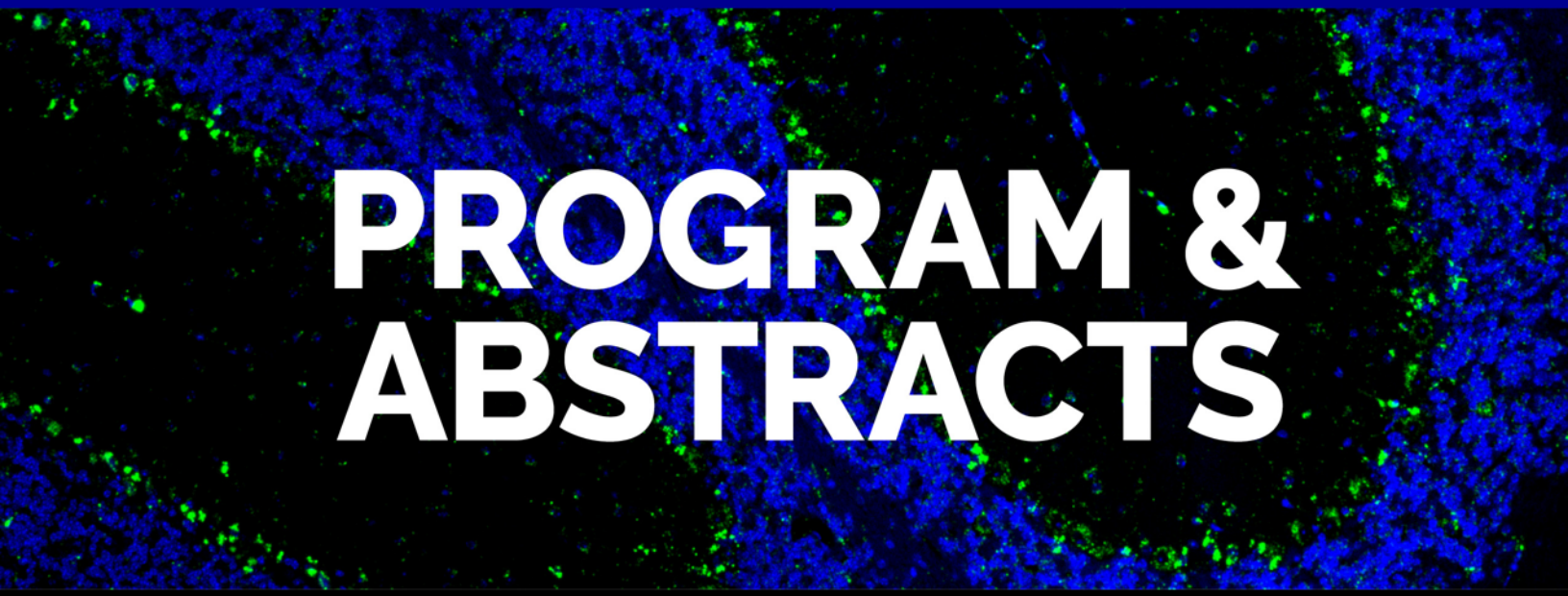
RNA Therapeutics Institute

UMASS MEDICAL SCHOOL

RNA Therapeutics: FROM CONCEPT TO CLINIC



PROGRAM & ABSTRACTS

JUNE 23-25, 2021

Virtual Symposium | UMass Medical School

www.umassmed.edu/RNATx

#RNATx2021

@UMassMedRTI

Dear Friends and Colleagues,

Welcome to the RNA Therapeutics Institute's third annual symposium, *RNA Therapeutics: From Concept to Clinic, held virtually* from the University of Massachusetts Medical School in Worcester, MA.

What a year it has been for all of us, but especially for RNA! Throughout the latter half of the 20th century, RNA was thought mainly to intermediate between DNA and protein. Over the last three decades, that view has given way to the understanding that RNA plays rich and complex roles previously ascribed only to proteins. From ncRNAs to RNAi, from CRISPR to circular RNAs, we have witnessed astonishing discoveries and equally amazing translation of these breakthroughs to therapeutics and now mRNA vaccines. We are delighted to feature speakers whose fascination with RNA biology has inspired them to explore the possibilities of harnessing RNA to create therapeutics.

This exceptional virtual experience will keep with our tradition of providing high-quality scientific sessions, an exhibit hall that will offer one-on-one opportunities to meet with our business partners, and social events to allow attendees to mingle, catch up with old friends, and forge new collaborations. It also promises two dynamic and inspiring keynote addresses, as well as a fun party that we know you will enjoy - all on a digital platform.

To stimulate dialogue, we've included time in the program for discussion during our welcome reception on Wednesday afternoon, after each talk, as well as during the poster sessions, coffee networking breaks, and our RNATx party on Thursday afternoon. The scientific sessions and posters, drawn from our international call for abstracts, offer a taste of the newest work in the field and provide new opportunities to forge collaborations with current and future thought-leaders in the RNA community.

We would like to thank everyone who has worked diligently to create this symposium, particularly the talented staff at the RTI. We especially thank our speakers for their enthusiastic participation. Finally, we are most grateful to our sponsors and exhibitors for their generous support.

Sincerely,

Phillip D. Zamore, PhD
Angela Messmer-Blust, PhD
RNA Therapeutics Institute
UMass Medical School

Symposium Co-Organizers

2021 RNA Therapeutics: From Concept to Clinic

Contents:

Cover Image: *This image of the mouse cerebellum in a murine model of ALS (C9ORF BAC) shows selective uptake of antisense oligonucleotides (ASOs; green) into purkinji neurons at the interface of grey and white matter. Nuclei are shown in blue. Image by Dr. Michael Moazami, Jonathan Watts Lab, RTI, UMass Medical School.*

Virtual Venue

VENUE: RNATx2021.vfairs.com

POSTERS & NETWORKING: https://spatial.chat/s/rnatx21
Tutorial & Info on Spatial Chat

REGISTRATION:
You will receive an email on how to activate your account for the virtual platform. Please email rnatx2021support@getvfairs.io if you have not received or are having trouble logging in. We recommend logging in a day prior to ensure connectivity.

TECHNICAL SUPPORT:
For technical support during the symposium rnatx2021support@getvfairs.io or call 508-380-6077.

CAREER WORKSHOP, EXHIBITORS, POSTER & SCIENTIFIC SESSIONS INFORMATION

Session	URL
Careers Under the Microscope Workshop (pre-registration required) Wednesday, June 23rd	RNATx2021.vfairs.com Enter Auditorium (if registered)
Keynote Sessions: Michael Rosbash and Kathy High Wednesday, June 23rd and Friday June 25th	RNATx2021.vfairs.com Enter Auditorium
Welcome Reception, RNATx & Sirnaomics Lobbies, sponsored by Sirnaomics Wednesday, June 23rd	https://spatial.chat/s/rnatx21
Scientific Sessions Wednesday - Friday, June 23-25th	RNATx2021.vfairs.com Enter Auditorium
RNATx 2021 Virtual Party sponsored by ChemGenes Thursday, June 24th	GO REMOTE LINK *(TURN OFF YOUR VPN)*
Poster Sessions sponsored by Agilent Wednesday - Friday, June 23-25th Spatial chat poster session info	https://spatial.chat/s/rnatx21
Exhibitors Wednesday - Friday, June 23-25th	RNATx2021.vfairs.com Enter Exhibit Hall
Coffee & Networking Breaks, sponsored by TriLINK Wednesday - Friday, June 23-25th	https://spatial.chat/s/rnatx21

2021 RNATx VIRTUAL PARTY
Please join the RNATx party to play an interactive Game Show sponsored by ChemGenes. Teams will play against each other in mini-games of trivia, puzzles, Pictionary, and more! Who will be the ultimate RNATx Game Show Champion?

EXHIBITORS
We are very happy to welcome our exhibitors who kindly support this meeting. Please take the time to visit them during the networking breaks and poster sessions.

POSTER SESSIONS

Poster Numbers	Session Date/Time	VOTE for the best poster	Note
Posters: 1 - 42 & 46	**Wednesday, June 23rd** *3.30 - 4.15pm*	**Session 1**	* ***These presentation times are for a Q&A session via Spatial Chat.***
Posters: 43 - 86	**Thursday, June 24th** *1.15 - 2.00pm*	**Session 2**	* ***The poster pdfs & 2 minute videos that you uploaded will stay up for the entirety of the meeting.***
Posters: 87 - 125	**Friday, June 25th** *1.15 - 2.00pm*	**Session 3**	

**NOTE: All posters will stay up for the entire symposium. The presentation times refer to the Q&A sessions in Spatial Chat only.*

SPEAKERS

All oral presenters are reminded to be in their respective sessions **no later than 30 minutes before the start of the session** in order to meet with the session chair and to finalize their setup of presentation visuals. **You will receive ALL links to your email via Zoom AND in a calendar invite.**

SOCIAL MEDIA

Official symposium hashtag: #RNATx2021
Symposium website: **www.RNATx2021.vfairs.com**
Twitter Account: **@UMassMedRTI**

The organizers encourage attendees to tweet about the remarkable science that they experience at the meeting. Please respect the following rules in your posts and tweets:

1. Be polite and respectful of others
2. Be respectful of those presenters that ask attendees to refrain from tweeting content from talks and posters.
3. If you're enjoying the conference, share it with your social media network!
4. Never post/tweet about unpublished data, and look for these icons on the speakers' slides:

Ok to screenshot, photograph, and/or share:

Not ok to screenshot, photograph, and/or share:

CONFERENCE HARASSMENT POLICY

2021 RNATx is dedicated to providing a harassment-free conference experience for everyone, regardless of gender, gender identity & expression, sexual orientation, disability, physical appearance, body size, race, age, or religion. We do not tolerate harassment of conference participants in any form. Conference participants violating these rules may be expelled from the conference (without a refund) at the discretion of the conference organizers. Our anti-harassment policy and how to report any violations can be found at: **https://bit.ly/30AMkQz**

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ORAL PROGRAM

Wednesday, June 23, 2021 (times listed in ET)

1.00 – 2.30pm	**Careers Under the Microscope Workshop** (pre-registration required; separate agenda)
2.40 – 2.45pm	Welcome and Keynote introduction, **Phillip D. Zamore,** *HHMI, RNA Therapeutics Institute, UMass Medical School*
2.45 – 3.30pm	**Keynote: Michael Rosbash,** *Brandeis University* ***More than 50 years of RNA: Plus ça change...***
3.30 – 4.15pm	**Virtual Poster Session and Exhibit Hall- VOTE for the best poster** *Poster Session Sponsored by **Agilent Technologies***
4.15 – 5.00pm	**RNATx Kickoff, Virtual Happy Hour via Spatial Chat Lobby,** *Sponsored by **Sirnaomics***

Thursday, June 24, 2021

8.55 – 9.25am	**Virtual Coffee & Networking, Spatial Chat Lobby**
Session I: Editing *Session Chair: Xiaolong Dong, RTI, UMass Medical School*	
9.25 – 9.30am	Welcome and Introduction, Phillip D. Zamore
9.30 – 9.55am	**Genome editing and single cell approaches to study early human development** **Kathy Niakan,** *Crick Institute*
10.00 – 10.25am	**In vivo gene editing in muscles and muscle stem cells** **Amy Wagers,** *Harvard University*
10.30 – 10.55am	**Development of gene-drives and a method to block them in A. gambiae** **Chrysanthi Taxiarchi,** *Imperial College of London*
11.00 – 11.25am	**Site-directed RNA editing: current strategies and future directions** **Joshua Rosenthal,** *Marine Biological Laboratory*
ON DEMAND	**Short Talks from abstracts – Vote for your favorite talk within the conference platform!**
11:30 – 12:00pm	**Virtual Networking & Coffee Break, Spatial Chat Lobby** *Sponsored by **TriLink BioTechnologies***
Session II: RNAs in Immunity *Session Chair: Kexin Zhang, RTI, UMass Medical School*	
12.15 – 12.40pm	**Innate Immune nucleic acid sensing pathways** **Kate Fitzgerald,** *UMass Medical School*
12.45 – 1.10pm	**N6-Methyladenosine in Immunity** **Ye Grace Chen,** *Yale University*
ON DEMAND	**Short Talks from abstracts – Vote for your favorite talk within the conference platform!**
1.15 – 2.00pm	**Virtual Poster Session and Exhibit Hall - VOTE for the best poster** *Poster Session Sponsored by **Agilent Technologies***
Session III: Regulatory RNAs *Session Chair: Amena Arif, RTI, UMass Medical School*	
2.00 – 2.25pm	**RNA Regulation in the Germline** **Ruth Lehmann,** *Whitehead Institute*
2.30 – 2.55pm	**Deciphering the role of the Argonaute proteins in mammalian gametogenesis** **Paula Cohen,** *Cornell University*
3.00 – 3.25pm	**piRNA guided heterochromatin formation** **Julius Brennecke**, *IMBA*
3.30 – 3.55pm	**Exploiting microRNAs to modulate cell senescence therapeutically** **Myriam Gorospe**, *National Institutes of Health*
4.00 – 5.00pm	**RNATx Mixer & Party, Sponsored by ChemGenes** (in GoREMOTE)

Friday, June 25, 2021

9.00 – 9.30am	Virtual Coffee & Networking, **Spatial Chat Lobby**
9.30 – 9.45am	***Accelerating RNA Therapeutics R&D with a Modern Informatics Solution*** **LIVE Short Talk: Prem Mohanty,** *Sponsored by Benchling*
	Session IV: Oligonucleotide Design & Delivery *Session Chair: Samantha Sarli, RTI, UMass Medical School*
10.00 – 10.25am	**Splice-switching antisense oligonucleotides for the treatment of disease** **Michelle Hastings**, *Rosalind Franklin*
10.30 – 10.55am	**Rapid synthesis and delivery of antisense oligonucleotides enabled by machine learning** **Bradley Pentelute**, *Massachusetts Institute of Technology*
11.00 – 11.25am	**Oligonucleotide Therapeutics to Treat Familial and Sporadic ALS** **Jonathan Watts,** *RTI, UMass Medical School*
11.30 – 11.45am	**Personalized splice-switching ASO therapy for a child with progressive blindness and insensitivity to pain** LIVE Short Talk: **Boxun Zhao,** *Boston Children's Hospital*
ON DEMAND	**Short Talks from abstracts – Vote for your favorite talk!**
11:45 – 12:15pm	**Virtual Networking & Coffee Break, Spatial Chat Lobby** *Sponsored by* **TriLink Biotechnologies**
	Session V: RNA Mechanisms & Therapeutics in the Era of COVID-19 *Session Chair: Eraj Khokhar, RTI, UMass Medical School*
12.15 – 12.40pm	**mRNA as Medicine** **Melissa Moore**, *Moderna Therapeutics*
12.45 – 12.55pm	**RNA-communication between viruses and their hosts** LIVE Short Talk: **Omer Ziv,** *University of Cambridge*
1.00 – 1.10pm	**Live-cell single-molecule investigation of localization and translation efficiency of m6A-modified mRNA** LIVE Short Talk: **Andreas Schmidt**, *University of Michigan*
ON DEMAND	**Short Talks from abstracts – Vote for your favorite talk within the conference platform!** **Voting for short talks & posters ONLY OPEN until 3pm ET TODAY**
1.15 – 2.00pm	**Virtual Poster Session 3 and Exhibit Hall - VOTE for the best poster** *Poster Session Sponsored by* ***Agilent Technologies***
	Session VI: Translating Discoveries *Session Chair: Shun-Qing (Simon) Liang, RTI, UMass Medical School*
2.00 – 2.25pm	**Engineering Antibody Oligonucleotide Conjugates (AOCs): Taking Receptor-Mediated Uptake One Step Further** **Art Levin,** *Avidity Biosciences*
2.30 – 2.45pm	***Ribosome inhibition by C9ORF72-ALS/FTD-associated poly-PR and poly-GR proteins revealed by cryo-EM*** **LIVE Short Talk: Anna Loveland,** *RTI, UMass Medical School*
2.45 – 3.10pm	**ASOs as individualized medicine: updates from the field** **Timothy Yu,** *Boston Children's Hospital, Harvard Medical School*
3.15 – 3.30pm	**Virtual Networking & Coffee Break, Spatial Chat Lobby** *Sponsored by* **TriLink Biotechnologies**
	Keynote Introduction: **Guanping Gao**, *Horae Gene Therapy Center, UMass Medical School*
3.30 – 4.15pm	**Keynote: Katherine High, *AskBio*** ***DNA Therapeutics: What have we learned from gene therapy drug development in the past decade?***
4.15 – 5.00pm	**The Road to the Clinic Panel Discussion** **Aimee Jackson**, *Atalanta Therapeutics* **Patrick Baumhof**, *CureVac* **Melissa Moore**, *Moderna Therapeutics* **T. Scott Johnson**, *Comanche Biopharma* **Dmitry Samarsky**, *Sirnaomics* Moderator: **Julia Alterman**, *RTI, UMass Medical School*
5.00pm	**Closing Remarks, Awards, and Thank You!**

ON-DEMAND SHORT TALK ABSTRACTS

CRISPR ribonucleoprotein - enhanced human adipocyte “browning” as cell therapy for metabolic disease

Emmanouela Tsagkaraki, Sarah M. Nicoloro, Tiffany DeSouza, Javier Solivan-Rivera, Anand Desai, Yuefei Shen, Mark Kelly, Adilson Guilherme, Felipe Henriques, Raed Ibraheim, Nadia Amrani, Kevin Luk, Stacy Maitland, Randall H. Friedline, Lauren Tauer, Xiaodi Hu, Jason K. Kim, Scot A. Wolfe, Erik J. Sontheimer, Silvia Corvera, and Michael P. Czech

University of Massachusetts Medical School, Worcester, MA, USA & University of Crete Medical School, Heraklion, Greece

Type 2 diabetes, a disruption of glucose and lipid homeostasis, is a major global health concern without a definitive treatment. Distinct adipose tissue depots differentially regulate systemic glucose and lipid metabolism. White adipose tissue (WAT) is abundant in humans and is mainly lipogenic whereas brown adipose tissue (BAT) is associated with an insulin-sensitive phenotype likely achieved through secreting beneficial factors and enhancing thermogenesis. Interventions that transiently cause “browning” of WAT to produce “beige” adipocytes enhance glucose metabolism, and transplantation of BAT or beige adipocytes in mice significantly improves glucose homeostasis. We aim to permanently convert white adipocytes into “beige” adipocytes by genome-editing with sgRNA-Cas9 ribonucleoprotein complexes (RNPs) under conditions that avoid uncontrolled integration of DNA, immune responses and off-target effects. Based on clustered regularly interspaced short palindromic repeats (CRISPR) gene editing, we developed a method that delivers Cas9 protein/sgRNA complexes ex vivo to strongly enhance a brown-like phenotype with virtually 100% efficiency in mouse and human adipocytes. This was achieved by targeting the thermogenesis suppressor gene NRIP1 in preadipocyte progenitors expanded from human and mouse subcutaneous adipose samples prior to their differentiation. Using our optimized ribonucleoprotein delivery in these cells, we targeted different genomic loci spanning the coding region of mouse and human NRIP1 gene and identified the precise target sequence that results in NRIP1 protein depletion and maximum expression of beneficial secreted and thermogenic factors including UCP1 (30 to 100 fold). We also confirmed that residual Cas9 protein and sgRNA were rapidly degraded. Implantation of the murine CRISPR-enhanced “beige” adipocytes into mice with diet-induced obesity ameliorated glucose intolerance and adiposity, prevented accumulation of liver triglycerides and decreased liver inflammation and increased energy expenditure. With similar procedures, implantation of CRISPR-enhanced human brown-like adipocytes into “humanized”, mice on high-fat diet enhanced glucose tolerance compared to mice implanted with unmodified adipocytes. These findings show efficacy of CRISPR-based genetic modification of human and mouse adipocytes to improve metabolic homeostasis in preclinical models and reveal a strategy for advancement of cellular therapies in metabolic disease.

Poly(I:C) Delivery Strategies to Selectively Activate TLR3 and MDA-5 Pathways for Immunotherapy

***Apoorv Shanker**#, Imane Bouzit#, Sandeep T. Koshy^, Janelle Velez^, Stephanie Schwartz^, and Paula T. Hammond#*

#Massachusetts Institute of Technology, Cambridge, MA, USA; ^Novartis Institutes for BioMedical Research, Cambridge, MA, USA

Among the various toll-like receptor (TLR) agonists, polyriboinosinic acid:polyribocytidylic acid (poly(I:C)), a synthetic mimic of viral dsRNA, has been shown to activate and boost antigen cross-presentation by dendritic cell (DCs), promote NK cell cytotoxicity, and effect direct tumor-killing to generate tumor-associated antigens for DC presentation. Poly(I:C) is recognized by the endosomal receptor TLR3 and the cytosolic receptors RIG-I and MDA-5. It activates the transcription factors NF-κB and IRF leading to the production of pro-inflammatory cytokines (via TLR3) and type-I interferons (via MDA-5), respectively. Despite its potent immunogenic properties, safety concerns over inadvertent immune over-stimulation and short half-life in vivo have limited the scope of poly(I:C). Several strategies including liposomal and polymeric encapsulation, polycationic complexes or polyplexes, and surface-assembly on nanoparticles (NPs) or microparticles (MPs) have been explored to overcome these issues.

Here, we present a comparative study of poly(I:C) polyplexes and layer-by-layer (LbL)-assembled NPs/MPs carrying poly(I:C) to selectively activate the TLR3 and MDA-5 receptors. Poly(I:C) delivery platforms were fabricated using three classes of polycations: polypeptides, polysaccharides, and synthetic polymers. Dynamic light scattering, zeta potential measurements, gel electrophoresis, fluorophore exclusion titration assay, circular dichroism, and isothermal titration calorimetry were employed to characterize the polyplexes and assess the structural integrity of poly(I:C) complexed with different polycations. LbL microparticles were built on a negatively-charged core using the polycations to electrostatically assemble poly(I:C) on the particle surface. Using the HEK-Blue mTLR3 reporter cell line, TLR3 activation by the polyplexes was found to be roughly inversely correlated with the poly(I:C) binding strength of the polycations. While poly(I:C)-loaded LbL particles were as effective as free poly(I:C) in activating TLR3 when in direct contact with the cells, significant loss of bioactivity was observed when the cells and the particles were separated using a trans-well. This was likely due to enhanced cellular uptake of the LbL particles or degradation of poly(I:C) released from the LbL particles in the latter case. The selectivity of polyplexes and LbL particles toward TLR3 and MDA-5 receptors was assessed in A549 dual cell line. Activation of the two pathways was further correlated with cellular uptake and trafficking of the polyplexes and LbL particles. We elucidate the role of the delivery mechanism and polycationic carriers in stabilizing poly(I:C) against serum nucleases, reducing cellular toxicity and selectivity toward endosomal or cytosolic receptors. The presented biophysical studies provide critical insight into the design of poly(I:C) delivery platforms toward more potent adjuvants.

miR-122-based Therapies Select for Three Distinct Resistance Mechanisms Based on Alterations in RNA Structure

Jasmin Chahal, Luca F. R. Gebert, Ian J. MacRae, and **Selena M. Sagan**

McGill University, Montreal, Quebec, Canada

Hepatitis C virus (HCV) is a positive-sense RNA virus that interacts with a liver-specific microRNA, called miR-122. miR-122 binds to two sites in the 5' untranslated region (UTR) of the viral genome and promotes HCV RNA accumulation. This interaction is important for viral RNA accumulation in cell culture, and miR-122 inhibitors have been shown to be effective at reducing viral titers in chronic HCV-infected patients. Herein, we analyzed resistance-associated variants that were isolated in cell culture or from patients who underwent miR-122 inhibitor-based therapy and discovered three distinct resistance mechanisms all based on changes to the structure of the viral RNA. Specifically, resistance-associated variants promoted riboswitch activity, genome stability, or positive-strand viral RNA synthesis, all in the absence of miR-122. Taken together, these findings provide insight into the mechanism(s) of miR-122-mediated viral RNA accumulation and provide novel mechanisms of antiviral resistance mediated by changes in RNA structure.

