understanding the gut-microbial ecology and mechanisms of recurrent *Clostridium difficile* infection
35	Donna L. White, PhD, MPH ☆ Assistant Professor Medicine - GI and Hepatology, BCM	Comparative evaluation of bioimpedance and anthropometric measurements as predictors of HCV-related fibrosis and inflammation risk
36	Lisa D. White, PhD Associate Professor Mol. & Human Gen	Whole Transcriptome RNA Sequencing: From Library Preparation to Sequencing

☆ Denotes past Pilot/Feasibility awardee

☆☆ Denotes 2012 Pilot/Feasibility awardee

Human Mesenchymal Stem Cells Attenuate Pancreatic Acinar Injury in Acute Pancreatitis in Mice

Michael Gonzales[1], Xuxia Gao[1], Yanna Cao[1,2], Hasen Xue[1], Judith F. Aronson[3], Shibani Pati[1], Tien C. Ko[1,2]

[1]Department of Surgery, The University of Texas Health Science Center at Houston, Texas; [2]Department of Surgery and [3]Department of Pathology, The University of Texas Medical Branch at Galveston, Texas

Introduction. Human mesenchymal stem cells (hMSCs) can modulate the host immune response against various diseases in animal models and in human with improved survival rate and organ function. Recently, the study on the rat acute pancreatitis models has shown a beneficial effect towards controlling pancreas organ damage. However, the underlying mechanisms of hMSCs' effects are unclear. Therefore, the objective of this study is to use a mouse acute pancreatitis (AP) model to evaluate hMSCs' effects for further investigation of the underlying mechanisms.

Experimental Design and Methods. In the mouse AP model, C57BL/6 mice (male, 8 wks old) were divided into 3 groups: control (PBS only, n=2), AP (cerulein and PBS, n=6), AP+hMSCs (cerulein and hMSCs, n=6). Cerulein was given via 12 hourly intraperitoneal injections (50 μg/kg), while the same amount and frequency of PBS was injected for control. The hMSC (1 million/0.1 ml, obtained from Lonza Inc.) or PBS was injected via tail vein at 24 and 48 hours following the first cerulein injection. The mice were euthanized 4 days after the first cerulein injections. The pancreas was harvested for H&E staining and histological assessment on edema, necrosis, inflammation, acinar injury (including distribution of lesions, glandular atrophy, pseudotubular complexes) and fibrosis.

Results. In respective to the groups of control, AP, AP+hMSCs, there are no significant differences in edema (1.25, 1.7, 2.3, p=0.14), necrosis (0, 0.3, 0.7, p=0.6), inflammation (0.25, 1.0, 0.9, p=0.06), and fibrosis scores (0, 1.7, 1.0, p=0.12). However, there are significant differences in combined acinar injury scores (0, 7.3, 3.2, $p<0.001$) including distribution of lesions (0, 3.2, 1.5, $p<0.001$), glandular atrophy (0, 2.0, 1.0, p=0.002), and pseudotubular complexes (0, 2.2, 0.7, p=0.001).

Conclusions. hMSCs treatment after AP induction showed no effect on edema, necrosis, inflammation, and fibrosis, but significantly reduced acinar injury shown by the decreased acinar injury scores. Our data demonstrate that hMSC treatment attenuates AP injury to pancreatic acini in a mouse AP model. The protective effects of hMSCs in AP may function through a reduced acinar injury or an accelerated acinar regeneration after AP induction. Future experimentation is required for the underlying mechanisms of hMSCs' effects, which may ultimately lead to the development of therapeutic strategies using hMSCs in AP and in other inflammatory diseases.

Identification of target genes in colorectal cancer cells directly regulated by the transcription factor SOX9

Chi-I Chiang, Zhongcheng Shi, Toni-Ann Mistretta, Angela Major, and Yuko Mori-Akiyama

Department of Pathology and Immunology, Baylor College of Medicine
and Texas Children's Hospital

BACKGROUND & AIMS: The transcription factor SOX9 is expressed in diverse cancers including most human primary colorectal cancer (CRC). The role of SOX9 in cancers has been a focus of many recent studies and there have been reports of both oncogenic and suppressive roles of SOX9 in various cancers, including breast, prostate, ovarian cancers, and melanomas. In CRC, some reports suggested an anti-oncogenic role for SOX9, while others demonstrated an oncogenic role. A recent genome-scale analysis of human CRC identified *SOX9* as one of the frequently mutated genes and the mutations of *SOX9* were either frame shift or nonsense mutations. Nevertheless, role of SOX9 in CRC has not been well studied and to date only a few direct targets of SOX9 in intestinal epithelial cells have been identified. In this study, we identified target genes of SOX9 and *in vivo* regulations of these genes by SOX9 were confirmed using two mouse models.

METHODS: Mice that ectopically express SOX9 throughout in the intestinal epithelium were used for identifying RNA profile. Chromatin immunoprecipitation (ChIP)-based assays that involve genome-wide mapping validated the direct binding of SOX9 on the potential target genes. The regulation of these genes by SOX9 was further confirmed in adenoma cells of *Sox9*-deficient Apc^{min} mice.

RESULTS: Combined analysis of RNA profiling by microarray using SOX9-overexpressing mouse intestine and genome-wide ChIP sequencing using human colorectal cancer cells revealed direct target genes of SOX9 that includeed anti-oncogenic as well as oncogenic genes. Our data suggested that SOX9 may potentially support cancer stem cells, however, SOX9 may suppress proliferation and progression.

Autophagy hijacked through viroporin-activated CaMKK-β signaling is required for rotavirus replication

Sue E. Crawford, Joseph M. Hyser, Budi Utama, and Mary K. Estes

Baylor College of Medicine

Autophagy is a cellular degradation process involving an intracellular membrane trafficking pathway that recycles cellular components or eliminates intracellular microbes in lysosomes. Many pathogens subvert autophagy to enhance their replication but the mechanisms these pathogens use to initiate the autophagy process have not been elucidated. This study identifies rotavirus (RV) as the first pathogen that uses its viroporin, NSP4, which releases calcium from the endoplasmic reticulum (ER) into the cytoplasm to activate a CaMKK-β and AMPK-dependent signaling pathway to initiate the autophagy process. RV hijacks this membrane trafficking pathway to transport viral proteins from the ER to sites of viral replication to produce infectious virus. This process requires PI3-kinase activity, autophagy-initiation proteins Atg3 and Atg5, and is abrogated by chelating cytoplasmic calcium or inhibiting CaMKK-β. These studies identify a novel mechanism of virus-mediated calcium-activated signaling that initiates autophagy and hijacks this membrane trafficking pathway to transport viral proteins to sites of viral assembly.

Bile acids induce myocardial dysfunction: candidate mechanism for cirrhotic cardiomyopathy

*****Moreshwar Desai[1], *Sayeepriyadarshini Anakk[2], Zeena Eblimit[1], Renan Orellana[1], Hernan Vasquez[3], Henry Cheng-Ju Wu[3], Heinrich Taegtmeyer[3], Daniel Penny[4], Saul Karpen[5], David D. Moore[2]

[1]Section of Pediatric Critical Care and Liver Center, [2]Dept. Molecular and Cellular Biology, [4]Dept. of Pediatric Cardiology, Baylor College of Medicine; [3] Dept. of Cardiology UTHSC; [5] Dept of Pediatric Gastroenterology, Emory, Atlanta, GA. [*equal contribution].

Introduction: Cardiac dysfunction in cirrhosis - manifesting itself as cirrhotic cardiomyopathy, is a life threatening complication of end-stage cirrhotic liver diseases with a prevalence approaching 50% of cirrhotic adults and children. Hearts of cirrhotic patients show electrocardiographic abnormalities and systolic dysfunction under stress. The pathophysiology is poorly understood, impairing prevention and treatment. Bile acid (BA) excess (cholanemia) is a pathognomonic feature of cirrhosis. It drives pathological processes in several extra-hepatic organs, but the cardiac effects are unknown. Using an established mouse model of cholanemia, $FXR^{-/-}/SHP^{-/-}$ (DKO) along with ex vivo BA perfusion of the whole heart, we tested the hypothesis that cholanemia impairs myocardial contractility, catecholamine response, metabolism and alters cell signaling pathways.

Methods: 12 week old male DKO mice (n=6) and their age matched littermate (WT) controls underwent echocardiography (ECHO), electrocardiography (ECG), treadmill stress tests and acute catecholamine (isoprenaline) challenge with stress ECHO. Hearts were analyzed for protein expression of various signaling pathways. To evaluate the direct effects of BA excess on contractility and metabolism, whole hearts (n=5/grp) were challenged with Taurocholic acid (TCA) [500µmol for 20 minutes] or vehicle ex vivo using Langendorff system.