Enhancing chemotherapy response through augmented synthetic lethality by co-targeting nucleotide excision repair and cell-cycle checkpoints

Yi Wen Kong, Erik C Dreaden, Sandra Morandell, Wen Zhou, Sanjeev S Dhara, Ganapathy Sriram, Fred C Lam, Jesse C Patterson, Mohiuddin Quadir, Anh Dinh, Ömer H. Yilmaz, Stephen J Lippard, H. Christian Reinhardt, Michael T Hemann, Paula T Hammond, and Michael B Yaffe

Massachusetts Institute of Technology, Cambridge, MA, USA

Targeting synthetic lethality holds great promise for the treatment of human cancers. We previously demonstrated a synthetic interaction between the cell cycle checkpoint kinase MK2 and the tumor suppressor p53, where loss of MK2 promotes sensitivity to DNA-damaging chemotherapy only in the context of a defective p53 pathway. Here we describe the concept of “augmented synthetic lethality” (ASL), where depletion of a third gene product enhances a previously established synthetic lethal combination. We show that loss of the nucleotide excision repair enzyme XPA markedly augments the extant synthetic lethality between MK2 and p53 to significantly improve tumor killing by cisplatin chemotherapy. Simultaneous delivery of siRNA-peptide nanoplexes targeting both MK2 and XPA to established p53-deficient tumors in a highly aggressive, immunocompetent mouse model of lung adenocarcinoma improved long-term survival and the anti-tumor response to cisplatin in vivo beyond those seen with the synthetic lethal p53 mutant/MK2 combination alone. Together, these findings establish the simultaneous co-targeting of DNA damage-induced cell cycle checkpoints and DNA repair pathways in vivo using RNAi nanocarriers and motivate the further exploration of augmented synthetic lethality as a generalized strategy to improve the response to frontline chemotherapy.

Expression-dependent RNA Transfer and its role in Complementation of a Human Disease Phenotype in a 2D co-culture model

Sandipan Dasgupta and Jeffrey E. Gerst

Weizmann Institute of Science, Rehovot, Israel

Intercellular RNA transfer is thought to play an important role in cellular communication in mammalian cells, whereby the transfer of both small- and micro-RNAs via extracellular vesicles (e.g. exosomes) between cells has been reported. In contrast, our lab previously demonstrated the contact-dependent transfer of full-length mRNAs through long, thin cytoplasmic connections called membrane nanotubes. However, information regarding intercellular transfer at the genome-wide level lacking. In this study, we characterized intercellular RNA transfer by unbiased and quantitative identification of the complete range of transferred mRNAs (i.e. the mRNA transferome) in one population of mammalian cells following co-culture with another population. By employing a simple 2D co-culture model of human and murine cells, we provide evidence of a low level of expression-dependent mRNA transfer across the entire transcriptome. Furthermore, we demonstrate that RNA transfer from a wild-type cell line can complement the phenotype of a mutant cell line. In this case, the peroxisome mutant phenotype of a patient-derived cell line underwent rescue when co-cultured with a wild-type cell line. Demonstration of this phenomenon expands our knowledge of the scope, regulation, and physiological impact of mRNA transfer. It also provides an intriguing example of a potential mechanism for the regulation of organelle biogenesis by intercellular communication.

Gapped Promoter Constructs Allow High Salt Transcription by T7 RNA Polymerase, Dramatically Reducing Primer Extension in High Yield Batch Syntheses

Kithmie MalagodaPathiranage, Elvan Cavaç, and Craig T. Martin

Department of Chemistry, University of Massachusetts, Amherst, MA USA

A key requirement in RNA therapeutics, and RNA research more broadly, is production of (only) the designed target RNA, at a variety of scales. T7 RNA polymerase is widely used to synthesize RNAs of any length, from mRNAs, to sgRNA, to lncRNAs, and siRNAs. Unfortunately, it is common that the final in vitro transcription reaction is contaminated by longer undesired products, from n+1 to much longer extensions that are difficult to fully remove post-synthesis, particularly for long RNAs. These products are derived primarily through cis-primed extension of the expected runoff RNA and are exacerbated by high yield conditions, where RNA re-binding to the polymerase competes with promoter binding. We show that increasing salt concentrations in the reaction can reduce or eliminate this re-binding, improving purity, but also reduces the promoter binding that leads to transcription, reducing yield. To counter this decrease, we show that promoter DNA constructs that are locally single stranded, mismatched, or nicked in the initially melted region of the promoter yield salt-resistant transcription. This derives from the nature of promoter binding by T7 RNA polymerase. We and others have shown that some of the binding energy from upstream duplex interactions is used to drive promoter melting - hence removal/reduction of the melting requirement strengthens promoter binding, even at high salt (we have previously shown that elongation is intrinsically salt resistant). To allow this approach to be applied in the synthesis of RNAs of any length, we show that the enzymatic introduction of a site-directed “gap” in PCR generated DNA templates is both facile and effective, similarly yielding salt resistant transcription, with very substantial increases in both purity and yield of the synthesized RNA.

Global CRISPR Patent Litigation - An Overview

James Velema

[*]Sponsored by Lathrop GPM, Boston, MA, USA

CRISPR editing technologies have enabled countless scientists to readily manipulate genomes, from basic research to *in vivo* gene therapy. However, underneath this Nobel prize winning technology, a patent war wages on. The battle between Berkley and the Broad Institute continues, with not one, but two interference proceedings at the USPTO. A lesser known, but equally important aspect of the fight has shaken up the Broad v. Berkeley perspective. Two new USPTO interference proceedings between the Korean biotech company, Toolgen, and Broad and Berkeley have recently been initiated as well. This discussion will also highlight challenges that the Broad and Berkeley have each faced in Europe.

A SARS-CoV-2 targeted siRNA-nanoparticle therapy for COVID-19

Alicia Davis

*Irell & Manella Graduate School of Biological Sciences, City of Hope Beckman Research Institute, Duarte CA, USA *Sponsored by IDT DNA*

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection in humans. Despite several successful vaccines, there remains an urgent need for therapies that can disrupt expression and replication of the virus. We designed 18 small interfering RNAs (siRNAs) to target the 5' untranslated region (5'UTR), RNA-dependent RNA polymerase, helicase, 7a-E, nonstructural protein (nsp) 8, or nsp9. These siRNAs were tested in SARS-CoV-2 infected Vero E6 cells and evaluated via plaque assay. siRNAs targeting the 5'UTR and the helicase domain demonstrated the highest antiviral activity. To deliver these siRNAs, we developed a novel lipid nanoparticle (LNP) formulation using scalable microfluidic mixing technology. We find that our LNPs traffic to the lung, liver, and spleen upon intravenous administration. As the lungs are the predominant site of viral infection and inflammation we moved forward with in vivo testing. Candidate siRNAs were encapsulated in LNPs and administered shortly after SARS-CoV-2 infection in K18-hACE2 mice with additional doses given on days 2 and 4. The siRNA-LNP treatment demonstrated robust repression of infectious virus in the lungs seven days post infection. We suggest that an siRNA-LNP therapeutic approach shows promise in treating COVID-19 and should be explored further.

LIVE SHORT TALK ABSTRACTS

Accelerating RNA Therapeutics R&D with a Modern Informatics Solution

Prem Mohanty

Benchling

RNA has emerged as an important therapeutic class recently. With technologies such as siRNA, antisense oligonucleotides, and mRNA, the pipeline of RNA therapies and vaccines continues to expand each year. However, the software tools to engineer these entities have been frustratingly limited. Scientists chemically modifying RNA oligos have had to contend with manual, low throughput, and disconnected software to complete the full workflow of designing, analyzing, and testing these complex biomolecules. There is need for a modern informatics solution that can handle complete hybrid molecule R&D with flexible, easy to use, and built-for-purpose capabilities so more RNA-based therapies can get to market as efficiently as possible. This presentation will highlight some of the critical considerations in evaluating an informatics solution to accelerate RNA therapeutics R&D.

Personalized splice-switching ASO therapy for a child with progressive blindness and insensitivity to pain

Boxun Zhao, Austin Larson, Francesca Bertino, Emily McCourt, Diana Chin, Casie Genetti, Chunguang Hu, Victoria Suslovitch, Deborah Chiabrando, E. Alice Lee, Timothy W. Yu

Division of Genetics and Genomics, The Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA, USA; Department of Pediatrics, Harvard Medical School, Boston, MA, USA

Posterior column ataxia with retinitis pigmentosa (PCARP) is an autosomal recessive neurodegenerative disease. It is characterized by pain insensitivity, incoordination, and progressive visual loss. The disease-causing gene is FLVCR1, which encodes an exporter of cytoplasmic heme. There are no existing treatments for this condition. Our 10-year-old patient presented with retinitis pigmentosa and pain insensitivity. She first came to ophthalmology at the age of 2 with a chief complaint of poor night vision and photophobia, and had a diagnosis of pigmentary retinopathy on clinical exam. Over time she has experienced decline in visual acuity and progressive visual field loss.

Whole genome sequencing analysis revealed that the patient was compound heterozygous for two pathogenic mutations in FLVCR1 gene: a paternally inherited missense mutation (c.1193A>G; p.Tyr398Cys), and a maternally inherited, ~3 kb ISG20L2 pseudogene insertion in intron 8. The pseudogene insertion was fully resolved at single-base resolution by Sanger sequencing, demonstrating hallmarks of L1-mediated retrotransposition via target primed reverse transcription (TPRT). The pseudogene insertion landed in an antisense orientation in the deep intron, accompanied by a 17 bp target site duplication (TSD), ploy-A tail, and a cleavage site at 5'–AT/AAGA–3', resembling the consensus L1 endonuclease motif 5'–TT/AAAA–3'. Splicing motif analysis predicted a novel 3' splicing acceptor site within the pseudogene insertion. Usage of this site was experimentally confirmed by RNA-seq, leading to incorporation of a nonfunctional pseudoexon with an early stop codon, truncating the FLVCR1 gene after exon 8.

We designed a series of phosphorothioate backbone, 2'-MOE-modified antisense oligonucleotides (ASOs) blocking the novel splicing acceptor and its branch site. Multiple rounds of screening in patient cells led to identification of a lead splice-switching ASO that is capable of 1) boosting normal-to-mutant splicing ratios and 2) restoring functional FLVCR1 protein in patient-derived cells, with pharmacologically favorable potency (IC50 = 16.6 nM and EC50 = 30.5 nM). In collaboration with the n-Lorem foundation, we are pursuing IND-enabling pre-clinical studies with a goal of gaining approval for experimental ASO treatment via intravitreal injection to forestall further vision loss.

RNA-communication between viruses and their hosts

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Bearing the largest single-stranded RNA genomes in nature, coronaviruses depend on short and long-distance RNA-RNA interactions to regulate their pathways inside the host cell. However, whether coronavirus RNA interacts with host RNA is unknown. Here we used the COMRADES method [Ziv et al, Nat Methods, 2018] to elucidate the in vivo RNA-RNA interactome of the SARS-CoV-2 genome and subgenomes [Ziv et al, Mol Cell, 2020]. We uncovered a network of RNA-RNA interactions spanning tens of thousands of nucleotides. These interactions reveal that the viral genome and subgenomes adopt alternative co-existing topologies and engage in conserved interactions with cellular RNA. We discovered a long-range RNA-RNA interaction - the FSE-arch - that encircles the programmed ribosomal frameshifting element of SARS-CoV-2. The FSE-arch is undergoing purifying selection and represents an intriguing case of convergent evolution with plant viruses. Our findings illuminate RNA-based mechanisms governing transcription and ribosomal frameshifting of coronaviruses and will aid future efforts to develop RNA-based antiviral strategies.

Live-cell single-molecule investigation of localization and translation efficiency of m6A-modified mRNA

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Post-transcriptional RNA modifications are important for cell viability and are emerging as crucial contributors to gene expression control. To date, over 170 posttranscriptional RNA modifications are known to occur in protein coding as well as non-coding RNAs. One such RNA modification is methylation of the exocyclic amine in the sixth position of the purine ring of adenine (N6-methyladenosine, m6A). m6A represents the most abundant internal modification of coding mRNAs in eukaryotes. Its importance is highlighted by the fact that m6A modification is crucial for many aspects of mRNA metabolism, including nuclear pre-mRNA processing, cytosolic export of mature mRNA, mRNA translation, mRNA interaction with RNA granules, and mRNA decay. RNA sequencing studies discovered that m6A modifications are highly enriched near stop codons and in 3' untranslated regions (3' UTRs) of mRNAs. The 3' UTR influences mRNA stability, translation efficiency, and subcellular localization. One example of a biologically important and functional 3' UTR methylation target is found in the sex determining region y-box 2 (SOX2) mRNA, which encodes a key transcription factor and oncogene. Its methylation status substantially shapes the fate of the mRNA and subsequently the cell's viability. However, a number of fundamental questions with respect to the influence of m6A modifications in the SOX2 3' UTR on translation efficiency and its recruitment to RNA granules remain unanswered.

Investigating a model m6A modified mRNA that includes the 3' UTR of SOX2, using a combination of live-cell single-particle tracking and mRNA translation visualization techniques revealed how the methylation status of 3' UTR shapes the translation efficiency, localization and partitioning into RNA granules. This study provides unprecedented insights into understanding m6A modification of mRNA as a significant part of the gene regulation processes collectively referred to as epitranscriptomics.

Ribosome inhibition by C9ORF72-ALS/FTD-associated poly-PR and poly-GR proteins revealed by cryo-EM

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Toxic dipeptide repeat (DPR) proteins are produced from expanded G4C2 hexanucleotide repeats in the C9ORF72 gene, which cause amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Two DPR proteins, poly-PR and poly-GR, repress cellular translation but the molecular mechanism of this repression remains unknown. We will present how poly-PR and poly-GR of ≥ 20 repeats inhibit the ribosome's peptidyl-transferase activity at nanomolar concentrations, comparable to specific translation inhibitors. Our high-resolution cryo-EM structures reveal that poly-PR and poly-GR block the polypeptide tunnel of the ribosome, extending into the peptidyl-transferase center. Consistent with these findings, the macrolide erythromycin, which binds in the tunnel, competes with the DPR proteins and restores peptidyl-transferase activity. Our results demonstrate that strong and specific binding of poly-PR and poly-GR in the ribosomal tunnel blocks translation, revealing the structural basis of their toxicity in C9ORF72-ALS/FTD.

POSTER PROGRAM

Posters are located within the virtual conference and will be up for the entirety of the symposium. Poster sessions will be held via **Spatial Chat for a LIVE Q&A session** during designated times.

Posters 1-42 will be available for a **Q&A session in spatial chat** on *Wednesday June 23rd, 3:30 - 4:15pm.*
Posters 43-86 will be available for a **Q&A session in spatial chat** on *Thursday, June 24 at 1:15 - 2:00pm.*
Posters 87-125 will be available for a **Q&A session in spatial chat** on *Friday June 25 at 1:15 - 2:00pm.*
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KEYNOTE BIOGRAPHIES

Katherine High, MD

President
AskBio

Dr. Katherine High joined AskBio in January 2021 as President, Therapeutics and member of the AskBio Board of Directors. Dr. High is responsible for driving the strategic direction and execution of the company's preclinical and clinical programs. Most recently, she was a Visiting Professor at Rockefeller University. Previously, she served as President, Head of Research and Development, and a member of the Board of Directors at Spark Therapeutics, where she directed the development and regulatory approval of Luxturna®, the first gene therapy for genetic disease to obtain regulatory approval in both the United States and Europe. She was a longtime member of the faculty at the University of Pennsylvania and medical staff at The Children's Hospital of Philadelphia, where she was also an Investigator of the Howard Hughes Medical Institute. She served a five-year term on the U.S. Food and Drug Administration Advisory Committee on Cell, Tissue and Gene Therapies and is a past president of the American Society of Gene & Cell Therapy. Dr. High received her bachelor's degree in chemistry from Harvard University, an MD from the University of North Carolina School of Medicine, a business certification from the University of North Carolina Business School's Management Institute for Hospital Administrators, and a master's degree from the University of Pennsylvania. She is an elected member of the National Academy of Medicine, the American Academy of Arts and Sciences, and the faculty of Pharmaceutical Medicine of the Royal College of Physicians (London).

Michael Rosbash, PhD

Peter Gruber Endowed Chair in Neuroscience, Professor of Biology, Nobel Laureate
HHMI, Brandeis University

Dr. Michael Rosbash is a Professor of Biology and the Peter Gruber Professor of Neuroscience at Brandeis University. He is also an Investigator of the Howard Hughes Medical Institute. Rosbash went to the Newton public schools in greater Boston and then to Caltech, graduating in 1965 with a B.S. in Chemistry. He spent the 1965-1966 academic year in Paris as a Fulbright Scholar in the lab of Marianne Grunberg-Monago and then entered the Ph.D. program at MIT in the fall of 1966. Rosbash worked there in the lab of Sheldon Penman and received a Ph.D. in Biophysics in 1970. After a brief stint at the University of St. Andrews, he was a post-doc in the lab of John Bishop in the Department of Genetics at the University of Edinburgh from 1971-1974. Rosbash joined the faculty of Brandeis University in 1974 and was promoted to Professor of Biology in 1986. He became a Howard Hughes Medical Institute Investigator in 1989. Rosbash has made fundamental contributions to our understanding of the post-transcriptional regulation of gene expression, especially RNA metabolism in yeast. He is best known however for his work in Drosophila that illuminated our current understanding of the molecular mechanisms that underlie circadian rhythms, the intrinsic clock that controls the cyclic behaviors of all animals. These same molecules, molecular machines and biological principles not only control Drosophila circadian clocks but also the ubiquitous process of circadian rhythmicity throughout the animal kingdom. This circadian clock also controls much of cell physiology and metabolism, again in all animals - from humans to Drosophila (fruit flies). Rosbash and his Brandeis colleague Jeff Hall as well as Mike Young of the Rockefeller University have received numerous awards for their circadian work, including most recently the 2017 Nobel Prize in Physiology or Medicine. They previously received the Shaw Prize in Life Science and Medicine (2013), the Wiley Prize in Biomedical Sciences (2013), the Massry Prize (2012), the Canada Gairdner International Award (2012), the Louisa Gross Horwitz Prize for Outstanding Basic Research (2011), and the Peter and Patricia Gruber Foundation Neuroscience Prize (2009). Rosbash also received the Caltech Distinguished Alumni Award (2001), and he is a Member of the National Academy of Sciences, a Fellow of the American Association for the Advancement of Sciences and a Fellow of the American Academy of Arts and Sciences.

SPEAKER BIOGRAPHIES

Julius Brennecke, PhD
Senior Group Leader
Institute of Molecular Biotechnology

Julius Brennecke, PhD. is the Senior group leader at the Institute of Molecular Biotechnology (IMBA), Vienna Austria. He earned his Ph.D. at the European Molecular Biology Laboratory, Heidelberg/Ruprecht-Karls University, Heidelberg, Germany. His research is focused on biology and mechanisms of small regulatory RNA pathways in animals; from controlling development to protecting the genome against selfish genetic elements.

Grace Chen, PhD
Assistant Professor
Yale University

Dr. Grace Chen received her undergraduate training in the College of Chemistry at UC Berkeley. She attended Harvard University for her PhD where she worked in David Liu's laboratory to discover and characterize novel RNA modifications. Her postdoctoral research was at Stanford University in Howard Chang's group, where she investigated circular RNA immunity. Grace Chen joined Yale University as a faculty in the Department of Immunobiology in 2019. Her research focuses on the functions and regulations of circular RNAs and RNA modifications in health and disease.

Paula Cohen, PhD

Professor; Associate Vice Provost for Life Sciences
Cornell University

Dr. Paula Cohen obtained her PhD in reproductive physiology in 1992 at the University of London, England, where she studied the endocrine regulation of implantation. In 1993, she took a Postdoctoral position at the Albert Einstein College of Medicine, New York, where she focused on regulation of gonadal function in males and females, and in maturation of the hypothalamic-pituitary-gonadal axis. During this time, she become interested in germ cell biology and genome integrity, and transitioned into this area to study the roles of DNA repair proteins in mammalian meiosis. She joined the faculty of the Department of Genetics at Albert Einstein College in 2000, and then in 2004 was recruited to Cornell University, within the Department of Biomedical Sciences. In 2007, she was promoted to the rank of Associate Professor with indefinite tenure, and then was promoted to full Professor in 2013. Since starting her own lab, she has received funding from NIH, the March of Dimes, the National Down Syndrome Society, and the Hereditary Diseases Foundation. More recently, she received a prized Grand Challenge grant from the Bill and Melinda Gates Foundation to investigate a new strategy for contraceptive development in men. In 2006, Dr. Cohen established the Center for Reproductive Genomics (CRG), the first Cornell-wide center encompassing clinicians and scientists from the College of Veterinary Medicine and from the Weill-Cornell Medical College, as well as participants from other colleges within Cornell University. In 2014, she successfully obtained a P50 grant to fund research within the CRG as part of the NICHD "*National Centers for Translational Research in Reproduction and Infertility*". She has co-authored over 60 papers, served as a regular and *ad hoc* member on several different NIH Study Sections, as well as several Special Emphasis panels for NICHD and NIGMS. Dr. Cohen has reviewed grants for international panels, including serving on the advisory board for the German Government's Research Foundation for their special priority program in "Genome Haploidization" for six years, as well as for the UK Medical Research Council, the Wellcome Foundation (UK), The Telethon Foundation (Italy), and for the European Research Council. In 2016, she was elected by her peers to become the vice-Chair for the 2018 Gordon Research Conference on Meiosis and the chair for the 2020 meeting. Dr. Cohen previously served as associate editor for the journal, *Chromosoma,* and currently serves in that role for *PLoS Genetics,* and previously served on the editorial advisory board of *Chromosome Research.* She has participated as a lecturer and lab leader in the *Frontiers in Reproduction* (FIR) course at the Marine Biology Labs at Woods Hole, MA, for the past fourteen years. In 2015, she contributed the first chapter on meiosis for the *4th Edition of Knobil and Neil's Physiology of Reproduction* (Elsevier Press). She has served as the Director of Admissions for the Graduate Field in Genetics and Development, and on the steering committee for that field as well as for the graduate field of Molecular and Integrative Physiology. While at Cornell, she has received numerous merit awards for her research contributions, the Provost's award for Distinguished Scholarship (2009), and the SUNY Chancellor's award for Academic Excellence (2017). In 2018, she became Associate Vice Provost for Life Sciences. In her own lab, Dr. Cohen has mentored 13 graduate students, 10 Postdoctoral fellows, 1 reproductive endocrinology fellow, and numerous undergraduates.

Kate Fitzgerald, PhD

Principal Investigator
UMass Medical School

Dr. Kate Fitzgerald received her B.Sc. in Biochemistry in 1995 from University College Cork, Ireland, and her Ph.D. in 1999 from Trinity College Dublin, Ireland. She was a post-doctoral fellow in the Department of Biochemistry at Trinity College Dublin working with Luke O'Neill (1999-2002). She joined the Division of Infectious Disease at the University of Massachusetts Medical School as a Wellcome Trust Fellow in 2002 and joined the faculty in 2004. She is currently Professor of Medicine, Director of the Program in Innate Immunity, The Worcester Foundation Chair in Biomedical Sciences and most recently elected into the National Academy of Sciences.

Myriam Gorospe, PhD

Chief, Laboratory of Genetics and Genomics
NIA, NIH

Dr. Myriam Gorospe did her undergraduate studies in Molecular and Cellular Biology (in Madrid, Spain) and earned her Ph.D. in Cell and Developmental Biology from the State University of New York at Albany. After post-doctoral training at the National Institute on Aging (NIA) of the National Institutes of Health (NIH), she became a Principal Investigator and head of the RNA Regulation Section (NIA, NIH) in 1998. She directs the Laboratory of Genetics and Genomics since 2014, where she studies post-transcriptional gene regulation in mammalian models of cellular stress, senescence, and aging. Her program has a long-standing focus on RNA-binding proteins and noncoding RNAs that influence gene expression programs in aging physiology and pathology.

Michelle Hastings, PhD

Associate Professor
Rosalind Franklin University of Medicine and Science

Michelle Hastings, PhD, is the Director of the Center for Genetic Diseases and an Associate Professor of Cell Biology and Anatomy at the Chicago Medical School at Rosalind Franklin University of Medicine and Science. Her research focuses on understanding genetic basis of disease and testing new therapeutic approaches by modulating the process of pre-mRNA splicing to alter gene expression. Her work has resulted in the discovery of effective means of targeting splicing with antisense molecules for the potential treatment of a number of neurodegenerative diseases including Batten disease, Usher syndrome, cystic fibrosis, Alzheimer's and Parkinson's disease. Dr. Hastings' studies on Usher syndrome led to the first demonstration that hearing and balance can be recovered in mice with a mutation that causes congenital deafness in humans, laying the groundwork for developing a treatment for Usher in humans. Her recent work has demonstrated that antisense technology can modulate gene expression pathways associated with Alzheimer's disease to mitigate learning and memory deficits in mouse models of the disease. A major focus of the lab currently is on developing approaches to treat different forms of Batten disease using antisense technology. Dr. Hastings holds a number of patents and has been supported by the National Institutes of Health and numerous Foundation grants. She was recognized as a 2019 Researcher to Know by the Illinois Science and Technology Consortium.

Ruth Lehmann, PhD

Professor of Biology; Director,
Whitehead Institute, MIT

Dr. Ruth Lehmann is a Howard Hughes Medical Institute investigator and the Director of the Whitehead Institute for Biomedical Research, succeeding David Page. She was previously affiliated with the New York University School of Medicine, where she was the Director of the Skirball Institute of Biomolecular Medicine, the Laura and Isaac Perlmutter Professor of Cell Biology, and the Chair of the Department of Cell Biology. Her research focuses on germ cells, the only cells in the body able to give rise to a new generation.
Born in Cologne, Germany, Lehmann studied biology at the University of Freiburg and Universität Tübingen (both in Germany) as well as the University of Washington, Seattle. She received her Ph.D. in the laboratory of noble laureate Dr. Christiane Nüsslein-Volhard at the Max Planck Institute for Developmental Genetics in 1985. After postdoctoral training at the Medical Research Council (MRC) in Cambridge, UK, she joined the Whitehead Institute and the faculty of the Massachusetts Institute of Technology in 1988. In 1996, Lehmann was recruited to the Skirball Institute of Biomolecular Medicine at NYU School of Medicine. Here, her lab expanded into the analysis of germ cell migration by showing how lipid signaling affects cell migration. Today, her lab uses systematic, unbiased genetic approaches and different imaging modalities in Drosophila to understand how germ cells are specified in the early embryo and how they maintain the potential for totipotency while differentiating into egg and sperm in the adult.

Lehmann is a member of the American Academy of Arts Sciences and the National Academy of Sciences. She was the 2011 recipient of the Edwin G. Conklin Medal of the Society of Developmental Biology and was elected a member of European Molecular Biology Organization in 2012. Christopher Burge, focusing on understanding the changes in mRNA splicing after immune response and developing methods to measure the dynamics of such processes. Athma assumed her current position as an Assistant Professor in the RNA Therapeutics Institute at the University of Massachusetts Medical School in January 2018. Her lab works on developing and applying methods to study the kinetics of mRNA processing and understand how various steps in RNA maturation are coordinated during the biogenesis of an RNA molecule.

Art Levin, PhD

Chief Scientific Officer
Avidity Biosciences

Dr. Art Levin has an unparalleled track record and reputation in the field of nucleic acid-based therapeutics. He serves as the Chief Scientific Officer at Avidity Biosciences. Previously he held that position at miRagen Therapeutics. Prior to that he held senior drug development roles at Ionis (formerly ISIS) Pharmaceuticals and Santaris Pharma.

He has played key roles in the development of numerous of oligonucleotides including the first approved antisense drugs, and the first microRNA-targeted therapeutic in clinical trials. He has a combined three decades of experience in all aspects of drug development from discovery through drug registration, both in large pharma and biotech companies. Dr. Levin has published over 60 scientific articles and several of the most cited reviews in the field. He is a key opinion leader in the field and is on the scientific advisory boards of multiple institutions. Art received a doctorate in toxicology from the University of Rochester, and a bachelor's degree in biology from Muhlenberg

Melissa Moore, PhD

Chief Scientific Officer
Moderna Therapeutics

In her role as Chief Scientific Officer of Moderna's research platform, **Dr. Melissa Moore** is responsible for leading all mRNA biology and delivery science research at Moderna. She joined Moderna from the University of Massachusetts Medical School (UMMS), where she served as Professor of Biochemistry & Molecular Pharmacology, Eleanor Eustis Farrington Chair in Cancer Research and Investigator at the Howard Hughes Medical Institute (HHMI). Dr. Moore was also a founding Co-Director of the RNA Therapeutics Institute (RTI) at UMMS, and was instrumental in the creation of the Massachusetts Therapeutic and Entrepreneurship Realization initiative (MassTERi), a faculty-led program intended to facilitate the translation of UMMS discoveries into drugs, products, technologies and companies. She is a member of the National Academy of Sciences.

Dr. Moore's research at UMMS and HHMI encompassed a broad array of topics related to the role of RNA and RNA-protein (RNP) complexes in gene expression and touched on many human diseases including cancer, neurodegeneration and preeclampsia. Prior to UMMS, she spent 13 years as a professor at Brandeis University, where she also received her Ph.D. in Biological Chemistry. Dr. Moore holds a B.S. in Chemistry and Biology from the College of William and Mary.

Upon joining Moderna, Dr. Moore has retained her academic affiliation with UMMS as a part-time faculty member.

Kathy Niakan, PhD

Group Leader
Francis Crick Institute

Dr. Kathy Niakan is a Group Leader at the Francis Crick Institute in London where her laboratory investigates mechanisms that direct how the first cell types become specialized in their fate and function in human embryos. Her laboratory was the first to use CRISPR/Cas9 genome editing techniques to study gene function in human embryos and discovered the role of a key gene in human embryo development. Research equipment and objects from her lab have been exhibited at the Science Museum in London and are part of the permanent collection. In collaboration with Mary Herbert and Doug Turnbull at Newcastle University, work from her laboratory was provided as evidence to the UK Department of Health ahead of a vote in 2015 in the UK House of Commons and House of Lords to change regulation allowing the clinical use of mitochondrial replacement therapy. Kathy is a Blavatnik Award UK Finalist in Life Sciences and was named Time Magazine's 100 Most Influential People. Kathy obtained a B.Sc. and B.A. from University of Washington, a PhD at University of California, Los Angeles and undertook postdoctoral training at Harvard University. She was a Next Generation Research Fellow at University of Cambridge.

Bradley Pentelute, PhD

Pfizer-Laubach Career Development Professor Associate Member,
Broad Institute of Harvard and MIT

Dr. Bradley Pentelute is currently an associate professor of chemistry at the Massachusetts Institute of Technology (MIT). He is also an Associate Member, Broad Institute of Harvard and MIT, an Extramural Member of the MIT Koch Cancer Institute, and Member, Center for Environmental Health Sciences MIT. He received his undergraduate degree in Psychology and Chemistry from the University of Southern California, and his M.S and Ph.D. in Organic Chemistry from the University of Chicago with Prof. Steve Kent. He was a postdoctoral fellow in the laboratory of Dr. R. John Collier at Harvard Medical School, Microbiology. His laboratory focuses on researching new methods in chemistry to selectively modify proteins and to deliver molecules to cells. His lab successfully modified proteins via “pi-clamps” made up of different amino acids, and delivered large biomolecules, such as various proteins and drugs, into cells via the anthrax delivery vehicle. Pentelute has also made several key contributions to automated synthesis technologies. This includes a recent invention of the world's fastest polypeptide synthesizer. This system is able to form more amide-bonds at a more efficient rate than standard commercial equipment and has helped in the process of understanding protein folding and its mechanisms. The primary goal of his endeavor is to use these processes to create biologics that can be used to treat diseases.

Joshua Rosenthal, PhD

Senior Scientist
Marine Biological Laboratory

Dr. Joshua Rosenthal is a Senior Scientist at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts. He received his Ph.D. in Biology from Stanford University and completed his postdoctoral training in biophysics and physiology at UCLA. Before coming to the Marine Biological Laboratory, he rose from Assistant to Full Professor at the University of Puerto Rico’s Medical Sciences Campus. Dr. Rosenthal’s research focuses on the process of RNA editing from a variety of angles. His group has shown that mRNA recoding is unusually active in cephalopods. They are interested in how cephalopod RNA editing can respond to environmental cues and how the underlying machinery for RNA editing in this taxon is able to drive such extensive recoding. The Rosenthal lab also pioneered systems to use RNA editing as a therapeutic in order to correct genetic mutations and engineer protein function. Dr. Rosenthal is a founder of Korro Bio, a biotech in Cambridge, MA that is developing RNA editing platforms to treat human disease.

Chrysanthi Taxiarchi, PhD

Postdoctoral Research Associate, Crisanti Lab
Imperial College of London

Dr. Chrysanthi Taxiarchi is a Postdoctoral Research Associate in Prof. Andrea Crisanti lab, at Imperial College London. Aiming at malaria eradication through mosquito genetic control, she has worked on transcriptomic analysis of the mosquito gametogenesis and the development of CRISPR-based genetic tools for population control. Recently the focus of her work has been on the development of technologies that could block the spread of gene drives in mosquito populations.

Amy Wagers, PhD

Co-Chair, Harvard Department of Stem Cell and Regenerative Biology
Harvard University

Dr. Amy Wagers is the Forst Family Professor of Stem Cell and Regenerative Biology at Harvard University, Senior Investigator in the Section on Islet Cell and Regenerative Biology at the Joslin Diabetes Center, an HHMI Early Career Scientist, and a member of the Paul F. Glenn Laboratories for the Biological Mechanisms of Aging at Harvard Medical School. Dr. Wagers received her PhD in Immunology and Microbial Pathogenesis from Northwestern University, and completed postdoctoral training in stem cell biology at Stanford University. Dr.Wagers' research seeks to understand how changes in stem cell activity impact tissue homeostasis and repair throughout life. Work from her lab provides evidence for the existence of a conserved systemic regulatory axis that modulates tissue maintenance and regeneration across a wide variety of tissues that vary significantly in their intrinsic repair capacity, and her ongoing studies have begun to identify the molecules responsible for age-variant regulation of regenerative potential. Dr. Wagers has authored more than 100 primary research and review articles, andher work has been recognized by awards from the Burroughs Wellcome Fund, Beckman Foundation, WM Keck Foundation, and Glenn Foundation, and National Institutes of Health. In 2013, she received the New York Stem Cell Foundation's Robertson Prize for outstanding achievement in translational stem cell research.

Jonathan Watts, PhD

Associate Professor
RNA Therapeutics Institute, University of Massachusetts Medical School

Dr. Jonathan K. Watts is an Associate Professor at the RNA Therapeutics Institute of UMass Medical School (Worcester, MA, USA). He completed his training at Dalhousie University (BSc), McGill University (PhD) and UT Southwestern Medical Center (Postdoctoral). After establishing his independent laboratory at the University of Southampton, UK, he moved to the RNA Therapeutics Institute in 2015. He has received over 20 awards including the 2013 OTS Young Investigator Award, the 2015 Vice Chancellor's award for teaching, and the 2018 Angel Award for ALS research.

Current work in the Watts lab is focused on the optimization of ASO chemistry for use in the CNS and lung, and on the chemistry of guides and donors for genome editing. The Watts group works on both platform technology and disease applications, and has contributed to the development of two drugs that have reached patients on a compassionate use basis. Jon is on the Board of Directors of the Oligonucleotide Therapeutics Society since 2015.

Timothy Yu, MD, PhD

Neurologist and Principal Investigator, Division of Genetics and Genomics, Boston Children's Hospital and Harvard Medical School

Dr. Timothy Yu is a neurologist and researcher at Boston Children's Hospital. He completed his MD-PhD at UCSF studying circuit development in C. elegans with Cori Bargmann, neurology residency at Massachusetts General Hospital and Brigham and Women's Hospital, and a postdoctoral fellowship in human geneticswith Christopher Walsh. He is an Assistant Professor at Harvard Medical School and an Associate Member of the Broad Institute of MIT and Harvard. An early pioneer inhigh throughput sequencing, he developed some of the first methods for genome-scale sequencing and wrote one of the very first bioinformatic pipelines for large-scale interpretation of human genomic variation. He has identified or contributed to the identification of over a dozen new human disease genes and co-founded a pediatric genomic diagnostic company. His lab applies diverse skills in genetics, neurobiology, and bioinformatics to study neurodevelopmental disorders and advance genomic medicine. Current projects range from computational analyses of tens of thousands of individuals with autism to identify disease genes, to investigations of genome sequencing for newborn screening and neonatal ICU care, to the development of rapid-turnaround, N-of-1 personalized therapies for rare pediatric disorders.

PANELIST BIOGRAPHIES

Patrick Baumhof, PhD
Vice President Formulation & Delivery
CureVac

Dr. Patrick Baumhof is currently Vice President Formulation & Delivery at CureVac GmbH, a privately held German biopharmaceutical company which develops mRNA based Therapeutics. He received his degree in organic chemistry from the University Leipzig. After an industrial postdoc at Qiagen, he joined CureVac in 2007 as a scientist in formulations and RNA stabilization. During this time he developed several different formulations for mRNA for several different applications and works on the translation of these from pre-clinical testing to clinical testing. His research interests are formation & characterization of mRNA based nanoparticles, their biological activity after local and systemic application, and their biodistribution and efficacy in several different animal species.

Aimee Jackson, PhD
Chief Scientific Officer
Atalanta Therapeutics

Dr. Aimee Jackson is currently the Chief Scientific Officer for Atalanta Therapeutics. Aimee has worked in the field of RNA interference and oligonucleotide therapeutics for nearly 2 decades, and has made numerous seminal discoveries in the field. She most recently served as the Vice President of Research for miRagen Therapeutics, where she advanced 3 microRNA programs from initial concept to First-in-Human clinical trials in less than 5 years. These programs encompassed diverse therapeutic areas and comprised both microRNA mimics and inhibitors. Two of these programs have advanced to Phase 2 clinical trials, including a fibrosis program for which Aimee served as the Product Development Team Lead. Prior to joining miRagen, she led preclinical research at Regulus Therapeutics and Merck/Rosetta, where she served as Molecular Profiling Lead for the development of siRNA-based therapeutics and played a lead role in the identification of siRNA design aspects for enhanced activity and specificity.

T. Scott Johnson, MD
Co-Founder and CEO
Comanche Biopharma

Dr. T. Scott Johnson is co-founder and CEO of Comanche Biopharma, a private company developing novel siRNA compounds to treat preeclampsia. Prior to Comanche, Scott was a co-founder of The Medicines Company where he served as director (1996-2009) until he joined the management team as chief medical advisor (2009-2021). In that role, he was responsible for evaluating the company's long-range global strategy and for the licensing or acquisition of innovative products, including inclisiran, for which the company was purchased by Novartis.Previously, Scott was a founding General Partner at JSB Partners, L.P., an investment bank with a focus on mergers and acquisitions, private financings and corporate alliances within the healthcare sector. From September 1991 to July 1999, Scott served as a founder and managing director of MPM Capital, LLC, a venture capital firm. Scott holds a B.S. in chemistry (Phi Beta Kappa) and an M.D. (Alpha Omega Alpha) from the University of Alabama. His academic career included faculty positions at Harvard Medical School, the University of Colorado Medical School, and the University of South Alabama. He has been Board Certified in Internal Medicine, Pulmonary and Critical Care Medicine and Sleep Disorders Medicine. He currently is Chairman, Board of Directors, MaxiVAX SA, a biopharmaceutical company focused on immuno-oncology.

Melissa Moore, PhD *(click to go to bio above)*
Chief Scientific Officer
Moderna Therapeutics

Dmitry Samarsky, PhD
Chief Technical Officer
Sirnaomics, Inc.

Dmitry Samarsky, PhD has been at the inception of the RNAi technology and drug development - starting in 2001 as a Director of Technology Development at Sequitur (acquired by Invitrogen) and (in 2005) as a Director of Technology Development at Dharmacon (now part of GE). He then served as a VP of Technology Development at RXi Pharmaceuticals, USA (2007-2011), an SVP of Technology and International Business Development at RiboBio, China (2011-2016) and, most recently, as a Chief Scientific Officer at Silence Therapeutics, Germany/UK (2016-2018). Dr. Samarsky has authored more than 40 scientific papers, articles, book chapters, patents and patent applications. He has been an invited speaker at more than 100 international conferences and served on the Scientific Advisory Boards for the OTS (Oligonucleotide Therapeutics Society), TIDES (Oligonucleotide and Peptide Therapeutics) and OPT (Oligonucleotide Precision Therapies) conference series. Dr. Samarsky received his doctorate in biochemistry and molecular biology from University of Massachusetts, Amherst (1998), followed by a postdoctoral position as an H. Arthur Smith Fellow for Cancer Research at Dr. Michael Green's lab at the University of Massachusetts Medical School (1998-2001).

Panelist Moderator:
Julia Alterman, PhD
Assistant Professor
RNA Therapeutics Institute, UMass Medical School

In her 14 years working in molecular medicine, Julia has received multiple distinctions. While working for six years in two small biotech companies, Resolvyx Pharmaceuticals and Anchor Therapeutics, she completed her master's degree at Harvard University, being awarded the Dean's Prize for Outstanding Thesis in the Field of Biology. Continuing to her PhD at the University of Massachusetts Medical School, Julia was awarded the Chancellor's Award for her research which developed a novel siRNA chemical scaffold exhibiting potent, long-term, and widespread delivery to the brain. This work was published on the cover of Nature Biotechnology and selected in the top 25 landmark papers of this journal over the past 25 years. Her work also inspired the founding of Atalanta Therapeutics to develop this technology for the treatment of neurological diseases. After aiding in the startup, Julia returned to UMMS to assist in an initiative to develop chemically modified siRNAs for the treatment of genetically defined diseases.

SYMPOSIUM ORGANIZERS

Phillip D. Zamore, PhD

Howard Hughes Medical Institute
RNA Therapeutics Institute, UMass Medical School

Phillip D. Zamore, Ph.D. has been an Investigator of the Howard Hughes Medical Institute since 2008. In 2016, he became the Chair of the RNA Therapeutics Institute, which was established at the University of Massachusetts Medical School in 2009. Dr. Zamore also is Professor of Biochemistry and Molecular Pharmacology, the department he joined in 1999, and he became the Gretchen Stone Cook Professor of Biomedical Sciences in 2005.

Dr. Zamore received his A.B. (1986) and Ph.D. (1992) degrees in Biochemistry and Molecular Biology from Harvard University. He then pursued postdoctoral studies on the role of the RNA binding proteins in Drosophila development at The Whitehead Institute for Biomedical Research, in Cambridge, Massachusetts.

Dr. Zamore's laboratory studies small RNA silencing pathways in eukaryotes and prokaryotes, including RNA interference (RNAi), microRNA, and PIWI-interacting RNA pathways. Dr. Zamore and his collaborators seek to use these insights to design therapies for human diseases, including Huntington's disease. Under Dr. Zamore's mentorship, the Zamore Lab has produced dozens of researchers working at top institutions both in the United States and abroad.

In 2015, Dr. Zamore was awarded the Chancellor's Medal for Excellence in Scholarship at the University of Massachusetts Medical School. To date, Dr. Zamore has more than 163 publications and has been among the most highly cited researchers for more than a decade. He serves on the editorial boards of numerous journals and is in demand as a presenter at conferences and institutions worldwide.

Dr. Zamore holds more than 20 patents, with other applications pending; he was elected a Fellow of the National Academy of Inventors in 2014. In 2002, Dr. Zamore co-founded Alnylam Pharmaceuticals (Cambridge, MA), a publicly traded biotech company which now has more than 1000 employees and multiple drugs in clinical trials. Alnylam's first drug, ONPATTRO, a first-of-its-kind RNAi therapeutic, for the treatment of the polyneuropathy of hereditary transthyretin-mediated (hATTR) amyloidosis in adults, was approved by the FDA in 2018. In 2014, he co-founded Voyager Therapeutics in Cambridge, MA. He serves on the scientific advisory boards of Alnylam, Voyager, and ProQR.

Angela Messmer-Blust, PhD

RNA Therapeutics Institute, UMass Medical School

Dr. Angela Messmer-Blust studied cell motility during her graduate studies at the University of Toledo where she received her Ph.D. (2009). She began her postdoctoral research at the CardioVascular Institute at Beth Israel Deaconess Medical Center, Harvard Medical School under Jian Li, investigating transcriptional regulation in diabetes and obesity. Following her postdoctoral studies, she held editorial positions at the Journal of Visualized Experiments, and later joined Cell Press as the director of conferences from 2012 - 2017. In 2017, Dr. Messmer-Blust joined the faculty of the RNA Therapeutics Institute as Senior Scientific Advisor to the department. Dr. Messmer-Blust co-organizes the RNATx symposium annually and recently co-founded the ScienceLIVE educational outreach program in the Worcester Public School district.