Results: DKO mice showed evidence of liver injury, cholestasis and cholanemia (Anakk et al . J Clin Invest. 2011 Jan;121(1):86-95). DKO mice showed significant bradycardia and prolonged PR interval on ECG. ECHO revealed a 30% decrease in cardiac output [CO] compared to WT controls (Table). On challenge with isoprenaline (200mcg/kg i.p), heart rate, ejection fraction and CO was significantly attenuated in the DKO mice and the time taken for peak response was twice that of WT controls. On the treadmill, DKO mice showed exercise intolerance as evidenced by lesser distance covered and earlier fatigue (Table). DKO hearts showed a robust 3 fold increase in AKT phosphorylation and a modest 1.5 fold increase in JNK phosphorylation. At the RNA level, there was a 50% upregulation of GLUT-1 and 75% reduction of PDK4, suggesting increased glucose uptake and oxidation, and a 50% downregulation of UCP3, m-CPT1, mCPT-2 and h-FABP suggesting decreased fatty acid oxidation. A 50% decrease in contractility with concomitant decrease in glucose and fatty acid oxidation was demonstrated in TCA perfused hearts ex vivo.

Conclusions: Cholanemia induces contractile dysfunction, catecholamine resistance, metabolic derangement and induction of stress mediated signaling in the heart. Further studies into myocardial remodeling and consequences of "pathologic" bile acid-myocardial interaction as chief driver for cirrhotic cardiomyopathy are warranted. [TCH startup funds (MD), CPRIT RP120138 (DDM)]

PARAMETERS	WT (n=6) [Mean±SD]	DKO (n=6) [Mean±SD]	P value (t-test)
Heart Rate (bpm)	750±25	700±50	0.04
PR interval (m-sec)	22±2	28±3	0.002
Cardiac Output (ml/min)	24±3.5	17±3.2	0.03
Peak Response (min)	20±10	40±12	0.007
Distance on treadmill (m)	425±50	300±25	0.007
Time to exhaustion (min)	18.5±2	15±1.5	0.04

Human Jejunal Enteroid Cultures as a Functional Model of Human Small Intestine to Study Infection with Human Enteric Microbes

Khalil Ettayebi[1], Xi-Lei Zeng[1], Sue E. Crawford[1], Joseph M. Hyser[1], James Broughman[1], Antone R. Opekum[2,3], David Y. Graham[3], Vadim Sherman[4], and Mary K. Estes[1]

(1) Department of Molecular Virology, Baylor College of Medicine
(2) Department of Pediatrics, Baylor College of Medicine
(3) Department of Medicine, Baylor College of Medicine
(4) Bariatric surgery at Methodist Hospital

One rate-limiting step in translational research is the absence of reliable pre-clinical models that adequately reflect relevant human physiology and disease pathology. The goal of the current study was to establish human enteroid cultures and validate them as functional pre-clinical models of clinically important diarrheal models. Methods: We successfully cultured human enteroids from jejunal tissues obtained from patients undergoing bariatric surgery. Jejunal human intestinal enteroids (jHIEs) have been established from 11 different individuals, maintained in culture for 5 months, and can be frozen and recultured. The enteroids contain multiple cell types, including enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. The epithelial cells, containing a number of ion channels, are organized around luminal cavities located within the core of the jHIEs. We tested the jHIEs as a new model for the cultivation and study of the gastrointestinal virus, rotavirus. Proof-of-principle analyses showed that jHIEs support replication of three rotaviruses, a lab strain RRV and two clinical isolates RV-35 and RV-08-42, based on detection of the nonstructural viral protein NSP4 by immunofluorescence, increased levels of viral RNA by quantitative reverse transcription-PCR (qRT-PCR), and production of infectious progeny virus. This work demonstrates that jHIEs offer a promising new model to study rotaviruses and they may be useful to study a variety of gastrointestinal-microbe interactions.

This study was supported by NIH grants (P30DK056338; PO1 AI057788, RO1 AI080656 and U18 NS080763).

Putative Histidine Decarboxylase Regulator (PhdR) Modulates Histamine Production and Immunomodulation by *Lactobacillus reuteri*

Hemarajata P[1,3], Gao C[1,3], Pflughoeft KJ[2,3], Thomas CM[2,3], Saulnier DM[2,3], Versalovic J[1,2,3].

[1]Department of Molecular Virology and Microbiology and [2]Department of Pathology and Immunology, Baylor College of Medicine; [3]Department of Pathology, Texas Children's Hospital

The human-microbiome-derived probiotic *Lactobacillus reuteri* ATCC PTA 6475 suppresses tumor necrosis factor (TNF) production by activated human myeloid (THP-1) cells. A gene encoding a hypothetical membrane protein PhdR was highly upregulated (>20-fold) in wild-type *L. reuteri* 6475 during the stationary phase of growth, when TNF inhibition is most potent compared to mid-log phase. Genetic inactivation of *phdR* resulted in a complete loss of TNF suppression in human myeloid cells and a diminished protective effect in a trinitrobenzene sulfonic acid (TNBS)-induced colitis mouse model compared to wild type. These findings suggested that *phdR* may regulate genes involved in *L. reuteri*-mediated immunomodulation. Histamine is one of the immunomodulatory factors produced by *L. reuteri* 6475 and histidine decarboxylase enzyme (HdcA) converts histidine to histamine. RNA-seq analysis, comparative proteomics and reverse transcription quantitative PCR yielded evidence of significant suppression of histidine decarboxylase (*hdc*) gene cluster expression, including that of *hdcA*, in the *phdR* mutant compared to wild-type. The *phdR* mutant produced less histamine compared to wild-type. A β-glucuronidase assay revealed diminished expression of a reporter gene *gusA* under the P_{hdcAB} promoter in the *phdR* mutant compared to wild-type. Moreover, histamine production, expression of the *hdc* gene cluster and P_{hdcAB}-driven *gusA* in the mutant were significantly lower compared to those of wild-type, and were not induced in the presence of supplemental L-histidine in growth media. These findings suggest that PhdR regulates the *hdc* gene cluster at the transcriptional level, resulting in regulation of HdcA expression and histamine production by the human microbiome. This study will provide a better understanding of mechanisms of immunomodulation by probiotics, which may lead to novel therapeutics for inflammatory bowel disease (IBD).

Increased miR-143 and decreased miR-31 expression in Apc(min/+) mouse adenomas and human FAP polyps

Pooja Prasad, Christina A. Sam MS, Toni-Ann Mistretta PhD, Yuko Mori-Akiyama MD, Milton J. Finegold MD, Preethi H. Gunaratne PhD, and Anne M. Hutson PhD

BACKGROUND: In Familial Adenomatous Polyposis (FAP) the development of polyps, lacking functional adenomatous polyposis coli (APC) tumor suppressor expression, precedes the development of colon cancer (CRC). Likewise, in sporadic CRC APC is the most frequently mutated gene. MicroRNAs (miRNAs), which usually repress gene expression, are dysregulated in all cancers and contribute to altered gene expression. We hypothesized that miRNA expression would be altered in the adenomas of Apc(min/+) mice and human FAP polyps.

METHODS: Small RNAs were extracted from Apc(min/+) adenomas and normal mouse tissues and formalin-fixed paraffin embedded FAP polyp and normal human tissue. Microarrays were performed with RNA from Apc(min/+) adenoma (ApcAd, n=3) and normal epithelia (ApcEpi, n=3) and littermate control epithelia (WtEpi, n=2) (LC Science). Normalized spot intensities were analyzed by one-way ANOVA (Partek). Small RNAs from ApcAd and WtEpi samples were also profiled by next generation sequencing (Illumina GAII). Reverse transcription (RT) followed by quantitative PCR validated microarray and sequencing results of 13 miRNAs in mouse samples (n=5), and tested the levels of two miRNA in human pediatric FAP and normal tissues (n=9).

RESULTS: Principle components analysis of microarray data indicated that ApcAd samples clustered separately from ApcEpi and WtEpi, thus normal tissues were combined for further analyses. By miRNAs microarray and sequencing we discovered 8 highly expressed miRNAs that were significantly upregulated and 17 downregulated ≥1.5-fold ($p<0.05$) in the ApcAd samples. As predicted, the tumor suppressors miR-16 and -15a were downregulated, and the oncomir miR-21 was upregulated in ApcAd. RT-qPCR confirmed enriched expression in ApcAd of miR-125b, and -143, and de-enrichment of miR-141, -30e, -22, -140*, -31, -378, -194, -215, and -192. The increased expression of miR-143 and decreased expression of miR-31 were unexpected, given conflicting results in human Stage II-IV CRC, thus we examined these miRNAs in human FAP polyps. Fifty-six percent of human polyps had higher expression of miR-143 compared to their controls (≥1.5-fold), and 67% had lower expression of miR-31.