SYMPOSIUM SURVEY

We hope you are enjoying the 2021 RNA Therapeutics symposium! We are very excited to be able to gather next year in person. We would love to hear from you on what went well, what you liked, and if there are suggestions for how to improve (both virtual and in-person). We take your suggestions very seriously and use them to continually improve year on year. We thank you for your time and effort to share your thoughts with us. Please fill out the survey once the symposium has completed:

https://www.surveymonkey.com/r/2021RNATx

POSTER ABSTRACTS

Developing Neisseria meningitidis Cas9 base editors (Nme2Cas9-BEs) for therapeutic applications

Han Zhang, Nathan Bamidele, Ogooluwa Ojelabi, Pengpeng Liu, Wen Xue, Scot Wolfe, and Erik Sontheimer

RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, USA

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Point mutations represent the largest class of known human pathogenic genetic variants. Base editors (BEs), which correct point mutations without generating double-strand DNA breaks, comprise a single-guide RNA (sgRNA) loaded onto a Cas9 nickase fused to a nucleobase deaminase enzyme. However, there are certain limitations to current BEs: 1) Targetable sites can be restricted by the protospacer adjacent motif (PAM) specificity; 2) The best-characterized SpyCas9—isolated from S. pyogenes—demonstrates significant off-target effects, which is the major cause of undesired editing by BEs; and 3) Most BEs have coding sequences exceeding the packaging limit (~5 kb) for adeno-associated-virus (AAV) vectors, which are among the most promising in vivo delivery approaches. Current dual-AAV delivery strategies often require high viral doses and are limited by co-transduction efficiency, and to our knowledge, no single-AAV in vivo BE delivery has been reported.

We previously identified and validated the genome editing activity of a compact Cas9 from Neisseria meningitidis (Nme2Cas9) that recognizes a distinct, short PAM (N4CC) and displays high targeting fidelity. Here, we constructed Nme2Cas9-BEs and defined their editing efficiencies, editing windows, and off-target activities in comparison with the widely-applied SpyCas9-BEs in cultured cells. Because of the compact size of Nme2Cas9, a Nme2Cas9-ABE with a sgRNA can be packaged into a single AAV vector. By optimizing the promoter and the nuclear localization signals, our design of the single-AAV vector construct for Nme2Cas9-ABE delivery can achieve a therapeutic level of in vivo base editing in the mouse model of hereditary tyrosinemia type 1. Lastly, we show that the activity of Nme2Cas9-BEs can be inhibited by anti-CRISPR proteins, which can be applied to achieve spatial control of base editing activity in vivo. In summary, we have developed Nme2Cas9-BEs to provide extra targetability to the existing BE toolbox, with improved safety and therapeutic potential.

Improving Genome Editing in the Central Nervous System by Complete Chemical Modification and Self-delivering of CRISPR RNAs

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P.2

Point mutations are the largest class of known human genetic disorder. Theoretically, those pathogenic alterations can be reverse by directly editing the mutated gene, thereby restoring its function in its natural context. CRISPR-based technology holds enormous potential as therapeutics offering an approach to permanently correct those disease-causing mutations. The initial therapeutic applications were performed on cells ex vivo with hematopoietic stem cells that were collected from patients. However not all tissue types are suited for ex vivo manipulation and most genetic diseases will require genome editing in vivo. Unfortunately, deploying CRISPR-based therapeutics directly into the human body remains a bottleneck for therapy development. Currently, there are three main delivery strategies under development for clinical genome-editing applications. (i) Adeno Associated Virus are the most commonly used clinical delivery vehicle for gene therapy. (ii) Nanoparticles such as cationic lipid polymers that can be used to encapsulate mainly mRNA and DNA to facilitate their cellular entry. (iii) Ribonucleoprotein which are purified protein and RNA.

Recent achievements in the delivery of nucleic acid therapeutics such as antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs) to relevant tissues, have depended on total chemical modifications, that enabled direct delivery to various tissues without nanoparticle formulation. Those chemical modifications have been shown to provide metabolic stability, improve biodistribution and reduce innate immune responses. Because of the nucleic acid components of CRISPR system we are able to use RNP for in vivo delivery and apply those same principles to improve the safety and efficacy of genome editing in vivo. We previously described our first generation of heavily modified (HM) crRNAs and tracrRNA that can support effective SpyCas9 activity in mammalian cells. We have now demonstrated that our HM crRNA/tracrRNA can also support efficient editing in vivo when delivered directly into the mouse CNS as SpyCas9 RNP. Moreover, we defined new variants of fully modified (FM) crRNA frameworks with improved Cas9 genome editing in mammalian cells and we are currently developing these guides for self-delivering in conjunction with AAV vectors that encode SpyCas9 and tracrRNA. This strategy is particularly useful for multiplex targeting, and elimination of the AAV construct after editing is achieved, minimizing off targeting and vector integration.

Development of Engineered Guide RNAs for Nme2Cas9 Genome Editing

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P.3

Gene editing using CRISPR-Cas9 technologies has dramatically advanced genetic research and promises to revolutionize gene therapy. Realizing this hope requires tools for safe, efficient editing and delivery. Oligonucleotide therapeutics require ribose and/or phosphodiester modifications to stabilize the compound in vivo, minimize immunogenicity, and enable delivery. Such chemical modifications have been vital to the clinical advancement of oligo-based therapies. Our laboratory and others have shown that chemically-modified Streptococcus pyogenes (SpyCas9) guide RNAs mediate efficient genome editing in mammalian cell culture in combination with RNP-, mRNA- and DNA-delivered SpyCas9 effectors. More recently, chemical modification of SpyCas9 guide RNA has been key in the development of RNP and mRNA delivery approaches ex vivo and in vivo based CRISPR-Cas9 therapeutics.

Our group also discovered a compact Type II-C Cas9 ortholog from Neisseria meningitidis (Nme2Cas9). Nme2Cas9 exhibits a unique DNA targeting motif (N4CC), high accuracy and the ability to mediate efficient ex vivo and in vivo gene editing in mammalian cells. Here, I explore chemical phosphate and ribose modifications for Nme2Cas9 guide RNA. For this study I used NmeCas9 crystal structures to design and assess the impact of chemically modified Nme2Cas9 guides on gene editing in mammalian cell culture. Using a fluorescent reporter cell line that is activated upon nuclease activity, I have found that chemically-modified Nme2Cas9 guide RNAs composed of ≥50% or more ribose modification demonstrates cleavage activity akin to unmodified guide RNAs. These findings will inform the development of heavily or fully modified Nme2Cas9 guide RNAs that may enable new delivery approaches for Nme2Cas9 in the context of RNP, mRNA, and viral co-delivery.

Targeting chromosome rearrangements in cancer with CRISPR

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P.4

Background: We are developing a novel precision and personalized cancer therapeutic approach to specifically target cancer cells using CRISPR technology. This approach is universal to all cancers and not dependent on the unique biology of the tumor. The innovation of this approach is that chromosome rearrangement junctions (CRJs), which typically exist in the hundreds in each tumor cell, are targeted by guide RNAs that bring together two parts of a dCas9-fused endonuclease, Fok1, leading to the induction of DNA double strand breaks (DSBs) at sites of the targeted CRJs in cancer cells specifically.

Materials and Methods: HCT116 colon cancer cells and UMUC-3 bladder cancer cells were engineered to express Fok1-dCas9 and two pairs of CRJ-targeting gRNA under doxycycline regulation. Induction of DSBs at CRJs were assessed by γH2AX foci and γH2AX ChIP-PCR and cell death was assessed by apoptosis (PARP1 & caspase cleavage and flow cytometry for sub-G1 DNA content), clonogenic survival and tumor growth in vivo.

Results: Induction of Fok1-dCas9 lead to the induction of 1-2 DSBs per cell and promoted cell death when accompanied with the expression of pairs of CRJ-targeting gRNAs. Furthermore, induction of these CRISPR reagents in vivo resulted in significantly reduced tumor growth and prevention of metastasis.

Conclusions: We have shown that we can direct two parts of a dCas9-fused Fok1 endonuclease to sites of CRJs in cancer cells using CRISPR gRNAs leading to the induction of DSBs and loss of cell survival and tumor growth in vivo. If successfully implemented, the impact of our universal approach targeting CRJs in cancer with CRISPR could be transformative for cancer therapy.

5′ Modifications Improve Potency and Efficacy of DNA Donors for Precision Genome Editing

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P.5

Nuclease-directed genome editing is a powerful tool for investigating physiology and has great promise as a therapeutic approach to correct mutations that cause disease. In its most precise form, genome editing can use cellular homology-directed repair (HDR) pathways to insert information from an exogenously supplied DNA repair template (donor) directly into a targeted genomic location. Unfortunately, particularly for long insertions, toxicity and delivery considerations associated with repair template DNA can limit HDR efficacy. Here, we explore chemical modifications to both double-stranded and single-stranded DNA-repair templates. We describe 5′-terminal modifications, including in its simplest form the incorporation of triethylene glycol (TEG) moieties, that consistently increase the frequency of precision editing events in Caenorhabditis elegans, zebrafish, mice, and in cultured human cells.

Impairment of host ribosome integrity by Pseudomonas aeruginosa

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P.6

Translation is an essential cellular process that is commonly targeted for inhibition in antagonistic organismal interactions. Pathogenic bacteria hamper translation in their eukaryotic hosts to fend off the host immune response and promote host damage. To dysregulate translation, bacteria produce virulence factors that elicit deleterious modifications to the host ribosome or translation factors.

Pseudomonas aeruginosa inhibits translation in many hosts. For insects and mammalian hosts, a main virulence effector is the exotoxin A protein, which ADP-rybosilates the translation elongation factor 2. However, for the infection of Caenorhabditis elegans by P. aeruginosa, the precise pathways and mechanism(s) of translational inhibition are not well understood. We found that upon exposure to P. aeruginosa PA14, C. elegans undergoes a rapid loss of intact ribosomes accompanied by the accumulation of ribosomes cleaved at helix 69 (H69) of the 26S ribosomal RNA (rRNA), a key part of ribosome decoding center. H69 cleavage is elicited by certain virulent P. aeruginosa isolates in a quorum sensing (QS)-dependent manner and independently of exotoxin A-mediated translational repression. Among P. aeruginosa strains, the bacteria's capacity to induce H69 cleavage strongly correlates with the presence of R-bodies, a multi-protein bacterial virulence effector that promotes H69 cleavage. Consistent with H69 cleavage resulting from an extracellular bacterial virulence factor that is transferred to the worm's tissues, the H69 cleavage is predominantly localized in the worm's intestinal cells, increases with time of exposure of live bacteria to the intestinal lumen, and requires the activity of the worm's endocytic uptake machinery.

Genetic and genomic analysis suggests that H69 cleavage leads to the activation of the worm's zip-2-mediated defense response pathway, consistent with translational inhibition. Indeed, H69 cleavage is antagonized by the zip-2 pathway, as well as by the worm's two other major host defense pathways defined by pmk-1 and fshr-1. Taken together, our observations suggest that P. aeruginosa deploys a novel virulence mechanism to cleave the host's large ribosomal subunit RNA at H69, and induce ribosome degradation, thereby impairing host translation and hence blocking antibacterial responses.

Building a foundation for the development of RNA-based therapeutics against fungal infections

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P.7

Fungi infect over 1 billion people annually and kill at least 1.5 million per year, more than tuberculosis or malaria. The most dangerous fungal pathogens are those capable of causing invasive infections, primarily from the genera Aspergillus, Candida, Pneumocystis, and Cryptococcus. Aspergillus fumigatus represents the most important airborne fungal pathogen and is a widespread filamentous fungus capable of producing huge numbers of aerosol asexual spores. Although typically cleared from healthy individuals by an efficient innate immune response, A. fumigatus spores can initiate devastating invasive infections in immunocompromised hosts. Unfortunately, limited diagnostic markers and therapeutic options are available for treatment. RNA-based therapeutics offer a potential pathway forward, but difficulties remain. Previous study has shown that the cell wall of A. fumigatus is a cationic, impermeable barrier that limits uptake of many different drugs and sequesters negatively charged nucleic acids. Compellingly, recent work revealed that host-derived extracellular vesicles produced by neutrophils are capable of delivering antifungal cargo across this cationic barrier to inhibit fungal growth, yet the RNA content of these vesicles remains unexplored. Our newly established group is interested in identifying these extracellular RNAs and defining the composition of the vesicles for the design of smarter therapeutic delivery options in the future. In addition, we have been characterizing the small RNA machinery of A. fumigatus to better understand how a delivered RNA will be processed once internalized by the fungus. We know that the RNAi pathway in A. fumigatus is active and consists of two dicer-like enzymes, two argonaute proteins, and two RNA-dependent RNA polymerases. After creating knockouts of each of these components in A. fumigatus, we were able to show that only a few of these proteins are required for silencing of an induced inverted-repeat transgene. The RNAi machinery is highly conserved across a large number of sequenced A. fumigatus genomes, suggesting that it may be exploitable for RNA-based therapeutics in the future. This concept has already proven fruitful in the treatment of plant fungal pathogens, as exemplified by spray-induced gene silencing. In the future, we hope that our increased understanding of the small RNA biology of A. fumigatus will facilitate the development of RNA-based therapeutics against fungal pathogens and serve as a new way to combat these terrible infections.

Human zinc finger RNA-binding protein coordinates post-transcriptional regulation of innate immunity

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P.8

The cellular response to infection is tightly regulated to effectively combat pathogen invasion while preventing deleterious hyperactivation of inflammatory responses. Pathways promoting transcriptional activation of type I interferons have been studied extensively; however, contributions of post-transcriptional regulatory mechanisms to innate immune signaling are poorly understood. We recently identified the human zinc finger RNA-binding protein (ZFR) as a potent negative regulator of the type I interferon response. Our work established ZFR as a major splicing regulator during monocyte to macrophage differentiation, including a particularly important role in preventing aberrant splicing and nonsense-mediated decay (NMD) of mRNAs encoding the histone variant macroH2A1. In the absence of ZFR, macroH2A1 expression is abolished, leading to hyper-activation of type I interferon signaling in macrophages and fibroblasts.

Using eCLIP, we find that ZFR binds large intronic domains flanking regulated exons, often spanning several kilobases. Many ZFR target exons are flanked by introns previously characterized as being spliced with slow kinetics. Among such "detained" introns, ZFR preferentially regulates cassette exons that determine transcript susceptibility to NMD, including a set of exons in splicing factor genes overlapped by ultraconserved elements (UCEs). ZFR, itself encoded by a UCE-containing transcript, systematically promotes the expression of the NMD-insensitive isoforms of genes containing ultraconserved exons. We further explore physical and functional interactions between ZFR and two additional RNA-binding proteins previously implicated in innate immunity, ILF2/NF45 and ILF3/NF90. Together, our data reveal a complex post-transcriptional regulatory network responsible for modulating innate immune responses.

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Delivery of pro-angiogenic anti-miRs from self-assembled wound dressings to promote diabetic wound healing

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P.9

Non-healing ulcers are a common complication of diabetes, resulting in decreased quality of life, elevated rates of amputation, and increased risk of mortality. Unfortunately, current treatments remain outdated and inadequate, as they do not directly address the underlying molecular abnormalities of these chronic wounds. In diabetes, neuropathy and microvascular changes in the skin lead to dysregulated molecular cues, resulting in reduced angiogenesis and impaired wound healing. Poor angiogenesis is particularly critical given the importance of the vasculature in supplying oxygen, nutrients, and systemic signaling molecules. Impairment of angiogenesis is in part driven by aberrant expression of non-coding microRNAs (miRNAs, miRs). Each miRNA can regulate the expression of hundreds of genes along defined, tissue-specific signaling pathways; thus, miRNAs prove ripe for targeting. One promising approach to alter the course of diabetic ulcers is to directly target the expression of upregulated miRNAs in the non-healing state using miRNA inhibitors (anti-miRs); however, delivery challenges prevent the translation of these anti-miR therapies to the clinic. To address these delivery challenges, we have developed and demonstrated self-assembled electrostatic deposition of nucleic acids through the layer by layer (LbL) technique, which leverages iterative adsorption of polyelectrolytes of alternating charge to create conformal coatings on wound bandages with tunable release kinetics. One critical advantage of the LbL approach is that it enables loading of anti-miR with a cationic polymer to aid transfection. Thus far, we have identified potential synergies of pro-angiogenic anti-miRs (miRNA inhibitors), as synergy between anti-miRs could increase the efficacy of treatments by targeting multiple complementary pathways. We have also begun investigating the effects of anti-miRs delivered from self-assembled LbL wound dressings on endothelial cell phenotype in vitro. Ultimately, this work will enhance the delivery of anti-miRs to promote angiogenesis and healing of diabetic ulcers.

Innate Immune Sensing of HIV-1 in Human Myeloid Cells

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P.10

Priming naïve T cells by dendritic cells (DCs) that are sufficiently matured to produce co-stimulatory cell surface molecules and cytokines is required for protective antiviral immune responses. HIV-1 challenge of isolated DCs ex vivo yields only low levels of productive HIV-1 infection. However, infected cells exhibit innate activation with upregulation of co-stimulatory molecules, cytokines, and type 1 interferon. Experimental bypass of blocks to HIV-1 entry and reverse transcription permits more efficient transduction and DC maturation, as indicated by production of type 1 interferon and increased expression of interferon stimulated genes. However, the mechanism by which HIV-1 is detected to activate innate immune signaling remains unclear.

HIV-1 produces a primary transcript which then exists as three main classes of mRNAs; unspliced, partially spliced, and multiply spliced RNAs. While the multiply spliced HIV-1 RNAs are exported from the nucleus via the canonical NXF1 mRNA export pathway, intron-containing singly spliced and unspliced mRNAs are exported via the CRM1-dependent RNA export pathway. Previously we showed that CRM1-dependent export of intron containing HIV-1 RNA is required for innate activation of DCs. To elucidate the mechanism by which intron containing RNAs are sensed and cause innate activation in DCs we initiated a knock-down screen. We suppressed an extensive list of innate signaling pathways and sensors, in addition to the factors that were previously shown to bind full length HIV-1 RNA. In our assay, we examine the changes in maturation markers as well as expression of ISGs via flow cytometry, seeking to identify genes that are essential for innate immune activation of both DCs and macrophages upon HIV-1 challenge. Our data elucidated that MDA5 signaling is required for sensing HIV-1 RNA and activating type 1 interferon response in human primary cells.

Identifying Novel Gene Targets for Wound Healing siRNA Therapeutics with Publicly Available RNASeq

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P.11

Chronic ulcers are a highly prevalent disease among diabetic patients and are characterized by potentially serious complications, such as amputation. Hallmarks of chronic wounds include a [AGB1] persistent aberrant inflammatory profile, stalled re-epithelialization, and impaired angiogenesis. On the molecular scale, changes in soluble mediators lead to the development of these chronic wound abnormalities. The two standard methods of care for diabetic chronic wounds are debriding the wound area to restart the wound healing process and dressing the wound to maintain a moist environment; however, neither of these treatment methods directly address the underlying molecular and cellular mechanisms driving chronic healing. A deeper understanding of the gene expression signatures of diabetic wounds can inform the selection of novel targets of small interfering RNA (siRNA) therapy, which could promote healing at the genetic or pathway level. We performed DEseq analysis on publicly available RNA sequencing data from 27 diabetic and nondiabetic patients to quantify differential gene expression between these two subgroups. After labeling genes by molecular function via gene ontology, we applied enrichment test analysis to determine significant gene functionality related to wound healing processes. Our results identified 49 upregulated genes as potential siRNA therapy targets and confirmed significant changes in molecular functionality in fibronectin binding, phospholipase activity, and growth factor binding. These results lay important groundwork for optimizing targets for siRNA therapy to the wound site. Ultimately, we believe that this work may advance chronic wound care of diabetic patients by identifying novel genes and gene pathways involved in chronic wound healing as potential targets for siRNA therapy.

Novel Stealth Oxime Ether Lipid Vesicles for Enhanced DsiRNA Delivery and Gene Silencing to Human Lung Cancer Mouse Xenographs

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P.12

Cationic lipids are considered the lead candidates as delivery agents for RNAi therapeutics. To this end, we have previously reported that oxime ether lipids (OELs), a class of cationic lipids bearing hydroxylated polar head groups boost efficient delivery of functional DsiRNA in cell cultures. In this study, we examined the in vivo utility of these hydroxylated OELs using OEL4 (lipid 4, C14/C14) as a prototype. Our studies show that the surface modification of the OEL4 formulations is essential for their accumulation in tumors of mouse xenographs. The surface-modified OEL4 formulations were developed by inclusion of the PEG lipid (distearoyl phosphatidyl ethanol amine (DSPE)) with varying contents of PEG (PEG350-2000). The resulting PEGylated OELs retained their ability to form stable vesicles (up to 5 mol % of the corresponding PEG lipids), as well as their subsequent association with DsiRNA duplexes. However, the vesicle stability and gene knock-down potential was found to be dependent on the PEG chain length. OEL4 containing DSPE-PEG 350 and DSPE-PEG1000 promoted gene silencing (eGFP & luciferase) in cell culture assays, whereas OEL4/DSPE2000 formulations impaired the gene silencing. In vivo studies were conducted in mice bearing human lung cancer (A549-luc2) tumors following intravenous injections. For biodistribution studies, a fluorescent lipid probe, DiR was included in the formulations. Our data show that OEL4 vesicles formulated using 3 mol% DSPE-PEG350 accumulate in tumors at high efficiency as compared to other formulations with a benefit of tumor to liver ratios. These vesicles also showed a statistically significant luciferase signal reduction in tumors as compared to untreated mice. Taken together, OEL4/DSPE-PEG50 formulation serves as a novel candidate for delivery of RNAi therapeutics including functionalized RNA architectures.

Therapeutic Oligonucleotides to Increase Cytarabine Efficacy in Acute Myeloid Leukemia

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P.13

Acute Myeloid Leukemia (AML) is a highly heterogeneous and aggressive cancer of the hematopoietic system affecting approximately 1,300 Canadians yearly. Cytosine arabinoside (ara-C) has been the front-line curative treatment for AML for over 50 years. However, approximately 80% of these patients eventually relapse and succumb to the disease and there is no salvage regimen that currently exists. Therefore, increasing the effectiveness of ara-C during the initial treatment is crucial for improving overall patient survival. Our aim is to design therapeutic oligonucleotides that can regulate endogenous gene expression and reverse the loss of ara-C sensitivity. We designed small activating RNAs (saRNAs) and small interfering RNAs (siRNAs) to interrogate genes responsible for loss of ara-C sensitivity. We confirmed differing expression levels of candidate genes using quantitative PCR in AML cell models. Our work will help identify predictive biomarkers for loss of ara-C sensitivity and can lead to better stratification of AML patients for alternative treatment regimens. If successful, we envision a personalized medicine approach where patients with low sensitivity to ara-C are identified at the time of diagnosis, and a suitable therapeutic oligonucleotide is prescribed as an adjuvant therapy that increases the efficacy of ara-C.

Advancing Peptide siRNA-Carrier Designs through L/D-Amino Acid Stereochemical Modifications to Enhance Gene Silencing for the Treatment of Cancer

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The 599 peptide has been previously shown to effectively deliver siRNAs to cancer cells, inducing targeted-oncogene silencing, with a consequent inhibition of tumor growth. Although effective, this study was undertaken to advance the 599 peptide siRNA-carrier design through L/D-amino acid stereochemical modifications. Consequently, the 599 peptide was modified to generate eight different peptide variants, incorporating either different stereochemical patterns of L/D-amino acids or a specific D-amino acid substitution. Upon analysis of the peptide variants, it was observed that these modifications, could in some instances, increase/decrease the binding, nuclease/serum stability, and complex release of siRNAs, as well as influence the gene silencing efficiencies of the complex. Modifications to the 599 peptide also affected cellular uptake and intracellular localization patterns of siRNA cargo, with one particular peptide variant, comprising a specific D-amino acid substitution, capable of mediating a more ordered binding of siRNAs to specific cellular projections, identified as filopodia. Interestingly, this particular peptide variant also exhibited the most enhanced delivery of siRNAs into cells and gene silencing, thus, implying that this specific change to the 599 peptide design could be responsible for directing a more efficient mode of siRNA drug delivery, resulting in the enhancement of gene silencing. Of note, measurements of the physicochemical properties of the 599 peptide variants in complex with siRNAs, including particle size, zeta potential, and polydispersity index, did not reveal any distinguishable characteristics between the complexes that could account for the observed differences in siRNA cellular uptake patterns or that would imply that these differences were a consequence of variances in particle aggregation properties. Thus, taken together, these data demonstrate the utility of peptide stereochemistry, as well as the importance of a key D-amino acid modification, in advancing the 599 peptide siRNA-carrier design for the enhancement of gene silencing in cancer cells.

Investigating the role of different HTT mRNA isoforms in Huntington's disease progression

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Huntington's disease (HD) is a rare autosomal dominant genetic disease caused by the expansion of a CAG trinucleotide repeat in exon 1 of the Huntingtin gene (HTT). CAG repeat expansion results in the production of a mutant Huntingtin (mHTT) protein that is prone to forming aggregates or "inclusions" in the nucleus and cytoplasm of neurons. mHTT inclusions cause neurodegeneration throughout the brain, particularly the striatum, leading to cognitive loss, mood swings, and chorea in patients. CAG repeat length is strongly associated with age of HD onset – individuals with 30-60 CAG repeats will experience later onset (30-40 years old), whereas those with >70 CAG repeats will have juvenile onset. Moreover, the CAG repeat expansion, which is somatically unstable, undergoes length increases over time Transcriptome Wide Association Studies have already identified a potential modifier of somatic expansion and HD progression– MSH3 (DNA mismatch repair co-factor), but other factors remain elusive.

The Bates group previously discovered that mHTT mRNA can be mis-spliced at the exon1-intron1 junction, producing a truncated protein, HTT1a. HTT1a production is positively correlated to CAG repeat length, with the highest HTT1a levels detected in juvenile HD brain.

The Khvorova lab developed divalent small interfering RNA (di-siRNA) – a novel siRNA chemical design that potently silences HTT mRNA in brain for 6 months after a single treatment.Our studies utilize the di-siRNA architecture, to selectively target MSH3, HTT1a and mHTT. We ICV injected these tools into the Q111 mouse model, which is known to form inclusions and undergo somatic instability, in the context of the mouse Htt gene. Our results show statistically significant silencing of MSH3 (80%) and mutHTT (94%) throughout the regions tested (Striatum, Medial Cortex, Posterior Cortex and Thalamus). We are the first to report that silencing of MHS3 complete arrests somatic expansion. RNAscope highlights that silencing of mHTT results in upregulation of HTT1a foci in the Striatum. Overall, our findings are leading to a better understanding of the mechanism of HD, as well as redefining the need for multiple clinical targets for an effective treatment of HD.

Selective Cancer Cell Killing by Dual siRNA Knockdown of CD320 and LRP2 Receptors

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RNA interference is often used to uncover synthetic lethality wherein knocking down expression of two or more genes results in cytotoxicity to cancer cells with little or no effect on normal cells. CD320 and LRP2 are two cell membrane receptors in the low-density lipoprotein receptor family. CD320 internalizes the complex of cobalamin (Vitamin B12) bound to its plasma chaperone protein transcobalamin II (TCN2) in nearly all cells. LRP2 also internalizes the cobalamin-TCN2 complex, in addition to more than 50 other ligands, and is strongly expressed in the kidney. CD320 and LRP2 are known to be overexpressed in some cancer cells. We studied the cytotoxic effect of simultaneously knocking down the cell surface receptors CD320 and LRP2 on cancer cells from diverse tissues and on normal fibroblasts. To this end, a panel of commercially available human cancer cell lines from lung (HCC15, A549), breast (MDA-MB-231, MCF-7), prostate (DU145, PC-3, LNCaP), brain (A172, U251), and skin (MDA-MB-435S), as well as normal human fibroblasts (GM05659) were transfected with siRNAs targeting mRNA for receptors CD320 and LRP2, singly and in combination. Knockdown of CD320 and LRP2 protein expression (up to 90%) was confirmed by western blotting. Simultaneous knockdown of CD320 and LRP2 in normal fibroblasts did not affect their cell proliferation, as measured by the Cell Titer-GLO® assay. However, knockdown of CD320 and LRP2 in cancer cell lines derived from diverse tissues inhibited cell proliferation or killed the cells by up to 80% relative to an irrelevant siRNA control. Interestingly, knockdown of either CD320 or LRP2 individually resulted in a concurrent increase in expression of the other receptor in some cell lines, suggesting that cells compensate for loss of either CD320 or LRP2 by increasing expression of the other receptor. Silencing both receptors was required for optimal cell killing. We conclude that simultaneous knockdown of CD320 and LRP2 targets a fundamental vulnerability that is present in diverse cancer cells but not normal cells. The mechanistic rationale for this vulnerability is currently under study.

Pre-clinical Assessment of SLN360, A Novel Short Interfering Ribonucleic Acid Targeting LPA, Developed to Address the Unmet Medical Need of Elevated Lipoprotein (a) in Cardiovascular Disease

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Introduction: SLN360 is a liver-targeted N-acetyl galactosamine-conjugated siRNA with a promising profile for addressing Lp(a)-related cardiovascular risk. Here we present the pre-clinical pharmacodynamic effects of SLN360 in female cynomolgus monkeys and findings from key pre-clinical safety studies.

Methods: SLN360 was tested in vitro in primary human hepatocytes for LPA knockdown and effects on hypothetical off targets identified through in silico screening. Healthy female animals were dosed subcutaneously with SLN360 at 3 and 9mg/kg (single dose) or 3 weekly doses at 3mg/kg. Liver biopsies were taken 2, 4 and 6 weeks after final dosing for mRNA analysis and serial serum samples were taken up to the end of the study on day 63 for biomarker analysis. In a GLP safety study, healthy cynomolgus monkeys received 5 once weekly subcutaneous injections of saline control or SLN360 (3 males / 3 females per group, up to 200mg/kg) followed by an 8-week recovery period (2 males / 2 females in saline and top dose). A standard battery of safety assessments was performed.

Results: In vitro, SLN360 reduced LPA expression in primary human hepatocytes with no effects on any hypothetical liver-expressed human off-target genes at concentrations >300-fold the human LPA IC50. In vivo, maximal liver LPA reduction was observed two weeks after final subcutaneous injection regardless of treatment schedule (up to 91%) and maintained 6 weeks after dosing in the 9mg/kg or 3x3mg/kg group (85% and 88% knockdown, respectively). Peak serum Lp(a) reductions (85->95%) were seen at day 21. At day 63 reduction of serum Lp(a) was still maintained (50->95%). No consistent effect on the expression of PLG or a panel of sensitive markers of altered liver lipid or lipoprotein metabolism was observed. In cynomolgus GLP safety studies, no clinical observations were observed following 5 subcutaneous doses of SLN360. Liver LPA mRNA levels were significantly reduced by 98% at day 30 and 95% after the 8-week recovery period, while serum Lp(a) was undetectable at both timepoints. Findings were restricted to the liver (increased organ weight and diffuse hepatocyte hypertrophy) and lymph nodes (vacuolated macrophages) at day 30 and considered non-adverse due to reversibility after recovery. No dose-related changes in other safety assessments were noted. The NOAEL was defined as 200mg/kg, >60-fold the active dose in cynomolgus monkeys.

Conclusions: The preclinical profile of SLN360 indicates suitability for entry into clinical studies. SLN360 has potential to address the unmet need of Lp(a) reduction in cardiovascular diseases.

Developing AAV-RNAi Mediated Gene Therapy for Adult Polyglucosan Body Disease and Lafora Disease

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Adult polyglucosan body disease (APBD) and Lafora disease (LD) are rare, autosomal recessive glycogen storage disorders that present mainly with neurological symptoms. APBD is caused by mutations in the glycogen branching enzyme (GBE1) gene and characterized by progressive upper and lower motor neuron dysfunction and premature death. LD is a fatal progressive myoclonus epilepsy disease caused by loss-of-function mutations in EPM2A or NHLRC1. In LD, symptoms typically start in previously healthy adolescents, usually with action and stimulus sensitive myoclonus. Initial symptoms rapidly evolve into progressive dementia, refractory epilepsy, cerebellar ataxia, respiratory failure, and patients die within about a decade of diagnosis. These clinically distinct diseases share a common pathology. Both APBD and LD are characterized by abnormal glycogen accumulation, namely the polyglucosan bodies (PBs) in various tissues, especially in the brain and central nervous system. PBs are poorly branched, insoluble, aberrant cytoplasmic glycogen inclusions, and are the principal driver of neurodegeneration and other brain abnormalities. Preventing formation of PBs by inhibiting glycogen synthesis presents a reasonable approach to treatment strategy for APBD and LD. We hypothesized that inhibiting glycogen synthase 1 (Gys1) thereby decreasing glycogen synthesis in the brain might prevent abnormal glycogen formation and rescue the neurological phenotypes in the diseases' mouse models. To test this hypothesis in a proof-of-principle study, we have pursued an adeno-associated viral vector (AAV) mediated RNA-interference (RNAi) dependent gene therapy approach. After screening multiple candidates in vitro, we packaged the artificial miRNA with highest Gys1 knock down efficiency in a recombinant adeno-associated virus (rAAV9). AAV9-RNAi was administered to the three diseases' mouse models at postnatal day 2 by intracerebroventricular (ICV) injection and mice were sacrificed at 3 months of age. The effects of treatment on PB quantity and neuroinflammation markers were assessed.

Administration of AAV9-RNAi resulted in approximately 15% reduction of Gys1 mRNA across the brain and approximately 40% reduction of GYS1 protein, of abnormal glycogen accumulation and of PB. Improvements in neuroinflammatory markers in all three mouse models were observed. Our results demonstrated that RNAi-mediated silencing of Gys1 mRNA provided benefit in APBD and LD mouse models by decreasing abnormal glycogen formation in the brain. Our work suggests a potential for benefit of a virally-delivered RNAi neurotherapeutic for APBD and a childhood-onset LD neurological disease.

Antisense oligonucleotides targeting FXN intron1 can activate FXN expression

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Friedreich's ataxia (FA) is the most common inherited ataxia, affecting 1 in 50,000 people in the United States. FA causes progressive damage to the nervous system, muscle, pancreas and heart, leading to significant morbidity and mortality.

FA is caused by an expanded GAA repeat in the first intron of the Frataxin (FXN) gene that induces increased R-loop formation and reduced FXN expression. Despite extensive ongoing efforts to develop various therapeutic modalities including small molecules, gene therapy, stem cell therapy and oligonucleotides, no disease-modifying therapy has yet been approved.

Previous work showed that GAA-repeat-targeted oligonucleotides could activate FXN expression in various disease models including fibroblasts and reprogrammed neural progenitor cells from FA patients. Here, we developed antisense oligonucleotides (ASOs) that can target non-GAA-repeat sequences of FXN intron 1 RNA and restore FXN mRNA to WT levels in two primary patient-derived fibroblasts with 330-540 GAA repeats. Interestingly, these ASOs can also further increase FXN mRNA levels in WT fibroblasts, 293T and U87 cells. These data suggest our ASOs activate FXN expression in a GAA-repeat-expansion independent manner and hold promise as a potential therapy to treat FA.

Cell type-specific gene silencing by tethered microRNAs

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P.20

Currently, no delivery strategy can silence disease-causing or viral mRNAs in some tissues but not others, irrespective of delivery specificity. Cell type-specific mRNA silencing requires siRNA formulation with tissue-specific nanoparticles or conjugation to specific ligands. siRNAs have only shown promise in human clinical trials for targets in hepatocytes. The competing needs to design siRNAs that effectively trigger RNAi in vivo, resist nucleolytic degradation, and accumulate in the correct cell type pose major challenges to the use of siRNAs to treat extra-hepatic diseases and viral infections. Our microRNA (miRNA) tethers elicit silencing in just one or a small number of tissues, irrespective of delivery specificity, because silencing occurs only in those cells that produce a specific miRNA. miRNAs are abundant regulators of mRNA expression, reaching ≥50,000 molecules per cell. The most abundant miRNAs are typically cell type-specific: miR-1 is restricted to muscle, miR-7 and miR-124 are limited to brain, while miR-122 is hepatocyte-specific. miRNAs can have increased expression as a result of a particular pathological state or viral infection, and these can be recruited to silence an mRNA whose expression drives the development of disease. Thus, miRNA tethers may delay the onset or impede progression of diseases in which expression of a miRNA is increased. miRNA tethering is thus the only therapeutic strategy that limits silencing to the targeted cell type even when the therapeutic agent is delivered systemically to many tissues. Our technology enables a new class of drug to silence mRNAs with increased specificity. We will present data from experiments that evaluate the modification chemistry and specificity of silencing of our tether oligonucleotides.

When less is actually more: in vitro assessment of the potential of anti-XYLT1 siRNAs to promote substrate reduction in Mucopolysaccharidosis type III

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Mucopolysaccharidosis type III (MPS III) refers to a group of four autosomal recessive neurodegenerative lysosomal storage disorders (LSD) caused by the incomplete lysosomal degradation of the heparan sulphate (HS) that accumulates in patient cells and triggers disease.

Degeneration of the central nervous system is the major hallmark of these disorders, resulting in mental retardation and hyperactivity. By their mid-teenage years most affected patients are dependent on their caregivers for all needs and death occurs at the end of the second or early in the third decade of life. The classical therapeutic approach for LSDs, enzyme replacement therapy, would hardly rise as a potentially successful tool to reduce the disease burden in MPS III patients due to the inability of the recombinant enzymes to cross the blood-brain barrier (BBB), having no impact in neuropathology. Thus, there is no effective therapy available, with treatment limited to clinical management of neurological symptoms. A tempting alternative, however, would be to block substrate accumulation upstream, by decreasing its synthesis. That concept is known as substrate reduction therapy (SRT).

In order to decrease HS storage inside the lysosomes, we designed and assayed in MPS III patients' fibroblasts a specific siRNA pool targeting XYLT1, a gene that encodes an enzyme involved in an early stage of the HS biosynthetic cascade.

Fibroblasts from MPS IIIA, B, C and D patients were transfected with the designed siRNAs pool to inhibit XYLT1. Cell pellets were collected 24/48/72 hours and 7 days post- transfection and total RNA extracted. Target mRNA levels were evaluated through qRT-PCR using the 2-ΔΔCq method. Additionally, the effect on HS accumulation was quantified 24 and 48h after transfection using a modified 1,9-dimethylmethylene blue assay.

The results showed a high inhibition of the XYLT1 gene mRNAs (around 80%) and a decrease in GAGs storage (only assessed for types C and D until now). Currently, we are evaluating the effect of that decrease on the overall GAGs storage 7 days post-transfection, also with promising results.

Here we present an overview on the current results of this project, while discussing its next steps, namely the development and evaluation of vectors for in vivo delivery. Our goal is to develop targeted stable nucleic acid lipid particles (t-SNALPs) coupled with different ligands, to promote cell uptake of the 'anti-GAG' siRNAs in a variety of cells, including neurons.

Computing siRNA Silencing Efficacy using Supervised Machine Learning

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P.22

The wide variation in small interfering RNA (siRNA) efficacy makes siRNA design a challenge. Extensive chemical modifications on siRNAs have ameliorated challenges with RNA-induced Silencing Complex (RISC) loading and product release, however effects of RISC entry and targeting on efficacy remain enigmatic and difficult to predict. Computational methods to predict siRNA efficacy are necessary primary screening tools for designing siRNAs both for therapeutics and research tools. These methods enable efficient identification of potent siRNA sequences, reducing the number of potential siRNA target sites among the potentially thousands possible. Current computational methods often fail to predict siRNA efficacy, necessitating large screens to identify potent siRNAs, a time consuming and costly process that can still fail to identify potent siRNAs. Here we develop a computational model utilizing only sequence preferences in the target mRNA to effectively predict siRNA efficacy. We consider the RISC target site as well as regions flanking it, to encapsulate sequence-specific effects of both RISC entry and targeting. We assess several computational models from simple linear regression to supervised machine learning models including random forest classification and support vector machines, to develop a method to accurately predict siRNA sequences for any mRNA target sequence. In addition to presenting an accurate siRNA selection method, we present an adaptable siRNA prediction method development pipeline. Our pipeline requires minimal data (siRNA sequences and their corresponding gene knockdown efficiencies) and can be adapted to any siRNA dataset to develop siRNA prediction algorithms tailored to specific dataset types and assay requirements.

Cationic Lipopolymers: A Versatile Class of Materials for Delivery of Gene Medicines

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P.23

Polyethylenimine (PEI) is commonly employed as a non-viral gene delivery vector due to high cationic charge density that is suitable for strong interactions with polynucleotides. The PEI's functional groups (amines) are also conducive for various chemical modifications and functionalizations. To design improved vectors, we have been exploring cationic lipopolymers prepared by grafting lipidic moieties onto small molecular weight (0.6 to 1.8 kDa) PEI. Such lipopolymers inherit the beneficial features of their building blocks such as low cytotoxicity, lipophilicity, buffering activity and strong nucleic acid binding and condensation [1]. These critical properties synergize to enable effective and versatile delivery of a range of polynucleotides, which are condensed by the lipopolymers into 'nano-sized' particles suitable for cell uptake. The efficiency of lipopolymers, however, were found to depend on (i) type of nucleic acid, (ii) nature and amount of lipid substituted, and (iii) cell phenotype intended for delivery.

Here, we provide a summary of synthesis of lipopolymers and typical outcomes of non-viral polynucleotide delivery. Nucleic acids used include plasmid DNA (pDNA) intended for protein expression, short interfering RNA (siRNA) intended for gene silencing, microRNA that regulates signaling pathways and messenger RNA (mRNA) for protein expression. The cell types include attachment dependent cells (typically breast cancer cells) and suspension growing cells (typically leukemia cells). While we present representative cell culture results, we encourage the readers to inspect our publications for the use of the lipopolymers in different animal models.

Therapeutic DNA/RNA Hybrid Nanoparticles

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P.24

Complementary hybrid DNA/RNA constructs can be designed to release embedded functional RNA solely upon reassociation through their toeholds. By manipulating the DNA length, hybrids can be programmed to re-associate through either DNA or RNA toeholds, according to the desired purpose. Generally, these hybrid constructs present several advantages over double stranded RNA: the release of the active RNAs is conditional to the hybrids reassociation, and they present low cytotoxicity and lower immunogenicity than either DNA or RNA. Here, we present RNA/DNA hybrid constructs with RNA toeholds functionalized for therapeutic purposes. We validated the hybrids in vitro, in cell culture, and in vivo. Hybrids formation, reassociation, and release of functional RNA, and stability in serum, were characterized in vitro by electrophoretic mobility shift assays. To study the potential of these hybrids as therapeutic agents, we incorporated Dicer substrate RNAs (DsiRNAs) targeting overexpressed genes in the apoptotic pathways: Polo-like kinase 1 (PLK1), B-cell lymphoma 2 (BCL2), and Survivin. The release of DsiRNAs should knock down gene expression, arrest cell cycle, and restore apoptosis. We chose human colorectal adenocarcinoma cell line HT29 as a cancer model for validating them in cell culture and in vivo. Hybrid toxicity and efficacy in HT29 cell culture were determined by flow cytometry, cell cycle assays and cell viability assays. We used xenograft mouse models to assess the hybrid toxicity and efficacy in vivo. The preliminary studies revealed that hybrids are not toxic at the studied doses and significantly slow down tumor growth compared to untreated mice. We confirmed the downregulation of targeted genes and induction of apoptosis through antibody staining and TUNEL assays ex vivo. We conclude that hybrids can successfully reduce tumor growth in vivo. The therapeutic potential of this technology can be explored further by incorporating a variety of therapeutic RNAs according to the overexpressed gene signature of any particular disease or cancer type.

siRNAs are efficient in switching Tau splicing isoforms in a cellular model of tauopathy

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P.25

Tauopathies are neurodegenerative diseases characterized by the abnormal processing of protein Tau, and its accumulation as insoluble neuronal deposits. Encoded by the MAPT gene, the Tau protein is implicated in several important neuronal functions: microtubule dynamics, neurite outgrowth, axonal transport.

MAPT pre-mRNA splicing is finely regulated. In particular, alternative splicing of exon 10 (E10) ensures that, in the adult human brain, neurons contain equally balanced amounts of Tau protein splicing isoforms, with three or four repeats of microtubule binding domains, respectively named the “3R” and “4R” isoforms.

FrontoTemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17) is a tauopathy caused by mutations in the MAPT gene. Approximately half of the mutations in MAPT affect splicing-regulating sequences, causing exon 10 aberrant inclusion, and aberrant production of 4R Tau. This, in turn, causes the tauopathy and results in the accumulation of Tau in protein aggregates called “neurofibrillary tangles” (NFT).

Aiming at developing an RNA therapy for FTDP-17, we designed fourteen exon-specific siRNAs to selectively degrade E10-containing MAPT mRNAs - with a caveat. RNAi is in fact known to also take place in the nucleus and the siRNAs might have in principle degraded the totality of the nascent pre-mRNA, thus not reaching the desired effect of re-establishing the correct isoform balance. By analysing the literature, we have in fact found very few examples of effective exon-specific siRNAs.