CONCLUSION: miRNA expression was altered in the adenomas of Apc(min/+) mice. During this early stage of CRC, we saw increased miR-143 and decreased miR-31 expression. In human FAP polyps, we found more similarity to mouse ApcAd expression of these miRNAs than to later stage human CRC tumors. Thus, these miRNAs may play a complex role during CRC progression.

Multiple calcium entry pathways are activated by the rotavirus viroporin NSP4

Joseph M. Hyser[1], Budi Utama[1], Frank T. Horrigan[2], Sue E. Crawford[1], Khalil Ettayebi[1], Doug Peters[1,3], and Mary K. Estes[1]

[1]Molecular Virology & Microbiology and [2]Molecular Physiology & Biophysics, Baylor College of Medicine; [3]Augustana College TMC Summer Research Internship Program, Rock Island, IL

A hallmark of rotavirus (RV) replication is disruption of cellular Ca^{2+} homeostasis, including an elevation in cytoplasmic Ca^{2+}, increased permeability of the endoplasmic reticulum (ER) to Ca^{2+} and activation of plasma membrane (PM)-localized Ca^{2+} entry channels. This elevation in cytoplasmic Ca^{2+} is essential for RV replication. We recently showed that the nonstructural protein 4 (NSP4) is a viroporin in the ER membrane and viroporin activity is the mechanism by which RV elevates cytoplasmic Ca^{2+}. We hypothesized that NSP4 forms a ion channel to release ER Ca^{2+} stores, which activates Ca^{2+}-release activated Ca^{2+} (CRAC) channels, as well as other Ca^{2+} entry channels, in the PM. Electrophysiological studies demonstrated that purified full-length NSP4 has intrinsic ion channel activity when reconstituted into planar lipid bilayers. Further, we recorded NSP4 ion channel activity using patch clamp of the outer nuclear envelope of Sf9 insect cells. Single channel conductance was 30 picoSiemens and the number of channels detected per patch increased concomitantly with the elevation in cytoplasmic Ca^{2+}. Using a cell line stably expressing YFP-tagged stromal interaction molecule 1 (STIM1-YFP), an ER transmembrane sensor of ER luminal Ca^{2+}, we found that wild-type NSP4, but not viroporin mutants, activates STIM1-YFP translocation to the PM and colocalization with the Orai1 CRAC channel. In addition to CRAC channels, we identified a voltage-activated Ca^{2+} channel ($Ca_V1.3$) expressed in human enterocytes and specific blockers of this channel (amlodipine and nifedipine) inhibited both NSP4-induced Ca^{2+} entry and RV replication. Thus, RV targets both store operated and voltage-activated Ca^{2+} entry pathways to acquire the Ca^{2+} needed to support virus replication. Since $Ca_V1.3$ blockers are FDA approved as anti-hypertensive drugs and are commonly used in young children, it is possible these drugs could be effective antiviral drugs to reduce the severity of rotavirus diarrheal disease.

Instrumental variable analysis on soluble receptor for advanced glycation end-products and risk of pancreatic cancer - a pilot study

Zhigang Duan, Guoqing Chen, Rachael Stolzenberg-Solomon, Stephanie Weinstein, Jarmo Virtamo, Demetrius Albanes, Liang Chen, Hashem B. El-Serag, Li Jiao

Background: We previously found a strong inverse association between prediagnostic serum levels of soluble receptor for advanced glycation end-products (sRAGE) and risk of pancreatic cancer in 255 cases and 485 subcohort controls from the Alpha-Tocopherol, Beta-carotene Cancer Prevention (ATBC) Study, a prospective cohort of Finnish male smokers. However, this novel association may be due to unrecognized confounding factors or biases. In the present study, we conducted a Mendelian randomization study to test the causality between log-transformed sRAGE and pancreatic cancer using rs2070600 (Gly82Ser, C/T), a single nucleotide polymorphism (SNP) of *RAGE*, as an instrumental variable. SNP rs2070600 has been shown in previous studies to determine the circulating levels of sRAGE.

Method: Genotype data were obtained from a GWAS of pancreatic cancer and were available on 184 cases and 186 controls. A multiplicative structural mean model was used to obtain a causal odds ratio (OR) for pancreatic cancer for genetically adjusted sRAGE variable. Age, body mass index, years of smoking, and serum levels of Nε-(carboxymethyl)lysine were the potential confounding factors adjusted in all multivariate models. First-stage F statistics greater than 10 indicates a strong instrumental variable.

Results: We observed the same inverse association between sRAGE and pancreatic cancer as previously reported (adjusted OR = 0.50, 95% CI: 0.23-0.68, for highest vs. lowest tertile). Thirty five study participants carried CT genotypes that significantly predicted lower levels of sRAGE (β = -51.2, *P* = 0.002) in multivariate models. There were no associations between rs2070600 and risk of pancreatic cancer (*P* = 0.88) or the confounding factors (*P* values > 0.30). The first-stage F-statistic of rs2070600 was 7.97 (unadjusted) and 28.2 in the multivariate model. However, the second stage of structural mean model did not generate a meaningful estimate in the multivariate model.

Conclusion: This pilot study suggested that SNP rs2070600 is potential a strong instrumental variable for a Mendelian randomization study to confirm the association between sRAGE and pancreatic cancer. However, a larger sample size is imperative for conducting a credible instrumental variable analysis.

Autologous Cell Therapy for Tyrosinemia Type 1: Exploring Human Cell Therapy in a Mouse

Claudia Kettlun Leyton, Xavier Legras, Mercedes Barzi Dieguez, Robert Kruse, Luis Ojeda, Karl-Dimiter Bissig

Center for Cell and Gene Therapy, Department for Molecular and Cellular Biology
Baylor College of Medicine

Tyrosinemia type I is an autosomal recessive disorder caused by mutations in the fumarylacetoacetate hydrolase (*FAH*) gene. When mutated this leads to a toxic accumulation of tyrosine catabolites in the liver. Clinically this is associated with severe liver and kidney disease and usually leads to liver cancer and early death.

To develop autologous cell therapy for this metabolic liver disease, we have generated induced pluripotent stem (iPS) cells by reprograming fibroblasts from a patient with tyrosinemia type I. The iPS cells expressed the pluripotency markers SSEA4 and OCT-4 and were able to form all three germ layers in a teratoma formation assay.

In order to correct the underlying disease, we have used zinc finger nucleases (ZFN) assisted homologous recombination. We have chosen two different approaches to do so. One approach, introduces an additional copy of the *FAH* gene into a "safe harbor" location (the *AAVS1* locus). The second approach is to correct the gene directly at the diseased locus. We have obtained several corrected FAH iPS cells and are currently evaluating FAH expression. Corrected iPS cells will be differentiated into hepatocytes by applying developmental clues (directed differentiation protocol).

Stem cell derived hepatocytes will then be used to explore cell-based therapy in the *fah-/-rag2-/-Il2rg-/-* (FRG) triple KO mouse. This tyrosinemic mouse model has been shown to be an excellent hepatocyte acceptor strain and bears the advantage that the mouse could be cured by a human cell therapy approach.

Investigating the immune modulatory properties of human Mesenchymal Stem/Stromal cells

Daniel Kota, Bryan DiCarlo, Scott Olson

Bowel perforation from a number of injuries and disease is a major source of infection and sepsis. Sepsis is a $24.3 billion problem and a major source of morbidity and mortality in the American health system. Mesenchymal Stem/Stromal cells (MSCs) are multipotent progenitor cells found in virtually all postnatal tissues. MSCs were first considered as an alternative therapy for tissue repair in many diseases because of their ease for in vitro expansion and differentiation capacity. Interestingly, MSCs have shown to reduce the burden of a variety of immune-based disease models by responding to inflammatory signals, being activated or licensed to suppress inflammatory/immune responses both in vitro and in vivo. The mechanisms involved in this new therapeutic paradigm are still elusive, largely due to differences regarding MSC sources - including the use of MSCs from different species, animal models and culture conditions. Determining the mechanism through which MSCs isolated from humans can inhibit inflammatory responses associated with sepsis will allow for the tailoring/harnessing of autologous or allogeneic MSC-based therapies for septic conditions and could possibly be translated to a variety of other inflammatory diseases. Here, we investigated the effects of human MSCs on different immune cells relevant to the pathology of sepsis.

High prevalence of HBV non-immunity in vaccinated pediatric liver transplant recipients

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Ryan Himes, Daniel Leung

Background: Infections represent a significant threat in solid-organ transplant recipients. Behind influenza and pneumococcus, hepatitis B virus (HBV) claims more lives than any other preventable illness. Long term protection afforded by childhood immunization assumes greater importance with increasing life span of transplant recipients. HBV vaccine durability in the post solid-organ transplant setting has not been well studied.

Methods: This was a prospective cross-sectional, single-center study evaluating HBV immunity in 117 pediatric liver transplant recipients. Patients with hepatitis B surface antibody (anti-HBs) levels <10 mIU/mL were considered non-responders. Screening data included demographics, time since transplant, anthropometrics, albumin, WBC differentials, fat soluble vitamin levels, immunosuppression, and PELD/MELD scores.

Results: All 117 subjects received the full HBV vaccination series prior to transplant. 77 (65.8%) were non-immune. Among the non-responders, the mean anti-HBs level was 2.7 (±2.1) and among responders, 95.7 (±180.3). Older age ($p=0.027$), longer time since transplant ($p<0.00001$), and lower WBC ($p=0.04$) were associated with non-immunity. 50%, 66.7%, 77.8%, and 76.5% of children at ages 2, 3, 4, and 13-17 years, respectively, had non-protective anti-HBs. Demographics, immunosuppression, markers of nutritional status, BMI Z-score, lymphocyte %, ANC, and PELD/MELD were not significantly different between immune and non-immune liver transplant patients.

Conclusions: This is the first U.S. study to prospectively evaluate the durability of hepatitis B immunity in pediatric liver transplant patients who were universally vaccinated prior to transplant. Only 34.2% of pediatric liver transplant patients were found to be immune to HBV following vaccination. Non-immunity was observed in the majority as early as 2-4 years of age. Markers of nutrition and immunosuppression were not predictors of non-immunity following vaccination, suggesting that a systematic approach may be warranted to detect HBV vaccine non-responders among pediatric liver transplant recipients.

Lactobacillus reuteri DSM 17938 inversely changes the effector and regulatory T cell population in a mouse model of necrotizing enterocolitis

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Background: Activated effector lymphocyte recruitment to sites of inflammation requires the sequential engagement of adhesion molecules such as CD44. The phenotype of $CD44^+CD45RB^{lo}$ separates effector memory from naïve cells ($CD44^-CD45RB^{hi}$). The $CD4^+CD44^+CD45RB^{lo}$ population contains $Foxp3^+$ regulatory T cells (Tregs). In the rat necrotzing enterocolitis (NEC) model, the frequency of $Foxp3^+$ Tregs in the intestine is significantly decreased compared to normal control intestine. Probiotic *Lactobacillus reuteri* DSM 17938 (LR17938), which reduces the incidence and severity of NEC, has the capacity to restore Tregs to normal levels. However, it is not clear whether effector $CD4^+CD44^+CD45RB^{lo}Foxp3^-$ T cells play a role in NEC. We hypothesized that this specific T cell subset participates in the inflammation associated with NEC and can be altered by administration of LR17938.

Aims: To determine if the frequency of effector T cells or $Foxp3^+$ Tregs changes in the intestine of mice with NEC compared to dam-fed controls, and to determine if this change can be reversed by gavage-feeding LR17938.

Methods: NEC was induced in 10-day old C57BL/6J mice by gavage feeding with special cow milk formula and exposure to hypoxia for 4 days. Groups studied included dam-fed controls, NEC, and NEC+ LR17938 (10^6 cfu/g. b.w. per day). Lymphocytes isolated from terminal ileum after digesting with collagenase V were labeled for CD4, CD44, CD45RB and intracellular Foxp3 and were analyzed by flow cytometry. After gating $CD4^+Foxp3^-$ cells, the frequencies of different $CD4^+$ T subsets were calculated.

Results: A significant increase in the percentage of $CD4^+CD44^+CD45RB^{lo}Foxp3^-$ effector T subset in the intestine was observed during NEC (8.2±0.4%, n=8), compared to dam-fed controls (3.9±0.8%, n=8) ($p<0.001$). Feeding LR17938 to mice with NEC significantly decreased this subset (5.1±0.7%, n=8) compared to samples from mice with NEC that did not receive probiotic ($p<0.05$). Surprisingly, we discovered that the subset $CD4^+CD44^+CD45RB^{hi}Foxp3^-$ T cells was also significantly increased in NEC (19.2±3.1) compared to dam-fed mouse intestine (2.9±0.6) ($p<0.001$), and decreased with LR17938 feeding during NEC (5.3±0.9) ($p<0.001$) compared to NEC without probiotic. The phenotype of this subset is a transitional stage of effector T cells from naïve ($CD44^-CD45RB^{hi}$) to memory ($CD44^+CD45RB^{lo}$) $CD4^+$ T cells. Concomitantly, the percentage of $Foxp3^+$ Tregs was decreased in the intestine of mice with NEC (2.1±0.4) compared to dam-fed controls (6.6±0.7) ($p<0.001$), which could be restored to normal (5.1±0.8, $p<0.05$) by LR17938 feeding.

Conclusions: $CD4^+CD44^+CD45RB^{lo/hi}Foxp3^-$ effector T cells and $Foxp3^+$ Tregs were inversely altered by LR17938 feeding in the intestine of mice with NEC. These T cell subsets might be potential biomarkers and new therapeutic targets during intestinal inflammation.

Purinergic Signaling in Hepatocellular Carcinoma

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Introduction: Hepatocellular carcinoma (HCC) is the third lethal cancer worldwide. Despite its increasing incidence the cellular and molecular mechanisms of pathogenesis are not well understood. Previous findings from our laboratory suggest that extracellular ATP via activation of P2Y2 purinergic receptors induce hepatocyte proliferation in response to partial hepatectomy and ATP treatment alone was sufficient to induce hepatocyte proliferation *in vitro*. The purpose of this study was to examine the role of purinergic signaling in the pathogenesis of HCC in patients and Mst1/2$^{-/-}$, a mouse model of HCC. Mst1/2 kinase activates the hippo signaling pathway which induces tumor suppression, via phosphorylation of Lats1/2 and Yap preventing nuclear translocation of Yap, which promotes transcription of pro-proliferative and anti-apoptotic genes.

Hypothesis: *Dysregulation of purinergic signaling facilitates aberrant cell proliferation underlying hepatocellular carcinogenesis.*

Methods: Mst1/2$^{-/-}$ and WT mice livers (1, 3, & 6 months) were analyzed by qRT-PCR and Western blotting for P2 purinergic receptor expression. HCC-derived Huh7 cells were treated with P2 receptor agonists; ATP γS, ATP, ADP, UTP (100 μM) for different time intervals. HCC patient samples (n=27) were evaluated by qRT-PCR for expression of all 15 P2 receptor isoforms.

Results: Mst1/2$^{-/-}$ mice which develop liver tumors (3-6 months) exhibit distinct differences in temporal profile of multiple P2 purinergic receptor isoforms, as compared to WT. As evidence for functional interaction between purinergic and hippo signaling pathways, ATP□S, ATP, ADP or UTP treatment alone was sufficient to induce early Mst1/2 phosphorylation in Huh7 cells. Indicative of inactivation of Mst1/2-mediated tumor suppression in Huh7 cells, ADP treatment decreased YAP phosphorylation, increased nuclear translocation of YAP protein and induced YAP downstream target, connective tissue growth factor (CTGF) mRNA expression, despite upstream activation of Mst1/2 and LATS1 phosphorylation.

In HCC patients, multiple P2 receptor isoforms were elevated ≥2-fold in liver tumors as compared to uninvolved areas in up to 50% of patients. P2 purinergic receptor upregulation was more prevalent among HCC patients infected with Hepatitis C Virus (HCV) as compared to non-viral groups (75% vs 20%; HCV vs non-HCV) identifying a unique subset of viral-induced HCC over expressing P2 receptors.

Conclusion: Our analysis of HCC patient and Mst1/2$^{-/-}$ mice livers has uncovered a potential role for purinergic signaling in the pathogenesis of HCC. We have identified extracellular nucleotide-mediated purinergic signaling as a novel upstream modulator of hippo tumor suppressor kinases Mst1/2 and LATS1 with dysregulation of purinergic-hippo signaling interactions in Huh7 cells. These findings highlight the potential for P2 purinergic receptors as potential biomarkers and novel therapeutic targets for HCC.