Nonetheless, we screened the fourteen MAPT exon 10-targeting siRNAs, using a dual-fluorescent MAPT splicing reporter minigene to evaluate their efficiency in reverting E10 inclusion. We also validated the results by RT-PCR and Western blots. Out of the fourteen siRNAs, we identified only three siRNAs which were effective in restoring the 3R/4R balance, and which we patented (WO 2016/151523). By RT-PCR, Western blots and immunofluorescence we validated the three siRNAs in neuronally differentiated human iPS cells bearing the FTDP-17- causing mutation E10+16 (Verheyen et al., 2018). We are now performing preclinical proof-of-concept studies in FTDP-17 mice models bearing human MAPT gene with mutation E10+16 (Umeda et al., 2013).

siRNAs Modified at the 2′ Position and with N6-Methyl Adenosine (m6A): Synthesis and RNAi Activity

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P.26

N(6)-methyladenosine (m(6)A) is a naturally occurring DNA and RNA modification that is important in epigenetic and epitranscriptomic regulation mechanisms. Such natural modifications may reduce undesired immune responses if used in synthetic oligonucleotide drugs such as siRNAs. We have studied the effects of this natural base modification within the same residue as common sugar modifications 2'-O-methyl (2'-OMe) and 2'-fluoro (2'-F) in the context of siRNA. The 2′-F- and 2′-OMe-modified m6A residues are thermodynamically less destabilizing in RNA:RNA duplexes than in DNA:DNA duplexes; this was expected based on the sugar-pucker of the 2′ modification. When incorporated at the terminus of an oligonucleotide, 2′-F m6A resulted in comparable stability in the presence of 5′-exonuclease stability to 2'-F adenosine, whereas 2′-F m6A and 2′-OMe m6A were less resistant to 3′-exonuclease than the parent 2'-modified analogs. Thus, phosphorothioate linkages were needed to maintain nuclease stability when m6A residues were incorporated at the 3′- termini of an siRNA. Interestingly, 2'-modified m6A did not adversely impact gene silencing in cell culture when incorporated at any single adenosine residue in either sense or antisense strand of ligand-conjugated siRNAs targeting three different gene targets (TTR, C5, and β-catenin). Thus, 2′-F- and 2′-OMe-modified m6A does not interfere with catalytic activity of the RNAi machinery.

Design, synthesis and evaluation of a PAD-4 siRNA in a rat model of COPD

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P.27

Chronic obstructive pulmonary (COPD) disease is defined as a chronic respiratory disorder that progresses slowly, it is characterized by an obstructive ventilatory pattern. Globally, it is estimated that 3.17 million deaths were caused by this disease in 2015 (that is, 5% of all deaths globally in that year), which makes it a public health problem. Although the pathogenesis of COPD is not fully understood, It's believed that the neutrophil and some other phagocytic cells are the main effectors of lung damage, in the case of neutrophils a dysregulated increase in the formation of neutrophil extracellular traps (NETs) has been reported in COPD. On the other hand, It´s necessary some enzymes produce the NETs, one of these is the protein is the arginine deiminase 4 (PAD-4) that catalyze changes in chromatin structure (chromatin decondensation). For that reason, we proposed the synthesis and evaluation of PAD-4 siRNA as a possible alternative treatment for COPD. We designed the siRNA using siRNA Wizard, RNAfold, and EMBOSS software and we synthesized the oligonucleotides in MERMADE 8 equipment. For the evaluation, we used a cadmium model (administration of 5 mg/kg in 5 daily doses of cadmium intraperitoneally), after 1 week of cadmium administration, we administrated the siRNA and after 2 weeks of cadmium administration, we determined the respiratory parameters by whole-body plethysmography, lung and heart weights and the expression of PAD4, TNFα, and BNP by RT-PCR. The results showed that the administration of cadmium significantly increases the levels of PAD4 and TNFα and decreases the BNP expression, lung and heart weight, and respiratory AUC compared with the non-cadmium group. Interestingly, the administration of siRNA decreases the expression of PAD4 and increases TNFα and BNP expression lung and heart weight and the AUC, significantly compared with the cadmium group. Our results suggest that the PAD4 siRNA could be an option for the treatment of COPD.

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Targeting “Undruggable” Transcription Factors using Therapeutic Oligonucleotides

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P.28

Acute myeloid leukemia (AML) is a highly heterogeneous and aggressive cancer of the hematopoietic system affecting >10,000 individuals world-wide. Approximately 30% of AML cases have an internal tandem repeat mutation within the juxtamembrane domain of the FMS-like tyrosine kinase 3 receptor (FLT3-ITD), leading to constitutive kinase activity promoting cell proliferation and survival in leukemic cells. Even with the current front-line treatments, AML patients with FLT3 mutations have an unfavorable prognosis and an increased risk of relapse than patients without the mutation. Importantly, certain essential transcription factors for hematopoiesis, such as PU.1, are dysregulated in AML cases with FLT3 mutations. These currently “undruggable” targets can be selectively modulated with the use of therapeutic oligonucleotides. To develop a treatment for AML, two strategies will be tested: 1) decreasing gene expression using duplex small-interfering RNA (siRNA) selectively targeting specific mRNAs for catalytic degradation; 2) increasing transcription initiation using small-activating RNA (saRNA) targeting a unique promoter sequence. Candidate siRNA and saRNAs were designed, transfected into cells using nucleofection and screened using RT-qPCR to evaluate changes in mRNA in cells lines derived from AML patients with FLT3-ITD mutations. By validating and optimizing new siRNA and saRNA drug candidates, the use of TOs in the clinic may significantly improve prognosis for AML and other diseases with “undruggable” targets.

Accumulation of AAV Vectors in the Pineal Gland After Focal Injections into the Brain or Spinal Cord

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In the course of studies of AAV-based gene modification for spinal cord injury repair, we made the surprising discovery that with injections of different AAV serotypes into the brain or spinal cord, AAV accumulates selectively in the pineal gland. We discovered this initially in studies in which AAVshPTEN/zsGreen was injected into the motor cortex in rats (AAV2/9 vector). Visualization of brains with epifluorescence revealed striking zsGreen labeling of the pineal gland in the majority of rats. Immunostaining with antibodies for cell types in the pineal gland revealed that zs Green was within pinealocytes. Similar labeling of the pineal gland was seen in the majority of rats following small injections of AAVshPTEN/zsGreen into the hippocampus. Using a different approach and vector, we found similar selective accumulation of AAV2 in the pineal gland following injections of AAV2/Cre into the spinal cord of stop-flox tdT reporter mice in which Cre-mediated recombination leads to activation of tdT expression. TdT expression was evident even when injections were made into the lumbar spinal cord many mm distant from the pineal gland. Possible explanations are that focal injections of AAV into CNS parenchyma leads to some leakage into the CSF, and then selective accumulation in pinealocytes of the pineal gland or uptake by some cell type at the injection site that subsequently migrates to the pineal. It remains to be seen whether other AAV serotypes have similar characteristics, especially those being developed as human therapeutics for neurological disorders. Together these findings suggest the need for further studies of functional consequences and possible adverse effects of AAV-driven expression of therapeutic genes in pinealocytes.

RNAi-based modulation of interferon-gamma signaling for the treatment of vitiligo

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P.31

Vitiligo is an autoimmune disease mediated by cytotoxic CD8+ T cells that attack pigment-producing melano-cytes, thus leads to white patches in the affected areas of skin. One reason for the poor efficacy of vitiligo treatments is due to the lack of well-defined pathogenic targets. It has recently become apparent that, during vitiligo progression, chemoattractants induced by interferon-gamma create a positive feedback loop for skin infiltration of auto-reactive CD8+ T cells and promote the killing of melanocytes. Here, in vivo delivery of fully chemically stabilized siRNA targeting interferon gamma receptor significantly reduced its downstream chemo-kine CXCL9 expression. In a vitiligo mouse model, the treatments prevented both local and systemic skin depigmentation. These results potentiate an effective, and new treatment paradigm for vitiligo and other auto-immune diseases.

Impact of scaffolding protein TNRC6 paralogs on gene expression and splicing

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P.32

TNRC6 is a scaffolding protein that bridges interactions between small RNAs, argonaute protein (AGO), and effector proteins to control gene expression. There are three paralogs in mammalian cells, TNRC6A, TNRC6B, and TNRC6C. These paralogs have ~40% sequence identity and whether they have unique or redundant functions is unclear. Here, we use knockout cell lines, enhanced crosslinking immunoprecipitation (eCLIP), and high-throughput sequencing to explore the roles of TNRC6 paralogs in RNA-mediated control of gene expression. We find that that the paralogs are largely functionally redundant and changes in levels of gene expression are well-correlated with those observed in AGO knockout cell lines. Interestingly, splicing changes observed in AGO knockout cell lines are observed in TNRC6 knockout cells. We do not observe a role for the TNRC6 paralogs beyond their role as scaffolding proteins for AGO. These data further define the roles of the TNRC6 isoforms as part of the RNA interference machinery.

Optimized Conditions for the Efficient Chemical Adenylation of DNA Oligonucleotides

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P.33

Adenylated DNA oligonucleotides are used as ligation donors in most small RNA sequencing library preparation protocols. Due to the high cost of purchasing pre-adenylated DNA, many labs purchase 5′ phosphorylated DNA and adenylate it themselves using either enzymatic or chemical methods. Enzymatic adenylation reactions provide a high yield but generally are done on a small scale and are expensive to scale up. Chemical adenylation uses an imidazole-activated adenosine monophosphate (ImpA), where the imidazole acts as a leaving group for the 5′ phosphate nucleophile of a DNA oligonucleotide. This chemical adenylation reaction is easily scalable but standard protocols give poor yields and require labor-intensive purification steps either through gel extractions or HPLC purification. Using standard molecular biology reagents, conditions were found that yield >90% adenylated DNA oligonucleotide product, negating the need for additional purification steps. This makes chemical adenylation of DNA adapters an easy, inexpensive, and scalable option.

RHAMMB-mediated bifunctional nanotherapy targeting Bcl-xL and mitochondria for pancreatic neuroendocrine tumor treatment

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P.35

The incidence of pancreatic neuroendocrine tumor (PNET) has continued to rise. Due to their indolent feature, PNET patients often present with incurable, metastatic diseases. Novel therapies are urgently needed. We have previously shown that Receptor for Hyaluronic Acid-Mediated Motility isoform B (RHAMMB) and Bcl-xL are upregulated in PNETs and both of them promote PNET metastasis. Because RHAMM protein is undetectable in most adult tissues, we hypothesized that RHAMMB could be a gateway for nanomedicine delivery into PNETs. To test this, we developed RHAMMB-targeting nanoparticle. Inside this nanoparticle, we assembled siRNA against Bcl-xL (siBcl-xL) and mitochondria-fusing peptide KLA. We demonstratsed that RHAMMB-positive PNETs picked up the RHAMMB-targeting nanoparticles. siBcl-xL or KLA alone only killed 30% of PNET cells. In contrast, a synergistic killing effect was achieved with the co delivery of siBcl-xL and KLA peptide in vitro. Unexpectedly, siBcl-xL induced cell death before reducing Bcl-xL protein levels. The systemically-injected RHAMMB-targeting nanoparticles carrying siBcl-xL and KLA peptide significantly reduced tumor burden in mice bearing RHAMMB positive PNETs. Together, these findings indicate that the RHAMMB targeting nanotherapy serves as a promising drug delivery system for PNET and possibly other malignancies upregulating RHAMMB. The combination of siBcl-xL and KLA peptide can be a therapy for PNET treatment.

Strategic Optimization of siRNA Chemistry for Safe and Enhanced Silencing of Placental sFLT1

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P.37

Pre-eclampsia (PE) is a serious and possibly fatal complication of pregnancy that is characterized by hypertension, proteinuria and eventual multi-organ failure, making it one of the leading causes of maternal morbidity and mortality in the world today. Affecting 5-8% of all pregnancies, PE also imposes a significant economic burden, with an annual cost of $2.18 billion in the U.S. alone. Previous studies have shown that maternal levels of circulating soluble vascular endothelial growth factor receptor FLT1 (sFLT1) are positively correlated with the development of PE. Building on previous work from our lab demonstrating that RNAi based modulation of placental sFLT1 is effective in reducing both sFLT1 levels and clinical signs of PE, we showcase key advancements in safety and efficacy of our PE therapeutic siRNAs.

Through strategic optimization of siRNA strand lengths, 2'-OMethyl/-fluoro composition, guide strand 5' chemical moiety and hydrophobic conjugate, we show that our advanced siRNA possess increased placental accumulation, improved sFLT1 silencing, reduced accumulation in bone marrow immune cells and a wider therapeutic index as evidenced by high dose toxicity studies compared to the first generation of PE siRNA therapeutics. These advancements represent a crucial step towards a clinical treatment for PE with the potential to supersede current symptomatic management approaches and significantly reduce pre-eclampsia related pre-term deliveries.

Activation of Vitamin E-modified siRNA for Gene Silencing Based on Tetrazine-Induced Bond-Cleavage Reaction

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Temporal activation of siRNA provide a valuable way to improve some properties (delivery efficiency, off-target effect, etc) of siRNA while keeping the activity of siRNA. However, current activation method mostly relies on light or endogenous stimuli. The light activation method are limited for its cell toxicity and poor tissue penetration. The endogenous responsive methods are limited for its unstability. Here we developed a new method for chemical activation of siRNA based on tetrazine-induced bond-cleavage reaction. We constructed chemical caged siRNA with 5' -vitamin E-benzonorbonadiene-modified antisense strand targeting green fluorescent protein (GFP) gene and mitotic kinesin-5 (Eg5) gene. In the GFP gene silencing experiments, we achieved activation of modified siRNA with tetrazine and found that the leakage of siRNA activity was related to the linker length which connected vitamin E and siRNAs. In the Eg5 gene silencing experiments, we found that caging effect was partially contributed by the biological activity of vitamin E, which might provide a new concept of caging strategy.

Gene Activation by Noisy Splicing Suppression using Antisense Oligonucleotides (ASOs)

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Alternative RNA splicing is a major source of genetic diversity across organisms. However, the choice of splice sites by the spliceosome is prone to errors, creating nonproductive transcripts that are likely decayed before translation. These nonproductive or “noisy” sites of pre-mRNA splicing waste cellular resources by creating non-functional transcripts that are prematurely degraded and have very brief half-lives. Re-direction of the spliceosome away from noisy sites can result in increased functional splicing, thereby upregulating gene expression. Using nascent RNA sequencing, these nonproductive splice sites can be enriched and hence, more easily identified and targeted. To prevent the spliceosome from using these sites, oligonucleotide steric blockers can be designed to physically occlude the splicing complex and prevent it from carrying out excision. To this end, we have designed steric-blocking oligonucleotides aimed at blocking sites identified as nonproductive in SLC6A1 and NF1 pre-mRNA for purposes of gene activation. Here we test several ASOs in KNS60 neuroblastoma cells, performing preliminary screens for RNA activation. Approaches such as these do not target specific mutations, but rather target the wild-type gene processing, thus removing genetic eligibility requirements. Additionally, these methods for gene activation can be customized into therapies for any target, particularly for patients with haploinsufficiency disorders.

2′-O-methyl at 3′ Guide Strand Termini Negatively Affects Target Silencing Activity of Chemically Modified siRNA

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Small interfering RNA (siRNA) have the potential to treat a broad range of diseases. siRNAs need to be extensively chemically modified to improve their bioavailability, safety, and stability in vivo. However, chemical modifications variably impact target silencing for different siRNA sequences, making the activity of chemically modified siRNA difficult to predict. We systematically evaluated the impact of 3′ terminal modifications (2′-O-methyl versus 2′-fluoro) on guide strands of different length and showed that 3′ terminal 2′-O-methyl modification negatively impacts activity for >60% of siRNA sequences tested in vitro, but only in the context of 20- and not 19- or 21-nucleotide long guide strands. However, this phenomenon was demonstrated for siRNAs with 21-nucleotide long guide strands in vivo. These results indicate that sequence, modification pattern, and structure may cooperatively affect target silencing. Molecular modeling analysis suggests that 2′-O-methyl modification at the 3′ end of 20- or 21-nucleotide long guide strands may impair guide strand interactions within the PAZ domain of Argonaute-2. Our findings emphasize the complex nature of modified RNA-protein interactions and contribute to design principles for chemically modified siRNAs.

Binding of 2'-5' linked aptamers to Amikacin: Evaluated using Isothermal Calorimetry Circular Dichroism Spectroscopy and Molecular Dynamics Simulation

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P.41

Aminoglycosides are a class of antibiotics that are used to treat bacterial infections. They selectively targets the decoding site (A-site) in prokaryotic 16s rRNA of the 30s ribosomal subunit. Development of new antibiotics plays a significant role in treating infections from bacteria that have developed resistance against current antibiotics. Natural and synthetic aminoglycosides have very similar structures that are very challenging to differentiate using traditional small-molecule protecting groups. As a result, the regioselective modification of these molecules usually requires long synthesis steps including tedious protection-deprotection strategies. To overcome this problem, we specifically applied 2′-5′ linked RNA aptamers which exists widely in nature and plays critical roles in many biological systems, as a non-covalent protective group to regioselectively synthesize a series of new aminoglycoside derivatives as potential antibiotics. We have chosen a structurally known RNA aptamer as the model complex system, where we can identify the key binding sites from, we then designed and incorporated different number of 2′-5′ linkages into different positions of the active binding sites. As a preliminary study, we screened all the designed aptamers with different aminoglycosides using isothermal calorimetry and studied the overall complex conformation by circular dichroism and found one of our 2′-5′ linked aptamers binds strongly to Amikacin than the 3′-5′ linked native one and the ultimate goal of this study is to expand the aptameric protection strategy to synthesize a few new aminoglycoside derivatives as the potential new antibiotics and evaluate their antibacterial activity.

Distinctive features of long non-coding RNA chromatin (dis-)association

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P.42

Long non-coding RNAs (lncRNAs) constitute a large heterogeneous class, with some being transcribed from enhancer-like regions and involved in regulation of gene expression in cis. Although lncRNAs in general are enriched in the chromatin fraction of the cell, to what degree this defines their broad range of functions remains unclear. In addition, the factors that contribute to lncRNA chromatin tethering, as well as the molecular basis of efficient lncRNA chromatin dissociation and its functional impact on cognate enhancer activity and target gene expression, remain to be resolved. Here, we combined pulse-chase metabolic labeling of nascent RNA with chromatin fractionation and deep sequencing from the chromatin-associated and nucleoplasmic fraction to follow nascent RNA transcripts from their co-transcriptional state to their release into the nucleoplasm. By incorporating physical and functional characteristics in machine learning, we build models to predict chromatin (dis-)association of lncRNAs and compare these to mRNAs. We find that parameters like co-transcriptional splicing efficiency of the worst processed intron per transcript contribute to lncRNA chromatin dissociation. Intriguingly, lncRNAs transcribed from enhancers display reduced chromatin retention, suggesting that, in addition to splicing, lncRNA chromatin dissociation may contribute to shaping cognate enhancer activity and target gene expression.

Sex-specific role for lncRNAs in depression: from molecular mechanisms to behavioral outcomes

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P.43

Major depressive disorder is a common, chronic and debilitating disorder. Depression strikes women twice more than men, yet the molecular mechanisms contributing to this sex difference are poorly understood. Epigenetic processes that mediate interactions between genetic predispositions and stressful life experiences are known to play a key role in depression risk. Identification of novel epigenetic targets will aid in developing sex-specific diagnostics and therapeutics for depression. Here, we explored the roles of long non-coding RNAs (lncRNAs), a class of epigenetic regulators, in depression in both sexes. To that end, we integrated cutting-edge molecular, bioinformatics, behavioral, and physiological approaches spanning both humans and mice. We found that lncRNAs are robustly regulated in a sex-specific manner in postmortem brain tissue from depressed humans compared to healthy controls. Utilizing a genome-wide "guilt-by-association" bioinformatics approach, we identified sex-specific lncRNAs linked to depression. One of these targets, LINC00473, is neuronal-enriched and is downregulated specifically in the prefrontal cortex of depressed females only. To determine a causal role for LINC00473 in depression, we virally expressed LINC00473 in mice of both sexes' prefrontal cortex neurons followed by a behavioral examination. This mirrored the human sex-specific phenotype; expressing LINC00473 induced stress resilience in females only, which was accompanied by changes in synaptic function and gene expression. Next, we utilized ChIRP assays to pinpoint depression-related genes, as well as mitochondrial proteins, to which LINC00473 binds to mediate its molecular functions. Another lncRNA identified (RP11-299D21.1) is upregulated in the depressed female brain, and we named it FEDORA (FEmale DepressiOn lncRnA). FEDORA is expressed in oligodendrocytes and neurons, thus, we utilized a cell type-specific viral expression system to test its role in both cell types. We found that expressing FEDORA promoted depression-like behaviors only in female mice in both cell types. However, these behavioral changes were associated with cell-type-specific transcriptional changes, as well as altered electrophysiological and morphological features. Finally, we found that circulating FEDORA levels reflect the brain expression profile: blood FEDORA levels are elevated in depressed women compared to controls and not in males, suggesting FEDORA as a potential sex-specific biomarker for a depression diagnosis. Together, this work establishes that lncRNAs play key roles in depression and contribute to the sex-differences of this disorder. These findings provide a new view of molecular adaptations that contribute to depression risk and point to promising targets that may serve as foundations for novel depression diagnosis and treatment.

Blood miR-144-3p: Novel Diagnostic and Therapeutic Tool for Depression

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P.44

Major depressive disorder (MDD) is an episodic form of mental illness that is characterized by mood disturbances, anhedonia, and alterations in physiological functions, cognition, and psychomotor activity. There is a clinical need for objective biomarkers for depression that can improve diagnostic precision and refine treatment assignment. MicroRNAs (miRNAs) are short noncoding RNA molecules, which can be detected in body fluids and have emerged as potential biomarkers of disease conditions, including depression. The present study explored the potential use of miRNAs as biomarkers for MDD diagnosis and for prediction of treatment response, as well as the ability to alter depression phenotype by manipulating a circulating miRNA. Here, we probed blood miRNA levels from male mice exposed to chronic social defeat stress (CSDS), a procedure that induces depression- and anxiety-like phenotypes. There are robust individual differences in response to CSDS: the majority of mice develop a behavioral syndrome termed susceptibility, while others show fewer behavioral deficit and are considered stress resilient. We collected blood at multiple time points: both before and after exposure to CSDS, as well as after either repeated imipramine or single ketamine doses. Using Nanostring analysis, we profiled the expression levels of ~600 blood miRNAs. We observed robust differences in blood miRNA signatures between resilient and susceptible mice 20 days after CSDS, but not 2 days after CSDS. Furthermore, treatment with ketamine, more than imipramine, re-established baseline miRNA expression levels in mice that responded behaviorally to the drug, but not in non-responders. We identified the red blood cell-specific miR-144-3p as a candidate biomarker in mice for stress susceptibility, which also predicts response to ketamine treatment. To probe the translational relevance of this finding, we used blood samples from a human MDD cohort and indeed validated that miR-144-3p is a predictor of depression severity, as well as of ketamine treatment response. Lastly, we demonstrate that systemic knockdown (KD) of miR-144-3p using an antagomir approach is sufficient to reduce depression-like behaviors in susceptible mice after CSDS. RNA-sequencing analysis of blood samples after CSDS and miR-144-3p manipulation revealed that the KD attenuated the molecular signature of CSDS on the transcriptome. Taken together, this study enhances our understanding of epigenetic processes accruing in the peripheral circulation in response to stress and antidepressants treatment. Specifically, we identified miR-144-3p as a candidate blood biomarker for depression diagnosis and prediction of treatment response, as well as a potential treatment.