Lymphotoxin Signaling is Important for Rotavirus Specific IgA Induction

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Intestinal IgA and B cells are critical to protection from rotavirus. TNF family members provide critical signals for T cell dependent and independent IgA antibody induction. To determine the signals that are required to induce intestinal IgA and protection from rotavirus infection, multiple strains of mice that lack specific TNF family member receptors or ligands (TNF, LT, CD40, BAFF) or wild type mice were orally infected with murine rotavirus. Mice were assessed for clearance of primary infection, development of intestinal and serologic rotavirus-specific antibody, and protection from re-infection with murine rotavirus. Significant differences in development of intestinal rotavirus-specific antibody, clearance, or protection were only observed in mice that lacked either lymphotoxin-alpha (LTα-/-) or LTβ-/-. Both LTα-/- and LTβ -/- mice had delayed clearance of a primary rotavirus infection compared to wild type mice. The resolution of fecal virus shedding occurred concurrently with induction of intestinal rotavirus specific IgA in LTα-/- mice. However, LTβ-/- mice failed to develop or maintain fecal IgA and were not protected from rotavirus challenge. In contrast, LTα-/- and wild type mice were protected from challenge. All strains cleared systemic rotavirus within 10 days and developed early and sustained serum anti-rotavirus antibody responses. Taken together, these data indicate that lymphotoxin, but not TNF, CD40, or BAFF, signaling pathways are important in timely induction or maintenance of rotavirus specific intestinal IgA and protection.

DNA Methylation and Microbiome Separation of Ulcerative Colitis in Treatment Naïve Children

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Background: Inflammatory bowel diseases (IBD, including Crohn disease [CD] and ulcerative colitis [UC]) are emerging globally supporting the hypothesis that environmental factors may play a role in their pathogenesis. Commensal microbes and epigenetic characteristics, such as DNA methylation can respond to environmental changes and have been implicated in triggering these diseases. Therefore, we evaluated the relationship between colonic mucosal microbiome and DNA methylation in untreated pediatric IBD.

Method: The mucosal microbiome was studied by 454 pyrosequencing of the bacterial *16S rRNA* gene in transverse colonic biopsy specimens from 26 controls, 16 untreated pediatric CD (15 treatment naïve), and 6 UC cases (5 treatment naïve). Genome-wide DNA methylation was examined by Infinium HumanMethylation450 BeadChip Kits in a subset of treatment naïve samples (10 controls, 10 CD, and 4 UC). Validation of the DNA methylation results was performed on an independent cohort (12 controls, 5 CD, 5 UC) and by bisulfite-pyrosequencing of select loci.

Result: There was no consistent DNA methylation association with CD. However, UC had a large number of significant DNA methylation associations (4277 shared CpG sites between discovery and validation cohort; false discovery rate: FDR<0.01). The UC-linked methylation changes associated with genes involved in cell adhesion and communication, defense and immune responses. UC microbiome separated from controls and CD (p=0.048). UC associated DNA methylation changes correlated with bacterial taxa abundance at reads with incomplete or uncertain genus assignment belonging to the families Eubacteriaceae and Lachnospiraceae.

Conclusions: Treatment naïve pediatric UC separates at the colonic mucosal microbiome and DNA methylome both from control and from CD. These findings may have etiologic, diagnostic and therapeutic relevance for IBD.

S-nitrosothiols: novel regulators of gut-microbial crosstalk

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The role of nitric oxide (NO) in gut homeostasis and inflammation has been extensively studied during the last decade. Growing evidence suggests that innate host responses to gut pathogens involve an upregulation of NO production. Posttranslational modification of exposed protein cysteine thiols by NO (S-nitrosylation) has been implicated in the regulation of diverse cellular functions, and aberrant S-nitrosylation plays a major role in disease-etiology. However, it is not known whether protective NO signals directed against enteric pathogens operate via S-nitrosylation of microbial target molecules.

We have recently reported that host-mediated S-nitrosylation plays a significant role in subverting toxin virulence during *Clostridium difficile* infection (Savidge, *et al*. Nature Medicine 17:1136-41 (2011)). *C. difficile* secretes two exotoxins (TcdA and TcdB) that cause diarrhea, inflammation, and necrosis of the colonic mucosa. After entering the colonocyte by endocytosis, the toxins are allosterically activated by cytosolic inositol *hexakis*phosphate which binds to the autocatalytic cysteine protease domain (CPD) of the toxin. By molecular characterization of this allosteric toxin virulence mechanism, we identified a novel CPD catalytic-motif that is also inhibited by host-mediated S-nitrosylation. To examine whether this motif is unique to the *C. difficile* toxins, we performed a systematic computational scan of all PDB entries for structural similarities with this allosterically regulated CPD. Besides the known CPD's from the *C. difficile* toxins TcdA and TcdB, *Vibrio Cholerae* RTX, and *Porphyromonas Gingivalis* Gingipain (RGPB), we identified several other potentially novel allosterically regulated microbial CPD's. Notable examples include *Bacillus Subtilis* Alkaline Phosphatase D, *Bacteroides Fragilis* putative Xylanase, *Clostridium Perfringens* GH95, *Enterococcus Faecalis* putative Mannonate Dehydratase, and *Propioni Bacterium* Acnes CLA-producing Fatty Acid Isomerase.

In conclusion, host-mediated S-nitrosylation may play a universal role in regulating toxin and enzyme-mediated microbial-host interactions at mucosal surfaces.

Generation of human iPSC by RNA Reprogramming and Novel 3D Culture and Differentiation of iPS Cells into Hepatocyte Like Cells

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Since the initial induced pluripotent stem cell (iPSC) work by Shinya Yamanaka and colleagues, significant progress has been made in identifying new strategies to enhance the reprogramming efficiency and clinical safety. Use of retroviral or even plasmid based methods brings the problem of insertional mutagenesis, which can affect cell function and promote tumor formation.

Thus, we have implemented the use of RNA-based reprogramming by transfecting messenger RNAs coding the genes *OCT4, SOX2, KLF4,* and *C-MYC* into human fibroblasts to generate iPSCs. This method eliminates the risks associated with the use of integrating viruses or DNA transfection methods. However, in our hands RNA reprogramming efficiency (<3%) remained lower than those observed for integrating viral methods. We are currently evaluating karyotype and pluripotency (teratoma assay) of the iPSCs generated by this RNA-transfection method.

Besides generating safer iPSCs, we have also used a novel method to grow and differentiate iPSCs in suspension culture. Embryonic stem cells (ESCs) and iPSCs are usually grown and differentiated in conditions where the cells are attached to the flask in a two-dimensional (2D) format. However, monolayer culture may hinder the differentiation of stem cells since such 2D conditions do not accurately reflect the complex nature of three-dimensional (3D) hepatocyte specification *in vivo*. Therefore, we grow stem cells (ESC and iPSC) in suspension culture which allows them to remain undifferentiated as shown by the expression of the glycolipid antigen SSEA-4. Further they retain their ability to form teratoma when injected into immune deficient mice. Our suspension culture facilitates a more physiological 3D differentiation of stem cells and, in addition the differentiated cell aggregates can be directly transplanted. This avoids the enzymatic or chemical separation of the cells from the plate that can affect cell viability, especially in the case of differentiated hepatocytes which are very sensitive to cell detachment procedures.

We apply a standard differentiation protocol on our suspension cultures and augment hepatic differentiation by timed expression of key developmental factors or master regulators of adult human hepatocytes. Suspension aggregates that undergo this differentiation procedure are evaluated for expression of hepatocyte markers such as alpha-fetoprotein. We are currently evaluating orthotopic transplantation of aggregates in immune deficient mice (FRG mouse).

Biochemical and biophysical evidence of calcium binding to coiled coil domain of rotavirus NSP4

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Rotavirus (RV) is the most common cause of life-threatening infantile gastroenteritis. RV nonstructural protein 4 (NSP4) is first described viral enterotoxin, and is a virulence factor that plays a role in the pathophysiology of RV-induced diarrhea. NSP4 is secreted from virus-infected cells and binds to non-infected intestinal cells where it can elicit a phospholipase C-dependent signaling cascade that elevates intracellular Ca^{2+} and subsequent chloride secretion. NSP4 is also thought to activate the enteric nervous system. Changes in cytoplasmic Ca^{2+} levels are sensed by a myriad of Ca^{2+} binding proteins that undergo conformational changes upon Ca^{2+} binding. Previous studies found that rotavirus RV NSP4 elevates cytoplasmic Ca^{2+} by viroporin activity and this in turn causes trafficking into vesicular puncta, suggesting that Ca^{2+} binding to NSP4 itself serves as a molecular switch. We have sought to identify and map Ca^{2+} binding sites (CaBS) in the cytoplasmic tail of NSP4. A conserved CaBS (residues E120 and Q123) was identified in crystal structures of the tetrameric NSP4 coiled-coil domain (CCD, aa95-146) but Ca^{2+} is absent in a recent pentameric structure of the CCD. The conservation of these residues suggests this CaBS serves an important role for NSP4 and may influence the oligomeric plasticity of this domain. We expressed the CCD of WT SA11 NSP4 and an E120A/Q123A mutant protein in *E. coli*. By analytical gel filtration, WT protein formed tetramer whereas mutant formed pentamer suggesting loss of CaBS influences the oligomeric state of NSP4. Using radioactive Ca^{2+} overlays and isothermal titration calorimetry, we found that WT NSP4 CCD binds Ca^{2+} with a K_a=500 μM, but the E120A/Q123A mutant lost this Ca^{2+} binding activity. We determined the crystal structures of both WT and the E120A/Q123A mutant and found that while WT forms a tetramer with bound Ca^{2+}, the E120A/Q123A mutant forms a pentamer. The mutant structure lacks Ca^{2+} at the core indicating that Ca^{2+} influences the NSP4 oligomerization state. These studies demonstrate that NSP4 is a functional Ca^{2+} binding protein and will generate insight into how Ca^{2+} regulates the pleiotropic functions of NSP4.

Roles of ERManI in liver disease associated with alpha1-antitrypsin deficiency

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Liver disease associated with alpha1-antitrypsin (A1AT) deficiency is largely due to inefficient clearance of mutant A1AT via endoplasmic reticulum-associated protein degradation (ERAD), a process initiated by ER alpha-1, 2-mannosidase (ERManI). Previously, we discovered that translational suppression of ERManI, which is caused by a single nucleotide polymorphism (SNP) in the 3'UTR of ERManI gene, is associated with early onset of end-stage liver disease associated with A1AT deficiency (Pan *et al.*, Hepatology, 2009). However, the molecular mechanism by which decreased ERManI contributes to the disease is not known. Following our recent findings on the Golgi-localization of ERManI (Pan *et al.*, MBoC, 2011), in this study, we discovered a direct interaction between Golgi-situated ERManI and γ-COP, a subunit of the COPI coat complex responsible for Golgi-to-ER retrograde transport of protein cargo. The direct interaction with γ-COP is required for overexpressed ERManI to accelerate the degradation of mutant A1AT, indicating that a functional partnership exists between ERManI and γ-COP in ERAD. Importantly, the mutant A1AT can associate with the ERManI/γ-COP complex. This association is apparently transient and required for sufficient degradation of A1AT since RNAi-mediated knockdown of γ-COP enhanced the association between ERManI and NHK while diminishing the efficiency of ERAD, implying that physical interaction with γ-COP enables Golgi-situated ERManI to participate in the release of captured ERAD substrates into COPI vesicles for retrograde transport back to the ER. Based on these findings, a model is proposed in which Golgi-situated ERManI brings mutant A1AT molecules into close proximity of COPI vesicles where they can be retrieved back to the ER prior to degradation.

Production of Gamma-Aminobutyric Acid by Intestinal Microbe *Bifidobacterium dentium* and Other Commensal Bacteria

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Recurrent abdominal pain, including but not limited to irritable bowel syndrome (IBS), is a common and costly health care problem. IBS is a condition manifested by abdominal pain of unknown etiology, but is attributed to visceral hypersensitivity. Evidence suggests an interaction between intestinal microbiota, the gut, and the central nervous system, often referred as microbiota-gut-brain axis. γ-aminobutyric acid (GABA) is primary inhibitory neurotransmitter in the body and therefore GABA-mediated neurotransmission regulates many physiological functions, including those in gastrointestinal tract. Here, we explore the ability of intestinal commensal microorganisms to produce GABA and alter the expression of visceral pain pathways via this secreted microbial stimulus.

Members of the intestinal commensal community, including *Lactobacillus* sp., *Bifidobacterium* sp. and *Alistipes* sp., were tested for their ability to secrete GABA using LC-MS method. Two *Bifidobacterium* sp. strains (*B. dentium* ATCC 27678 and *B. angulatum* ATCC 27535) and one *Lactobacillus plantarum* ATCC 14917 strain, when grown on MRS, produced 176.8, 61.4 and 21.4 µg/ml of GABA, respectively. Once MRS was supplemented with 1% w/v L-glutamate, GABA production by *B. dentium* ATCC 27678 increased 6.6 fold and reached a concentration of exceeding 1 mg/ml. Putative *gadB* gene of a predicted *gad* gene cluster has been identified on a *B. dentium* ATCC 27678 chromosome. Our hypothesis is that GadB is responsible for GABA production. The *gadB* gene of the *gad* operon of *B. dentium* ATCC 27867 is, to the best of our knowledge, the first described bifidobacterial glutamate decarboxylase with L-glutamate as a substrate. These findings indicate that neurotransmitter production by the intestinal microbiome deserves exploration as a mechanism of neuromodulation of the gut mucosa.

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Loss of enterocyte derived argininosuccinate lyase results in increased incidence of necrotizing enterocolitis

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Background: Nitric oxide (NO) is a mediator in the pathogenesis of necrotizing enterocolitis (NEC). Argininosuccinate lyase (ASL) is the only enzyme in the body capable of generating arginine, the substrate for NO. Previously, we have shown that ASL is required for the assembly of the NO synthesis complex and, loss of ASL results in decreased NO production. Enterocytes are the principal sites expressing ASL during the neonatal period. Understanding the contribution of cell specific NO production to the development of NEC has been limited by redundancies of the nitric oxide synthase (NOS) isoforms and the temporal and spatial regulation of NO production. Hence, we have generated an enterocyte-specific *Asl* cKO mouse, a novel, and better model to study the role of NO in causation of NEC in a cell-specific manner.

Objective: We hypothesize that the deficiency of enterocyte-derived NO secondary to loss of ASL contributes to the development of NEC.

Design/Methods: Utilizing Cre-Lox technology, we generated an enterocyte-specific knockout of *Asl* (Ent cKO). NEC was established in these mutant and control mice by subjecting premature mouse pups to exclusive formula feed, hypoxia, and hypothermia. The severity of NEC was graded based on histological changes. Characterization of the model was performed by intestinal histomorphometry, immunostaining, nitrosylation studies, cytokine expression and microarray analysis.

Results: Ent cKO showed histomorphological changes including significant reduction in length and increase in breadth of villi. Significant loss of ASL in the enterocytes was demonstrated by RT-PCR, western blot and immunostaining. There was a strong trend towards decreased NO production by enterocytes in the Ent cKO mice. The expression of *eNOS, IL-6,* and *BAX* were significantly elevated in the Ent cKO. The incidence of NEC was significantly higher ($p=0.003$) in the Ent cKO [29/46(63%)] as compared to controls [32/84 (38%)].

Conclusions: Loss of ASL in the enterocytes resulted in increased incidence of NEC. This cell-specific loss of ASL was associated with altered histomorphometry, decreased NO production and, increased expression of cytokines and apoptosis markers. Thus, our studies suggest that enterocyte-derived NO is protective against the development of NEC. Hence, manipulation of enterocyte ASL may be of translational value in prevention or treatment of NEC.

The VP8* of Neonatal Rotavirus Strain G10P[11] Binds to Type II Precursor Glycans

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Naturally occurring bovine-human reassortant rotavirus strains with a P[11] VP4 type appear to exhibit a restrictive tropism for neonates. There is recent evidence for the interaction of the VP8* domain of VP4 of human rotaviruses with non sialylated glycoconjugates including histo blood group antigens (HBGAs). We sought to determine if the bovine rotavirus-derived VP8* of the reassortant neonatal G10P[11] virus interacts with hitherto uncharacterized glycan partners using a glycan array screen comprised of 611 glycans and whether this interaction is significant for infection. VP8* P[11] showed specific binding to glycans with the Galβ1-4GlcNAc motif, that forms the core structure of H type II HBGA. Infectivity of G10P[11] in cell culture was significantly enhanced by the expression of H type II HBGA in CHO cells. The expression of the bovine VP4 was confirmed as critical for the increased infectivity in CHO cells through infectivity assays with laboratory-derived reassortant viruses derived from sialic acid binding simian rotavirus SA114F and bovine G10P[11] rotavirus B223. Neonatal rotavirus infections are unique in comparison to that in older children and often involve unusual strains. The binding to a core glycan unit has not been reported for any rotavirus VP4. Core glycan synthesis is constitutive in most cell types and modification of these glycans is thought to be developmentally regulated. These studies provide the first molecular basis for understanding neonatal rotavirus infections, indicating that glycan modification during neonatal development may mediate the age-restricted infectivity of neonatal viruses. Glycan expression and specificity may be important to consider in settings where neonatal immunizations are being evaluated to improve vaccine efficacy.