Suppression of LBR by miR-340 disrupts chromatin, promotes cell senescence, and enhances senolysis

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P.45

One of the cellular processes influenced by microRNAs is senescence, a state of indefinite growth arrest triggered by sublethal cell damage. Here, through bioinformatic analysis and experimental validation, we identified miR-340-5p as a novel miRNA that foments cellular senescence. miR340-5p was highly abundant in diverse senescence models, and miR-340-5p overexpression in proliferating cells rendered them senescent. Among the target mRNAs, miR-340-5p prominently reduced the levels of LBR mRNA, encoding Lamin B Receptor (LBR). Loss of LBR by ectopic overexpression of miR-340-5p derepressed heterochromatin in lamina-associated domains (LADs), promoting the expression of DNA repetitive elements characteristic of senescence. Importantly, overexpressing miR-340-5p enhanced cellular sensitivity to senolytic compounds, while antagonization of miR-340-5p reduced senescent-cell markers and engendered resistance to senolytic-induced cell death. We propose that miR-340-5p can be exploited for removing senescent cells to restore tissue homeostasis and mitigate damage by senescent cells in aging human pathologies.

The RNA-binding protein GTSF1 potentiates the endonuclease activity of PIWI proteins

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In the animal germline, PIWI-interacting RNAs (piRNAs) direct PIWI-clade Argonaute proteins to silence complementary target RNAs via transcriptional and post-transcriptional mechanisms. piRNAs repress transposons in all animal germ cells, but in placental mammals, a specialized class of piRNAs, called pachytene piRNAs, regulates the expression of mRNAs encoding proteins required for meiosis and spermiogenesis. In mice, pachytene piRNAs bind the PIWI-clade proteins MIWI and MILI, which are essential for completion of spermatogenesis and production of functional sperm. Unlike miRNAs and siRNAs, how pachytene piRNAs bind and repress their RNA targets remains poorly understood. Pachytene piRNA sequences are rapidly diverging and poorly conserved, confounding computational prediction of their targets. To define the biochemical properties of pachytene piRNAs, we generated cell lines stably expressing MIWI and MILI. Recombinant MIWI and MILI purified from these cells programmed with a synthetic piRNA guide to form an active piRNA-induced silencing complex (piRISC) cleaved highly complementary target RNAs. However, piRNA-guided PIWI cleaved the targets far more slowly than mouse AGO2. Here, we report efficient target cleavage by MIWI or MILI requires gametocyte specific factor 1 (GTSF1), a small, conserved, zinc-finger protein known to be essential for spermatogenesis and male fertility. GTSF1 specifically potentiates the endonuclease activity of PIWI proteins, showing no enhancement of target cleavage catalyzed by siRNA-loaded AGO2. Mutations that disrupt GTSF1 RNA binding prevent it from functioning as a PIWI auxiliary factor. Insect GTSF1 homologs similarly potentiate target cleavage by insect, but not mammalian PIWI proteins. Finally, in both mice and insects, GTSF1 paralogs are specific for distinct PIWI proteins. GTSF1 is the first example of a protein that stimulates the endonuclease activity of an Argonaute protein. Our data provide a model for how GTSF1 participates in piRNA biogenesis and function in animals.

Developing tools to understand piRNA-precursor trafficking

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In animals, reproductive fitness depends on small RNAs called PIWI-interacting RNAs (piRNAs), which silence transposable elements to ensure genome stability. piRNAs direct PIWI proteins to cleave the transcripts from piRNA clusters, producing a 5′ monophosphorylated pre-pre-piRNA, which can be loaded into another PIWI protein. The endonuclease Zucchini then cleaves the pre-pre-piRNA 27–35 nucleotides from its 5′ end, yields a PIWI protein-bound pre-piRNA and a new 5′ monophosphorylated pre-pre-piRNA. Subsequent 3′-to-5′ trimming and 3′ terminal 2′-O-methylation generates a mature piRNA. The mechanism of piRNA biogenesis has been conserved among animals for at least 900 million years.

Many proteins required for the initiation of piRNA production, including PIWI proteins and the DEAD-box helicase Vasa, localize to perinuclear, membraneless organelles called nuage. However, Zucchini resides on the outer mitochondrial membrane. Nuage and mitochondria are physically separate, requiring a PIWI protein-bound pre-pre-piRNA to transit from one subcellular location to the other. The RNA-binding ATPase Armitage (Armi) resides in both nuage and on the outer mitochondrial membrane and is thought to deliver PIWI-bound pre-pre-piRNAs to Zucchini. Thus, individual piRNA precursors appear to follow a defined spatial pathway—from nuage to the outer mitochondrial membrane.

To understand the spatial regulation of piRNA processing, I propose to develop a sequencing-based method to map the journey of individual RNA molecules as they move from one intracellular compartment to another. This technique will involve two RNA-modifying enzymes: APEX2, an engineered ascorbate peroxidase that generates biotin–phenol radicals that covalently bind nearby nucleic acids (~20 nm); and ADAR, an adenosine deaminase that converts adenosine to inosine. Both enzymes will be expressed as fusions to piRNA processing proteins (Vasa-ADAR and Zucchini-APEX2) in lepidopteran Hi5 cells—a germ cell line immortalized from cabbage looper (Trichoplusia ni) ovarian germ cells. The Zamore lab has recently sequenced, assembled, and annotated the genome and piRNAs of Hi5 cells, establishing Hi5 cells as a germ-cell model for studying piRNA biogenesis.

I will isolate the biotinylated RNAs from Hi5 cells that co-express Vasa-ADAR and Zucchini-APEX2 and use high-throughput sequencing to identify sequences that are both biotinylated and A-to-I edited. (Inosine is read as guanosine.) Biotin-labeled pre-pre-piRNAs., with the sequence A-to-G transitions can be inferred to have moved from nuage to the outer mitochondrial membrane. Once I have established this dual RNA-labeling system, I will use CRISPR to disrupt piRNA processing factors to study how they regulate the trafficking and processing of piRNA precursors. Together these studies will reveal the spatial dynamics of piRNA precursor trafficking and processing at single-molecule resolution. The proposed RNA-labeling approaches should also be useful for studies of RNA trafficking in other cellular contexts.

Visualizing Ribosome Biogenesis in Intact Cells

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The rate of cell growth is determined by the number and activity of ribosomes. Ribosome biogenesis is therefore a tightly regulated and highly efficient process in diverse cell types, and its dysregulation is commonly associated with disease. In yeast, the production of the large (LSU) and small (SSU) ribosomal subunits occurs progressively in three subcellular compartments –the nucleolus, the nucleus and the cytoplasm– and requires >200 ribosome biogenesis factors to correctly fold and assemble the four ribosomal (r)RNAs and 79 ribosomal proteins, making it one of the most complex processes in biology. Despite this complexity, actively growing yeast cells are capable of producing an astonishing ~2,000 ribosomes per minute. Spatial organization is required to achieve this high level of efficiency, however, beyond a broad characterization of intermediate steps as nucleolar, nuclear or cytoplasmic, a more detailed understanding of the spatial organization of ribosome biogenesis is lacking. A new method in active development in the Grigorieff lab, termed 2D template matching (2DTM), has made it possible to locate molecules in electron cryo-microscopy (cryo-EM) images of cells to within a few angstroms using their internal atomic signatures. Using molecular models representing 5 stages of LSU biogenesis, I determined the location and orientation of different states of maturation within the yeast nucleolus, nucleus and cytoplasm. The locations and orientations are consistent with their expected distribution in the cell. By comparing the cross-correlation score between the model templates and the target molecules, I was able to differentiate between related intermediate states. Further, the population of nuclear intermediates shifted towards a more mature state when nuclear export was inhibited, consistent with the detected targets representing on pathway intermediates of ribosome maturation in cells and demonstrating the sensitivity of this approach. Using this method, we can now directly visualize LSU biogenesis, from an early intermediate in the nucleolus to more mature intermediates before export from the nucleus, to mature LSUs in the cytoplasm. Our results demonstrate that 2DTM is a potent tool to study the structural details of entire biological pathways within cells.

Is the Gene Silencing of Endothelin-1 an Option for the Treatment of Pulmonary Arterial Hypertension?

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P.49

Pulmonary arterial hypertension (PAH) is an orphan disease characterized by an increase in pulmonary arterial pressure greater than 20mmHg, in which patients have a poor prognosis, the mechanisms associated to this pathology include increase in vascular resistance and pulmonary arterial pressure with right ventricle remodeling, among the pathways involved in this alterations is endothelin-1 which is a polypeptide of 21 amino acids that participate in the regulation of vascular tone, the increase of endothelin-1 causes vascular resistance in the pulmonary arteries, which elevates pulmonary arterial pressure. Recently, one of the most studied therapies is the gene therapy based on RNA silencing, due to its ability to developt selective drugs. For that reason, the aim of this work was to design, synthesize and evaluate a siRNA against endothelin-1 in a rat model of monocrotaline-induced pulmonary hypertension. We used male Wistar rats which were administered monocrotaline (60 mg / kg i.p.) to induce PAH. After four weeks, the systolic blood pressure, right ventricle mean arterial pressure, weight, ratio of RV / LV + S and the relative expression of mRNA endothelin-1 by RT-PCR were determinated. The siRNAs were designed using the Wizard v3.1, RNAfold and EMBOSS software programs, the selected sequences were synthesized using a MERMADE 8 oligos synthesizer. The results showed a significantly increase in the ratio of RV / LV + S, right ventricle mean arterial pressure, levels of endothelin-1, BNP and TNF-α in the right and left lung, as well as in the right ventricle and a decrease in the weight compared with the non hypertensive rats (controls). The administration of endothelin-1 siRNA in rats with monocrotaline produced a significant decrease in the relative expression of endothelin-1 mRNA, BNP and TNF-α, as well as hypertrophy and right ventricular pressure. For this reason, we suggest that the siRNA against endothelin-1 could be a therapeutic option for the treatment of PAH.

The Germline KH Protein, TOFU-7, engages the HSP-90 chaperone and PRP-19 spliceosome components to promote piRNA-dependent epigenetic silencing.

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Genetic studies in eukaryotes have implicated spliceosome components in Argonaute-mediated silencing. Little is known however about how this occurs and whether or not it involves direct participation in small RNA biogenesis and downstream silencing, or more indirect effects for example on the expression of Argonaute pathway proteins. Here, we show that the C. elegans germline KH protein TOFU-7, previously reported as a piRNA-related factor, interacts by yeast 2 hybrid with SFTB-2, CDC5L and M03F8.3., components of the PRP-19 splicing complex. TOFU-7 also interacts with HSP-90, a chaperone required for loading piRNA guides onto PIWI. These factors are all required for piRNA-mediated establishment of silencing, yet their genetic behavior suggest they may function at different steps of the piRNA pathway. In tofu-7 mutants PRG-1 and mature piRNAs are completely absent. In contrast, depletion of HSP-90 or CDC5L using an auxin-inducible system results in rapid disappearance of TOFU-7 protein and a mobility in shift in PRG-1. The tofu-7 phenotype is epistatic to the degron depletion of HSP-90 or CDC5L resulting in absent rather than mobility shifted PRG-1. Our findings support a model in which TOFU-7 engages HSP-90 and PRP-19 components to enable PRG-1 loading and stability. Future studies will also explore the possibility that PRP19 complex recruits the spliceosome to promote piRNA biogenesis.

High Throughput Analysis of Circular Forms of ANRIL, a lncRNA in the CDKN2A/B Locus with Implications in Metabolic Disease

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Circular RNAs (circRNAs) are gaining attention recently due to the mysterious nature of their production, regulation, and function. In this study, we used Next Generation Sequencing (NGS) to investigate the linear and circular isoforms of a long non-coding RNA named ANRIL (antisense non-coding RNA in the INK locus), which originates from the CDKN2A/B (INK4-ARF) locus. As misregulation of this locus is implicated in diseases such as coronary artery disease (CAD) and diabetes, it is important to understand the array of linear RNA and circRNA produced which may have downstream regulatory effects. Because the regulation of ANRIL is implicated in the development of Type II diabetes (T2D) through decreased beta cell mass, we isolated RNA from five different pancreatic islet cell donor preps which contain a mix of alpha and beta cells. To compare linear and circular isoforms of ANRIL, we sequenced total RNA with and without RNase R treatment to enrich for circRNAs and identified back-spliced junctions indicating of circRNAs. Using a comprehensive master isoform accounting for all annotated ANRIL isoforms, we identified all ANRIL splice sites and exons involved in ANRIL circRNAs. We validated a number of circular forms with divergent PCR primers and Sanger sequencing. To understand the mechanisms leading to circular ANRIL formation and potential consequences, we investigated various genetic and molecular features associated with circular ANRIL, including regions flanking circular-associated splice sites with self-complementarity, enriched RNA motifs, and enrichment for miRNA target sites. Finally, using allele specific circular ANRIL expression analyses, we identified single nucleotide polymorphisms (SNPs) between individuals within ANRIL that are associated with increased circRNA production.

Why are piRNA 3′ ends trimmed and 2′-O-methyated?

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P.53

In animals, piRNAs silence transposons, fight viral infections, and regulate gene expression. piRNA biogenesis concludes with 3′ terminal trimming and 2′-O-methylation. Both trimming and methylation influence piRNA stability. Here, we report that trimming and methylation protect mouse piRNAs from different decay mechanisms. In the absence of 2′-O-methylation, mouse piRNAs with extensive complementarity to long RNAs become unstable. In flies, 2′-O-methylation similarly protects both piRNAs and siRNAs from complementarity-dependent destabilization. In contrast, trimming protects mouse piRNAs from a separate degradation pathway unaffected by target complementarity but sensitive to the 3′ terminal, untrimmed sequence. Because distinct sets of mouse piRNAs are protected by each of these mechanisms, loss of both trimming and 2′-O-methylation causes the piRNA steady-state abundance to collapse, demonstrating that these two small RNA modifications collaborate to stabilize piRNAs.

Through the Looking Glass: Mito-Nuclear Retrograde Communication via Mitochondrially Derived Long Non-coding RNA

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As the mitochondria began its transition from bacteria to a eukaryotic cell organelle, a portion of the mitochondrial genome was integrated into the nuclear genome. Although the rest remains a physically separate genome within the organelle, these two genomes are intertwined; a single point mutation in mitochondrially associated genes can have catastrophic effects on the cellular, tissue, organ and organismal levels.

The integration of these genomes as well as the function of the mitochondria as a “first responder” to metabolic change and role as an integral player in apoptosis suggest retrograde regulation occurs. Prior elucidation of cross-talk between the nucleus and mitochondria has primarily focused on nuclear control over mitochondrial gene expression. While the regulatory effect of the mitochondrial metabolic products on nuclear gene expression has been observed, information on retrograde communications via mitochondrial non-coding RNA (ncmtRNA) is sparse.

Oncological research has indicated these ncmtRNA may be viable targets to halt tumor growth via RNAi; however, how these transcripts fit into the greater interactome has not been established. We propose combining RNA sequencing techniques with single molecule imaging technology to explore these proposed retrograde signals and their association with nuclear gene expression and cell cycle regulation.

Molecular Mechanisms of Target Recognition by the microRNA-guided protein Argonaute1 in Drosophila melanogaster

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P.55

Background and aim: In Drosophila melanogaster, gene silencing is directed by different classes of small RNAs that guide distinct members of the Argonaute protein family to specific RNA targets: small interfering RNAs (siRNAs) are loaded into Argonaute2 (Ago2), while microRNAs (miRNAs) are loaded into Argonaute1 (Ago1). Ago2 divides its RNA guide into domains with distinct biochemical properties, such that the seed region, nucleotides g2–g8, is pre-organized into an A-form helix. Once bound to target, the extent of complementarity beyond the seed region determines whether or not Ago2 cuts the target. In flies, Ago2 dissociates so slowly from extensively complementary target RNAs that nearly all binding events end with target cleavage. In contrast, when fly Ago2 is paired to a target RNA solely through the seed sequence of the small RNA guide, it binds 80 times less tightly. Here, we report a detailed characterization of the rules for stable target binding by Drosophila Ago1, the homolog of the mammalian miRNA-guided Ago2 protein.

Results: Like mammalian Ago2, fly Ago1 binds its target through both its seed sequence and four additional nucleotides, usually positions g13-g16, that form 3′ supplementary base pairs with the target. Using competition assays with fly Ago1 isolated from embryos, we find that central mismatches (g8g9, g10g11) have no detectable effect on binding affinity, consistent with miRNAs being centrally unpaired with their targets. In contrast, mismatches at positions g4 and g5 of the seed reduced binding 150-fold. Interestingly, extensive base pairing beyond the seed compensated for imperfect pairing in the seed. Using RNA-Bind-n-Seq (RBNS) to test all possible 20 nucleotide sequences, we find that let-7-programmed Drosophila Ago1 binds with highest affinity via canonical seed pairing.

Conclusion: Our data suggest that fly Ago1-RISC and Ago2-RISC function by distinct molecular mechanisms.

Defining the binding properties of each of the four mammalian Argonaute paralogs

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In eukaryotes, Argonaute (AGO) proteins loaded with ~21–23 nt RNA guides form the RNA-induced silencing complex (RISC). Mammalian AGO proteins consist of four Argonaute paralogs (AGO1–4), which are expressed in different proportions across various cell types. MicroRNAs (miRNAs) tether AGO1–4 proteins to their targets, triggering mRNA degradation or repressing translation. miRNAs bind targets through their seed sequence—guide bases g2–g7. As few as six base pairs can mediate high affinity interactions, but some RISCs gain considerable binding energy from non-seed nucleotides. Moreover, AGO proteins can function cooperatively over short distances. In addition to this “miRNA” binding mode, extensively complementary sites allow miRNAs to act like siRNAs, directing AGO2 to cleave the target at a single phosphodiester bond across from the center of the miRNA. Recent studies have defined the binding and slicing properties of AGO2, but comparable data is unavailable for AGO1, AGO3 and AGO4, precluding accurate prediction of their targets. We are using RNA Bind-n-Seq and Co-localization Single Molecule Spectroscopy to define the target interactions of the four mammalian Argonaute proteins. Our data should lead to quantitative targeting rules for individual miRNAs bound to AGO1, AGO2, AGO3, or AGO4 and facilitate the development of siRNA, miRNA and antagomir therapeutics with high target specificity.

Assessing the Function of Pachytene piRNA Loci in Mouse Spermatogenesis

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In nearly all animals, PIWI-interacting RNAs (piRNAs), 23–32 nt long, guide PIWI proteins, a specialized class of Argonaute proteins that defend cells against viruses, transposons, and other selfish genetic elements. In the male germ cells of post-pubertal placental mammals, the abundance of pachytene piRNAs rivals that of ribosomes, yet we do not know why they are required for the successful production of sperm. Despite comprising nearly a million distinct sequences, few individual pachytene piRNAs have been shown to have a biological function. In mice, pachytene piRNAs derive from 100 non-coding genes co-regulated by a common transcriptional circuit. Three pachytene piRNA genes—pi2, pi9, and pi17—are most productive loci and contribute 22% of the total piRNA pool. To investigate the function of pachytene piRNAs, we deleted the promoter of each locus in C57BL/6 mice and evaluated the impact on spermatogenesis. Individually, each of the three single mutants (pi2–/–, pi9–/–, pi17–/–) exhibited normal fertility. However, males lacking two (pi9–/–pi17–/–) or three (pi2–/–pi9–/–pi17–/–) were subfertile. Sperm from doubly or triply mutant males fail to fertilize oocytes in vitro. Mutant sperm populations contained fewer motile, progressive, and hyperactive spermatozoa. pi2–/– and pi9–/– mutant males, while reproductively normal, also demonstrate a reduction in progressively motile sperm. RNA-seq analysis of FACS-purified pi9–/–pi17–/– primary spermatocytes identified 30 genes whose steady-state abundance was >2-fold greater in double mutants than in C57BL/6 primary spermatocytes. The transcripts from twenty of these genes are predicted to be cleaved by a pachytene piRNA specific to pi9 or pi17. Our data suggest that (1) piRNAs do not regulate their target RNAs by microRNA-like mechanisms; and (2) most pachytene piRNAs have no detectable role in regulating meiotic mRNA stability.

Understanding the spatial organization of the somatic RNAi response

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RNA interference (RNAi) was first discovered in C. elegans and is a well conserved mechanisms that regulates gene expression and fights pathogens like viruses. Gene expression is tightly controlled in the nuclear space and the genome has been shown to spatially segregate into active “euchromatin” and silenced “heterochromatin”. Heterochromatin is often localized to the nuclear periphery, while euchromatin is more likely to be found in the nuclear space. Our preliminary results indicate that the RNAi silencing response is able to initiate a reorganization of the nuclear space of the germline. Upon silencing initiation, RNAi silenced mRNA and DNA localize to one germ granule at the nuclear periphery, which can be visualized by smRNA and DNA FISH. Mutants deficient for RNAi, fail to concentrate the silenced DNA and/or mRNA at the granule, suggesting that the reorganization has functional implications. We are currently analyzing if the somatic RNAi silencing response leads to a similar restructuring of the nuclear space.

Somatic cells possess somatic granules that can be visualized by GFP tagged to the DEAD box RNA helicase, RDE-12, which also localizes to germ granules. DEAD box RNA helicases trigger the unwinding of RNA molecules, which is required for many RNA processing steps. In an effort to uncover functional properties of somatic granules, we mutated different RDE-12 DEAD box helicase motifs by CRISPR. Mutations of different RDE-12 helicase motifs lead to RNAi dysfunction and RDE-12 mislocalization in germ granules. One mutation in the DEAD box domain, which likely prohibits RNA substrate release, provokes an aggregation of RDE-12 specifically in somatic granules. Our results suggest that RDE-12 plays distinct roles in the germline and somatic tissue.

The N-terminal domain of dyskerin plays crucial role in post-transcriptional regulation of scaRNA13

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Background: Mutations in the DKC1 gene that encodes dyskerin cause rare genetic disorders, including dyskeratosis congenita (DC). Dyskerin combines with other proteins (NOP10-NHP2-GAR1) to stabilize box H/ACA noncoding RNAs, including the telomerase RNA component (TERC). H/ACA noncoding RNAs include small nucleolar RNAs (snoRNAs) and small Cajal body-specific RNAs (scaRNAs) that act as guides for dyskerin to catalyze the conversion of uridine residues to pseudouridine in ribosomal and spliceosomal RNAs. A consistent finding in DC due to pathogenic DKC1 mutations is decreased steady-state levels of TERC RNA. However, impacts on other H/ACA RNAs are variable, for poorly understood reasons.

Objective: To elucidate the effects of different DKC1 mutations in DC patients on noncoding RNAs, in order to define disease mechanisms beyond impaired telomere maintenance.