GLP-2 receptor is required for the growth of intestinal crypts *ex vivo*

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Glucagon-like peptid-2 (GLP-2) is secreted from endocrine L cells in the gut, and is required for intestinal growth and adaptation. GLP-2 has recently been proved to treat short bowel syndrome and inflammatory bowel disease. GLP-2 receptor (GLP-2R) is expressed in endocrine cells, neurons, and myofibroblasts in the gut; and thus proposed via a pancrine manner to stimulate epithelial cell proliferation. However, GLP-2R cellular action and signaling network are still unknown due to the lack of *Glp2r*-expressing cell lines and cell-specific *Glp2r* knockout mouse models. The objective of this study was to determine if GLP-2R stimulates epithelial cell proliferation through a direct action on intestinal stem cells. First, we revealed that GLP-2R was unexpectedly expressed in putative crypt base columnar stem cells in the mouse gut. Then, we showed that GLP-2 (s.c. injected for one week) stimulated cell proliferation (indicated by BrdU incorporation) in the gut crypts of *Glp2r* widetype (WT) mice, and this action was negated in *Glp2r* knockout (KO) mice. Importantly, GLP-2 increased the number of intestinal crypts in the WT mice, but not in the KO mice. To further address if GLP-2R directly modulates the intestinal stem cell homeostasis, we established a primary culture of intestinal crypts, and finally demonstrated that [1] *Glp2r* was required for the enteroid growth. Budding on the enteroids was decreased from the *Glp2r* KO mouse gut (5.16 ± 2.07 vs 2.39 ± 1.37, respectively, for the WT and KO mice). [2] The enteroids from the *Glp2r* KO gut could barely survive for 3 days after passage, while those from the WT gut grew well. [3] GLP-2 enhanced the enteroid growth from the WT mice, but not from the KO mice. [4] GLP-2 protected enteroids from death (apoptosis) induced by 5-fluorouracil exposure. And [5] GLP-2R-promoted growth of intestinal crypts was associated with enhanced glycolysis. Further analysis of GLP-2R action on the renewal and apoptosis of intestinal stem cells is going on in the laboratory. In conclusion, our data suggest that GLP-2 stimulates cell proliferation probably by a direct action on the intestinal stem cells. Moreover, *Glp2r* is required for the growth of intestinal crypts *ex vivo*.

SOX9 directly activates *IGFBP-4* and suppresses proliferation in *Apc*$^{min/+}$ adenomas and colorectal cancer cells

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BACKGROUND & AIMS: In the normal intestinal epithelium, SOX9 expression is localized in the crypt cells. SOX9 is also expressed in diverse cancers, including colorectal cancer (CRC). Our previous loss-of-function study showed increased proliferation in *Sox9*-deficient crypts, indicating SOX9 suppresses cellular proliferation. We examined role of SOX9 in proliferation of normal intestinal epithelium, *Apc*$^{min/+}$ mouse adenomas, and CRC cells.

METHODS: *Sox9*-deficient *Apc*$^{min/+}$mice were generated to investigate role of SOX9 in tumorigenesis. Crypt epithelial cells isolated from *Sox9*-deficient mice were also used to identify the potential target genes of SOX9.

RESULTS: *Sox9* deficiency in *Apc*$^{min/+}$ mice resulted in increased tumor burden relative to *Apc*$^{min/+}$ control mice. Insulin-like growth factor-binding protein 4 (IGFBP-4), a well documented inhibitor of the IGF/ IGFR axis, was significantly downregulated in *Sox9*-deficient intestinal epithelial cells as well as in adenoma cells of *Sox9*-deficient *Apc*$^{min/+}$ mice. Co-staining experiments revealed IGFBP-4 is colocalized with SOX9 in mouse and human intestinal epithelial cells as well as primary CRC specimens. Reporter assays and chromatin immunoprecipitation (ChIP) demonstrated direct binding of SOX9 to *IGFBP-4* promoter. Overexpression of SOX9 attenuated cellular proliferation, which was restored following treatment with a neutralizing antibody against IGFBP-4, suggesting that SOX9 requires IGFBP-4 to suppress cellular proliferation.

CONCLUSION: SOX9 suppresses cellular proliferation in intestinal epithelium, adenoma cells of *Apc*$^{min/+}$ mice, and CRC cells through direct activation of IGFBP-4.

Loss of TGF- β Adaptor β 2SP Activates Notch Signaling and SOX9 in Esophageal Adenocarcinoma

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TGF-β and Notch signaling pathways play important roles in regulating self-renewal of stem cells and gastrointestinal carcinogenesis. Loss of TGF-β signaling components activates Notch signaling in esophageal adenocarcinoma (EA); but basis for this effect has been unclear. Here we report that loss of TGF- β adapter β2SP (SPNB2) activates Notch signaling and its target SOX9 in primary fibroblasts or EA cells. Expression of the stem cell marker SOX9 was markedly higher in EA tumor tissues than normal tissues, and its higher nuclear staining in tumors correlated with poor survival and lymph node invasion in EA patients. Downregulation of β2SP by lentivirus shRNA increased SOX9 transcription and expression, enhancing nuclear localization for both active Notch1 (ICN1) and SOX9. In contrast, reintroduction into EA cells of β2SP and a dominant-negative mutant of the Notch co-activator mastermind-like (dnMAN) decreased SOX9 promoter activity. Tumorsphere formation and invasive capacity in vitro and tumor growth in vivo were increased in β 2SP-silenced EA cells. Conversely, SOX9 silencing rescued the phenotype of EA cells with loss of β 2SP. Interaction between Smad3 and ICN1 via Smad3 MH1 domain was also observed, with loss of β2SP increasing the binding between these proteins, inducing expression of Notch targets SOX9 and C-MYC, and decreasing expression of TGF- β targets P21(CDKN1A), P27 (CDKN1B) and E-cadherin. Taken together, our findings suggest that loss of β2SP switches TGF- β signaling from tumor suppression to tumor promotion by engaging Notch signaling and activating SOX9.

Key words: TGF-*β*; Notch; β2SP; SOX9; Stem cells; Barrett's esophageal adenocarcinoma.

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Pangenomic analysis of *Lactobacillus reuteri* highlights the evolution of a human-specific ecotype

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Probiotic *Lactobacillus reuteri* is autochthonous to humans and other vertebrate organisms. Previous work has demonstrated host-specificity among strains, and preliminary genome comparisons suggest that the species is approaching a closed pangenome. We expanded the pangenome analysis to include ten *L. reuteri* strains, incorporating three newly sequenced organisms derived from murine and porcine hosts. The 10 genomes range in size from 1.9 – 2.3 Mbp, average 2,028 genes per genome, and contribute 3,961 genes to the pangenome with 1,175 core genes. The average nucleotide identity (ANI) shared between all strains fell within the >95% ANI standard for species designation, with the genomes sharing greater than 70% of their total gene content. The human-derived strains, however, clustered at >98% ANI and shared >90% of gene content, suggesting that they are highly conserved at the nucleotide level and are more ecologically constrained than those derived from other hosts. Features that distinguish the human-derived lineage were identified using the sequence clustering program, CD-hit. While our analysis confirms *L. reuteri* is nearing a closed pangenome model, it also identified 542 genes present in human-adapted strains and absent in all others. Only 17 of the 542 genes were found common to all human strains, and include genes involved in cell signaling, secretion, cell envelope biogenesis and amino acid metabolism, or hypothetical proteins. Current efforts are focused on determining whether the human *L. reuteri* strains have diverged as a result of periodic selection or genetic drift, and how their co-evolution with the human host impacts human health

P2Y2 Purinergic Receptor Signaling Plays a Key Role in Diet-Induced Obesity in Mice

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Background: Obesity epidemic is alarmingly widespread among adults and children increasing the risk for metabolic syndrome and Type 2 diabetes. Adipose tissue hypertrophy and excessive release of free fatty acids, cytokines and chemokines initiate a low-grade systemic inflammation central to the pathophysiology associated with diet-induced obesity. P2 purinergic receptor activation has the potential to influence acute and chronic inflammatory responses but their influence on low-grade chronic inflammation orchestrated in metabolic tissues and obesity remains unknown. Therefore, the purpose of this study was to test the **hypothesis** that P2Y2 purinergic receptor activation plays a critical role in the induction of diet-induced obesity.

Methods: Wild type (WT) and P2Y2 knockout (KO) mice (5 weeks) were fed a high fat/high sucrose diet (42% calories each from fat and sucrose; HF), or a standard chow (SC) diet for 10 weeks. Mice were subjected to Glucose tolerance tests (GTT; 6 weeks), Dual-emission X-ray Absorptiometry (DEXA; 8 weeks) to determine percentage body fat and body mass. Total RNA isolated from adipose tissues harvested at 10 weeks were analyzed by qRT-PCR for inflammatory cytokines (TNFα), chemokines (MCP-1, MIP-2), and macrophage infiltration (F4/80, CD206, CD11c). Total proteins isolated from livers were analyzed by Western blotting for key mediators of ER stress (c-Jun, c-Fos), lipid synthesis (PPAR□, phospho-ACC, ACC), and lipid droplet formation (ADFP). Hepatic triglyceride (TG) accumulation was assessed by thin layer chromatography (TLC) and CD36 mRNA expression (fatty acid uptake) was analyzed by qRT-PCR.

Results: WT on HF diet exhibited hallmarks of diet-induced obesity, whereas P2Y2 KO on HF diet had attenuated increase in total body weight (0.8), fat mass (0.7), liver and adipose weight/body weight ratios (0.8, 0.6) and increased lean mass (1.1), as compared to WT on HF diet (1.0). Glucose tolerance curves were comparable between P2Y2 HF and SC controls, whereas WT on HF diet exhibited impaired glucose tolerance. Reflecting adipose tissue inflammation and macrophage infiltration, WT mice on HF diet had elevated TNFα, MCP-1, MIP-2, F4/80, CD206, and CD11c mRNA expression in epididymal fat as compared to SO controls, whereas, P2Y2 KO mice exhibited an attenuated increase (0.4, 0.5, 0.5, 0.4, 0.6, 0.4 respectively) as compared to WT (1.0). In the liver, HF diet-induced TG accumulation (0.6), induction of CD36 mRNA (0.6), c-Jun (0.5), c-Fos (0.3), PPAR□ (0.3), phospho-ACC(0.8) and ADFP (0.7) proteins were all attenuated in P2Y2 on HF diet as compared to WT on HF diet (1.0)

Conclusions: High fat diet-induced changes in body composition, glucose tolerance, adipose tissue inflammation and hepatic steatosis were all attenuated in P2Y2 KO on HF diet, suggesting a role for P2Y2 purinergic receptor signaling in the pathogenesis of low-grade metabolic inflammation and diet-induced obesity.

A multi-omics approach to understanding the gut-microbial ecology and mechanisms of recurrent *Clostridium difficile* infection

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Background: The number of hospitalized patients with a *Clostridium difficile* infection (CDI) discharge diagnosis has increased dramatically due in part to the emergence of the epidemic strain BI/NAP1/027. Vancomycin and Metronidazole have long been preferred treatment options in CDI, but neither is fully effective as evidenced by up to 35% clinical recurrence and significant fatality rates. Medical treatment and hospitalization associated with CDI burdens the U.S. health care system with up to $3.8 billion in excess costs each year, with much of the expense attributable to disease recurrence. Reduction in recurrence, therefore, is a priority clinical need and major market opportunity.

Results: Despite a known correlation between antimicrobial disruption of protective gut microflora and the development of symptoms in infected individuals, there is still a major gap in our understanding of why certain patients are susceptible to disease recurrence by *C. difficile*. To address this question, we profiled stool microbial community composition and the metabolome from 55 patients with and without CDI, and found that patients who subsequently develop recurrent disease have significant shifts in microbial populations and biochemical composition. Patients with recurrent disease were identified with decreased abundances of taxa belonging to the families Bacteroidaceae and Prevotellaceae, and increased abundances of taxa belonging to the families Peptostreptococcaceae (including *C. difficile)* and Clostridiaceae. Notably, in patients with recurrent CDI, the Pepostreptococaceae and Clostridiaceae accounted for ≥ 40% of all 16S rRNA sequence recovered. Concurrent with changes in microbial community structure, significantly elevated levels of γ-aminobutyric acid (GABA) and precursors of GABA synthesis were also detected. GABA is synthesized by gut bacteria and is a potent amino acid neurotransmitter that has various physiologic effects throughout the body. GABA and its agonists, induce neural modulation as a consequence of their interaction with specific binding sites for each of these classes of neuroactive substances on the GABA receptor complex of postsynaptic neurons. Using agonists and antagonists of the GABA transaminase and receptor respectively, we demonstrate that GABAergic signals directly regulate *C. difficile* neurotoxin activity and cytokine secretion in experimental CDI. These processes may contribute to increased CDI severity and recurrence.

Conclusion: We demonstrate that integration of global metabolomics and metagenomics into ecosystem network models provides an organizational and analytical framework for the discovery of new diagnosis and treatment in CDI. The significance of this multi-omics approach is our identification of a novel microbe-neuroimmune signaling mechanism in patients with recurrent disease.

Comparative evaluation of bioimpedance and anthropometric measurements as predictors of HCV-related fibrosis and inflammation risk

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Background: Relative adiposity, particularly BMI and more recently waist and hip circumference, is a well-recognized risk factor for NAFLD-related liver disease. However, other than BMI, its role in advanced HCV-related liver disease is not well- established. Bioelectric impedance analysis (BIA)-based measurements of adiposity offer advantages over widely used anthropometric calculations or skinfold evaluation including less potential for inter-observer variability or bias.

Methods: HCV+ male veterans with confirmed viremia, mono-infection, and without ascites or decompensated disease were prospectively recruited at a VA medical center. In addition to anthropometric measurements (waist, hips, height), we obtained direct 8-segment multi-frequency bioelectric impedance analysis (BIA) measurements of body composition with a Biospace Inbody scale (98% DXA correlation). The FibroSURE test was used to assess hepatic pathology. ANOVA was used to compare mean BIA and anthropometric measurements with advanced compared to mild fibrosis (F3/F4-F4 vs. F0-F3), and with advanced compared to mild inflammatory activity (A2-A3 vs. A0-A1/A2).

Results: We recruited 198 HCV+ male veterans. Average age was 56; most were African-American or non-Hispanic White (52% and 43%, respectively). BIA-based % body fatness (%BF) and BMI were significantly associated with advanced fibrosis and inflammation in both univariate and multivariate analyses. However, waist and hip circumference were not significantly associated with fibrosis and both were only marginally significant for inflammation. Similarity in total body water and in lean body mass in advanced compared to mild disease sub-groups supports that observed associations with %BF and BMI are primarily associated with differences in relative fatness and not in water retention or muscular atrophy. The correlation between BMI and %BF was significant and moderately strong (Kendal tau-b=64%), with similar results for both BMI and %BF with waist and hip circumference, respectively.

Conclusion: BIA measured %BF and BMI both predict advanced fibrosis and inflammation in HCV+ male veterans. Given correlation among measures, additional research is needed to clarify their respective joint and individual contributions in risk of advanced liver disease.

Whole Transcriptome RNA Sequencing: From Library Preparation to Sequencing

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RNA Sequencing, or whole transcriptome sequencing, converts total RNA to adapter-ligated cDNA, allowing researchers to gain valuable information about RNA at the nucleotide level. RNA-Seq can be used to study gene structure and expression, and also provides information about differentially expressed genes, alternatively spliced transcripts, non-coding RNA, gene fusions, post-transcriptional editing, and single nucleotide variations (SNV). When designing an RNA sequencing project, decisions about library preparation and sequencing length, depth and coverage must be discussed. Protocols for creating libraries from RNA must begin with removal of abundant RNA and include methods that either isolate RNA (ie. pull out RNA using poly(A) selection and random priming) or deplete highly abundant transcripts from total RNA (ie. remove rRNA or normalization of transcript concentrations). A poly(A) selection method separates coding and noncoding RNA using the 3' polyadenylated tail of mature, eukaryotic RNA whereas depletion of rRNA from total RNA will leave behind polyadenylated and non-polyadenylated messages, including microRNA and other noncoding RNA. To be presented (see poster) is a description of library preparation kits commonly used at the Genomic and RNA Profiling Core Facility (GARP), along with advantages and disadvantages of each preparation type.

Texas Medical Center Digestive Diseases Center
4th Annual *"Frontiers in Digestive Diseases"*: Stem Cells in GI Health & Disease

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