Results: By studying somatic and induced pluripotent stem cells (iPSCs) from three DC patients with DKC1 mutations (del37L, A386T, A353V), we found that the N-terminal domain of dyskerin regulates 3´-end maturation of scaRNA13. In del37L iPSCs but not A386T or A353V iPSCs, nascent scaRNA13 transcripts showed a discrete population of 3´-extended forms by RNA-ligation mediated RACE (RLM-RACE), a pattern seen in the setting of DC-causing mutations in the PARN (polyA-specific ribonuclease) gene. Because del37L is found in an N-terminal cluster of DC mutations, we used CRISPR-Cas9 mediated mutagenesis of the endogenous DKC1 locus in normal male hemizygous iPSCs to recapitulate these mutations. As expected, only iPSCs carrying in-frame dyskerin mutations were viable and able to be cloned and propagated. Amongst the iPSCs generated to carry a variety of N-terminal dyskerin mutations, we consistently observed 3´-end processing defects of scaRNA13, as seen in del37L patient cells. Deep sequencing of 3´ RLM-RACE products showed that extended scaRNA13 forms were composed of genomically-extended and post-transcriptionally oligoadenylated species. Increasing evidence indicates oligo-adenylation of certain box H/ACA RNAs is mediated by PAPD5 (poly (A) polymerase domain containing 5), a non-canonical polymerase, which in turn is opposed by PARN. In keeping with this, we found that genetic deletion of PAPD5 or its pharmacological inhibition using a dihydroquinolizinone molecule, RG7834, rescued scaRNA13 end processing defects in iPSCs with N-terminal dyskerin mutations.

Conclusion: Our data provide genetic evidence that N-terminal residues of dyskerin regulate scaRNA13 post-transcriptional processing by the PARN/PAPD5 axis. These findings have important implications for our broader understanding of noncoding RNA biogenesis alterations in DC pathogenesis and therapeutics.

Regulation of Gene Expression by Mouse Pachytene PIWI-Interacting RNAs

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In adult mouse testes, ~30nt pachytene PIWI-interacting RNAs (piRNAs) comprise >80% of all small non-coding RNAs. Despite their extraordinary abundance and sequence diversity, the biological significance and mechanisms of pachytene piRNAs remain open questions. Unlike microRNAs (miRNAs), the base-pairing rules for piRNA targeting are poorly defined, hindering a clear understanding of in vivo targets of pachytene piRNAs and how these small RNAs mechanistically perform biologically meaningful functions. Previously, we reported the generation and characterization of a mouse model that does not produce a defined population of pachytene piRNAs from a source locus on mouse chromosome 6 (pi6). Using animal experiments, in vitro assays, and computer-assisted sperm analysis, we demonstrated for the first time that pachytene piRNAs are directly required for male mouse fertility. Specifically, pi6 piRNAs are crucial for the final steps in mouse sperm maturation ("capacitation") that are essential for fertilization. Using small RNA-seq, RNA-seq, and 5′-phosphorylated RNA (degradome) sequencing, we identified transcripts whose steady-state abundance and piRNA-directed RNA cleavage are associated with siRNA-like base-pairing between the cleavage site sequences and specific pi6 piRNAs. Together, our data suggest a gene regulatory network centered around the cleavage function of mouse pachytene piRNAs; they cleave not only mRNAs important for sperm function, reducing their steady-state abundance, but also pachytene piRNA precursors, initiating production of piRNAs from other pachytene piRNA loci in trans. To further understand how regulation of transcriptome by pachytene piRNAs directly contributes to specific biological pathways, one critical step is to link the abundance of piRNA target mRNAs to their encoded proteins. However, correlation between mRNA and protein abundance in cells is typically poor, presenting an obstacle in elucidating mouse piRNA functions. To meet this challenge, we use isobaric labeling-based mass spectrometry and ribosome footprint profiling to survey the translation landscape in male mouse germ cells. Here, we report our preliminary results of these two complementary experimental approaches.

Trichoplusia ni Cell Lines as Models for Germline and Somatic piRNA Pathways

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P.64

Pioneering studies of the fruit fly ovary and mouse testis largely influence our view about piRNAs: germline-specific guardians fighting against transposons. However, many aspects of the fly piRNA pathway are not present in other orders of arthropods. Moreover, most arthropods produce somatic piRNAs, unlike flies. The Lepidopteran Trichoplusia ni (cabbage looper) provides a novel model for piRNA biology, because (1) its immortalized cell lines enable the study of both germline and somatic piRNAs ex vivo, which is infeasible in most other animals and (2) the T. ni piRNA pathway contains many of the features thought to have been present in the last common ancestor of arthropods. To systematically study the genetic makeup of the piRNA pathway in the cabbage looper, we are developing tools to conduct genome-wide CRISPR screens using a piRNA-regulated reporter gene. These tools will also enable studies of gene regulation by microRNAs and the siRNA innate immune pathways. This study paves the way for the systematic identification of piRNA pathway genes in a novel model organism.

DiscFull_Lnc: discover full-length long noncoding RNAs without transcriptional initiation profiles

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P.65

Long noncoding RNAs (lncRNAs) are long (>200 nucleotides) linear RNA transcripts that do not encode proteins. LncRNAs have been found to play important roles in mammalian development and disease. Trimethylation of histone H3 at lysine 4 (H3K4me3) is a histone modification known to mark transcription start sites of expressed genes, including those of lncRNAs. Hence, H3K4me3 profiles have been applied to lncRNA identification. However, due to the cost and time of generating H3K4me3 profiling data (ChIP-seq) and sample material availability, H3K4me3 profiles are not available in many tissues, cell types, and conditions of interest. Without H3K4me3 profiles, it becomes a challenge to identify full-length lncRNA transcripts which include their transcription start sites.

To meet that challenge, here we develop a machine-learning-based computational method, Discovering Full-length LncRNA tool (DiscFull_Lnc), to identify full-length lncRNAs directly from raw RNA sequencing (RNA-seq) data without H3K4me3 profiles from the corresponding sample. DiscFull_lnc uses a logistic regression model built with six features: number of exons; genomic location; expression level; length of transcripts; promoter signature; public H3K4me3 profiles from relevant samples. Trained using millions of putative lncRNA transcripts initially found in 61 human datasets (each coupled with H3K4me3 profiles), DiscFull_Lnc achieves an accuracy of 0.82 without H3K4me3 profiles, and can improve its accuracy to 0.88 if H3K4me3 profiles in similar samples are available. Using DiscFull_lnc, we are building a comprehensive catalog of full-length lncRNA transcripts across 17 human primary cell types and cell lines. We are also improving our approach by using larger training datasets and implementing a software suite and web interface for the community.

Spatiotemporal regulation of piRNA production and target silencing during Drosophila melanogaster spermatogenesis

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The Piwi-associated RNA (piRNA) pathway is a small RNA-mediated interference mechanism that silences repetitive and selfish genetic elements, particularly in the germline. Intense studies have identified the molecular mechanisms of piRNA biogenesis. piRNAs can be produced de novo by cleavage of specific long non-coding RNAs into mature 23-29nt piRNAs. This process, the primary pathway, is mediated in Drosophila melanogaster by the endonuclease Zucchini (Zuc; MitoPLD in mammals) and Armitage (Armi; MOV10L1 in mammals). piRNAs generated by this mechanism are loaded onto an Argonaute protein Aubergene (Aub), which recognizes target mRNAs via nucleotide complementarity and cleaves them. Target cleavage generates sense-strand piRNAs, which are subsequently loaded onto another Argonaute protein, Ago3. Sense-piRNA-loaded Ago3 further cleaves piRNA precursor long non-coding RNAs to generate more anti-sense piRNAs, leading to a feed-forward amplification loop called the 'ping-pong cycle'. Although the ping-pong cycle is a powerful mechanism to amplify piRNAs, it requires pre-existing piRNAs to initiate the amplification process. The original source of pre-existing piRNAs can be synthesized de novo in an Armi/Zuc-dependent manner or maternally deposited.

In contrast to a widely-held view of piRNA production, whereby primary piRNA production and ping-pong cycle occur within the same cell to achieve robust amplification of sense and anti-sense piRNAs, our study reveals that in the Drosophila melanogaster testis de novo generation of Su(Ste) anti-sense piRNAs by Armi and Zuc are spatiotemporally separated from Aub/Ago3-mediated ping-pong mechanism with the former specifically occurring in early germ cells (germ line stem cells (GSCs) and early spermatogonia (SGs)) and the latter mostly occurring in spermatocytes (SCs), where Stellate (Ste), the major target of the piRNA pathway in male germ line, starts to express. We show that this de novo synthesis of primary piRNAs that precedes days before the expression of the target (Ste) is crucial for efficient repression of the target. We further show that the requirement of de novo synthesis in early germ cells can be compensated by allowing mothers (XXY females that have Y-linked Su(Ste) locus) to deposit maternal Su(Ste) piRNA into sons. Taken together, we propose that concerted actions of de novo piRNA production and maternally-deposited piRNAs complement each other in providing defense against selfish elements.

Improved and Linear-Time Stochastic Sampling of RNA Secondary Structure with Applications to SARS-CoV-2

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Many RNAs fold into multiple structures at equilibrium. The classical stochastic sampling algorithm can sample secondary structures according to their probabilities in the Boltzmann ensemble, and is widely used. However, the current sampling algorithm, consisting of a bottom-up partition function phase followed by a top-down sampling phase, suffers from three limitations: (a) the formulation and implementation of the sampling phase are un- necessarily complicated; (b) the sampling phase recalculates many redundant recursions already done during the partition function phase; (c) the partition function runtime scales cubically with the sequence length. These issues prevent it from being used for very long RNAs such as the full genomes of SARS-CoV-2. To address these problems, we first adopt a hypergraph framework under which the sampling algorithm can be greatly simplified. We then present three sampling algorithms un- der this framework, two of which eliminate redundant work in the sampling phase by caching. Finally, we present LinearSampling, an end-to-end linear-time sampling algorithm that is orders of magnitude faster than the standard one. For instance, LinearSampling is 176× faster (38.9s vs. 1.9h) than Vienna RNAsubopt on the full genome of Ebola virus (18,959 nt). More importantly, LinearSampling can scale up to the full genome of SARS-CoV-2, taking only 69.2 seconds on its reference sequence (29,903 nt). It finds 23 regions of 15 nt whose accessibilities are larger than 0.5, i.e., they are more likely to be opening than closing. Some of the regions are overlapped, resulting in a total of 9 separate accessible regions. Among the 9 regions, two are in ORF1ab, one in ORF3a, one in the M gene, three in the N gene, and two in the S (spike) gene, whose proteins can recognize and bind with receptor. These regions can be potentially used for COVID-19 diagnostics and drug design. The code is available at: https://github.com/LinearFold/LinearSampling

Linear-Time RNA Structural Alignment and Conserved Structure Prediction with Applications to Coronaviruses

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P.68

RNAs play important roles in multiple cellular processes, and some of their functions rely on the folding of specific structures. To maintain their functions, secondary structures of RNA homologs are conserved across evolution. Meanwhile, conserved structures involve compensating mutations, where two bases change across evolution and the base pair still exists, for instance, a GC base pair is replaced by an AU or a CG pair in homologous sequences. Therefore the compensatory mutations provide strong evidence for conserved structures. Besides, the structural alignment is more accurate than the sequence alignment when the structure is more conserved than the sequence identity and it is a useful tool for the covariation analysis.

To solve this issue, TurboFold II, one of the SOTA algorithms, iteratively folds and aligns RNA homologs, but it scales cubically with sequence length, which limits its application for long sequences. We propose LinearTurboFold, a linear-time version of TurboFold II without sacrificing accuracy on secondary structure and multiple sequence alignment predictions. LinearTurboFold is orders of magnitude faster than TurboFold II, e.g., 372× faster (12 minutes vs. 3.1 days) on a group of five HIV-1 homologs with an average length of 9,686 nt. The linearization of LinearTurboFold enables it to scale up to SARS-CoV-2 full-length genomes (about 30,000 nt).

We run LinearTurboFold on a group of complete genomes consisting of SARS-CoV-2 genomes and several SARS-related betacoronavirus to detect conserved structures, which are potential targets for anti-viral treatments. We verify our results by comparing with several well-characterized structures, and list the conserved base pairs across SARS-CoV-2 and SARS-related betacoronaviruses. We observe the genome cyclization which involves the long-range interaction between 5' and 3' UTRs conserved among all the genomes.

Furthermore, to locate both conserved and exposed regions, we combine LinearTurboFold and LinearSampling. LinearSampling is a linear-time statistical sampling algorithm and can get accessibility for regions with different lengths by independently sampling thousands of structures. We discover some regions, which are both with high accessibilities and fully conserved among all the SARS-CoV-2 genomes. These regions are potentially critical targets for the siRNA drug design.

Predicted Spike-ORF8 Genomic RNA-RNA Interaction Unique to SARS-CoV-2 May Allosterically Impact the Rate of Nucleocapsid Sub-genomic RNA Synthesis

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The current pandemic situation of COVID-19 due to SARS-CoV-2 virus has affected the life of billions. SARS-CoV-2 is related to severe acute respiratory syndrome coronavirus (SARS-CoV-1) which was highly lethal but disappeared after intense public health measures. Some studies have identified differences between SARS-CoV-1 and SARS-CoV-2 that may be related to the potential of wide spread of the latter virus and immune evasion. Using a series of in silico RNA structure prediction tools, we found a long-range RN-RNA interaction between Spike and ORF8 genomic regions of SARS-CoV-2 RNA, not seen in SARS-CoV-1. Compensatory mutations observed in the population of SARS-CoV-2 sequences downloaded from the Global Initiative on Sharing All Influenza Data (GISAID) supported our prediction. Although it may be very well possible that the variations in Spike region are solely related to Spike protein evolution, we raise the possibility of genomic RNA structural evolution in favor of long-range base-pairing interaction between Spike and ORF8 regions. We hypothesize that the Spike-ORF8 genomic interaction could impact the rate of the nucleocapsid (N) gene sub-genomic RNA synthesis, which is a structural protein that forms complexes with genomic RNA, interacts with the viral membrane protein during virion assembly and plays a critical role in enhancing the efficiency of the virus transcription and assembly.

Evaluation of potential therapeutic ASO's to prevent SARS-CoV2 non-endosomal entry

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COVID-19 is a viral respiratory illness caused by a new coronavirus called SARS-CoV-2. The World Health Organization declared the SARS-CoV-2 outbreak a global public health emergency since its emergence affected more than 117 million people and led to more than 2.6 million deaths worldwide in little more than a year, equally rapid has been the progress in vaccine development, however, RNA viruses are characterized by a high mutation rate, up to a million times higher than that of their hosts, mutation rate drives viral evolution and genome variability, thereby enabling viruses to escape host immunity and to develop drug resistance. Therefore, this work aimed to design and evaluate TMPRSS2 and ACE2 ASO's as potential therapeutic targets to hamper SARS-CoV-2 enter in the cell host.

Methods: A bioinformatic search of the TMPRSS2 and ACE2 mRNA in rat and human was carried out, then a human-rat alignment was performed to identify homologous regions; after that, their secondary structures were evaluated. The selected sequences were synthesized by the BioAutomation Mermade-8 for its subsequent purification. The ASO's were administrated in the jugular vein, after three days the relative expression of mRNA TMPRSS and ACE2 were measure by RT-PCR.

Results: The administration of two TMPRSS2 ASO's intravenously led to a decrease in the relative expression of mRNA of TMPRSS2 in the lung rats three days after their administration, on the other hand we obtained that the administration of ACE2 ASO's increase the expression of ACE2 protein in the right lung nor in the left lung.

Conclusion: The mechanism involved in the increase of the expression of ACE2 in the right lung are not clearly understood, however, TMPRSS2 ASO's seems to be a potential treatment to prevent the entrance of SARS-CoV2 in human airway epithelial cells, nevertheless further studies are needed to confirm its real effectiveness.
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Modified-mRNA SARS-CoV-2 vaccine development for targeted delivery

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P.72

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic presents a tremendous challenge to lives and economies worldwide. As of 8th March 2021, SARS-CoV-2 has spread around the globe with over 116 million infected people and more than 2.5 million deaths. In an effort to control the pandemic, during 2020 and at the beginning of 2021 several vaccines were developed and received emergency use authorization. These first-generation vaccines are based on different platforms including nanoparticle-encapsulated mRNA, viral vectors, subunits, inactivated-virus, and live attenuated virus vaccines.

Inspired by the FDA approval of the first chemically modified RNA drug (siRNA GIVLAARI TM), here we propose a method for vaccine development based on chemically modified mRNA for targeted delivery to immunocompetent cells.

Click chemistry is herein used to modify in vitro transcribed (IVT) mRNA coding for the nucleocapsid (N) protein of SARS-Cov-2 to possibly enable its use in the context of mRNA-based vaccination. We have already demonstrated the efficient enzymatic incorporation of 3'-Azido-2',3'-ddATP (AzddATP), which can be then conjugated to sugar moieties. We therefore decided to use mannose- and GalNAc-derived molecules to target C-type lectin receptors for uptake into immunocompetent cells, such as dendritic cells (DC).

A genome-wide screen of siRNA molecules against SARS-CoV-2

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P.73

The world urgently needs a wide arsenal of anti-viral therapies in order to mitigate covid-19 and to prevent future pandemics. Multiple studies have shown that nasally-administered RNAi triggers are an effective prophylactic treatment for respiratory viruses, including SARS-CoV, influenza, and RSV. However, siRNA synthesis is relatively expensive, necessitating hyper-potent siRNA triggers to reduce dosage and associated costs.

Here, we harnessed a new framework called Sens.AI to find hyper-potent RNAi triggers against SARS-CoV-2. Sens.AI utilizes a cost-effective massively parallel assay for screening RNAi triggers, using advanced synthetic biology and bioinformatics tools. We used Sens.AI to screen >16,000 RNAi triggers against SARS-CoV-2, testing virtually every amenable site in the virus genome. We performed multiple validations to ensure the quality of our screen. We found high consistency between the rankings of two biological replicates ($R>72\%$, $P<1e-9$), and between our screen results and prediction algorithms ($R>90\%$, $P<1e-20$). We additionally verified that our top-ranked siRNAs are significantly enriched for sequence motifs known to be associated with potent RNAi ($P<1e-20$).

To validate our results, we selected 10 of the top-ranked siRNAs for further experimentation, using (1) a flow cytometry reporter assay, which identified 5 hyper-potent siRNAs with $IC50<30pM$; and (2) live virus tests, which identified 5 siRNAs that decreased viral load in Vero E6 cells by >95%. Encouraged by these results, we evaluated 14 cocktails spanning pairs of our top siRNAs with live virus. We identified four cocktails that exhibit synergistic effects and further reduce viral load by 2-3x using the same concentration. Importantly, each of these four cocktails targets every known SARS-CoV-2 strain with at least one of its two siRNAs. We selected two promising cocktails, chemically formulated them to prolong their effect and in-vivo durability, and verified that these formulations do not affect their efficacy. We are currently testing these chemically modified cocktails using a hamster model.

In conclusion, Sens.AI can screen complete viral genomes to solve the needle in a haystack problem of finding hyper-potent RNAi triggers. Our platform enables using siRNAs as a new type of cost-effective prophylactics beyond vaccines for respiratory viruses.

Preclinical & Clinical Immunogenicity Characterization of ARCT-021 SARS-CoV-2 Vaccine

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P.74

Arcturus Therapeutics is a global clinical-stage mRNA medicines company focused on the development of infectious disease vaccines and opportunities within rare liver and respiratory diseases. ARCT-021 is being developed for prevention of coronavirus disease 2019 (COVID-19), the disease caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) virus infection.

ARCT-021 is a self-replicating RNA based vaccine developed to illicit protective immunity against SARS-CoV-2 infections. The RNA encodes for a Venezuela Equine Encephalitis Virus (VEEV) replicase set of proteins that have been optimized to maximize expression of the spike glycoprotein (Sgp). The RNA is packaged in a cationic lipid formulation that protects the RNA from being degraded prior to cell entry and facilitates uptake and intracellular release into the cytosol. Mouse immunogenicity studies were conducted, and results were compared to an mRNA expressing the full length spike glycoprotein and formulated with the exact same lipid formulation as the VEEV Sgp RNA. In Balb/c mice RNA dose dependent production of anti-Sgp IgG persisted for 60 days post prime vaccination whereas anti-Sgp IgG induced by Sgp mRNA was significantly less and plateaued at day 10. Neutralizing antibody titers for VEEV-Sgp significantly increased from day 30 to day 60 post prime vaccination whereas no neutralizing antibody titers were observed after single vaccination of Sgp mRNA. D 30 post vaccination end point titers showed equivalent titers were obtained for S1, S2 and RBD domains of the full length spike.

RNT70 Neutralizing Antibody Titers for D 30 and D 60 Post Vaccination, and Convalescent Sera from COVID-19 Patients. , Approximately, 100 plaque forming units (pfu) were incubated with serially diluted serum from vaccinated or control animals before inoculation onto VeroE6 cell culture. The titer of serum required to reduce the number of plaques in the inoculum by 70% was then calculated to determine the PRNT70 titer, which indicates how much neutralizing antibody is elicited by vaccination.

Cell mediated immune response was evaluated in C57BL/6 mice for Sgp specific T lymphocytes and for Th1 and Th2 response by intracellular cytokine staining. VEEV-Sgp produced an RNA dose dependent increase in CD8+IFN-γ+T lymphocytes whereas the % CD8+ IFN-γ+ T lymphocytes were less, and no RNA dose dependent increase was observed. ELISPOT analysis identified Sgp specific T lymphocyte response to the RBD and S1-NTD domains of the Sgp. The VEEV Sgp gave at ≥ 4 fold higher % of positive T lymphocytes than Sgp mRNA. Th1 and Th2 response were measured by %CD4+ IFN-γ+/CD4+IL-4 T helper lymphocyte ratio in C57BL/6 mice. The results supported a Th1 mediated immune response that was not skewed by Th2 response. The Th1 mediated immune response was further supported by assaying the anti-Sgp IgG2a/IgG1 ratio in Balb/c and C57BL/6 mice. The 0.2 μg RNA dose showed that both the VEEV-Sgp and Sgp mRNA had ratios >1 indicating a Th1 immune response. However, the anti-IgG2A/IgG1 ratios for the 2 μg and 10 μg doses between 8 and 10 whereas the ratios for the Sgp mRNA vaccination were <1. These results show that the ARCT-021 SARS-CoV-2 vaccine elicits a robust humoral and cell mediated immune response after a single vaccination and is currently being tested in a Phase 1/2 clinical trial in Singapore.

An RNA degradation complex required for silencing of Polycomb target genes

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P.76

Polycomb Repressive Complex (PRC) 1 and 2 are histone-modifying and chromatin-binding complexes that are required for silencing of developmental regulatory genes and genes that control cellular proliferation. Their gene silencing functions are thought to involve chromatin compaction and condensate formation but whether other mechanisms contribute to silencing is unknown. Here we show that the rixosome, a conserved RNA degradation complex with roles in ribosomal RNA processing and heterochromatic RNA degradation in fission yeast, associates with human PRC complexes, is recruited to promoters of Polycomb target genes in differentiated cell lines and embryonic stem cells, and is required for efficient silencing of Polycomb target genes. These findings reveal an unanticipated role for RNA degradation in Polycomb-mediated silencing.

Termi-luc: a versatile assay to monitor termination and readthrough of stop-codons

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P.77

Premature stops in mRNA lead to premature termination and truncated proteins. Premature stops account for more than 10% of all human genetic diseases. Simple and reliable systems are required to identify and characterize drugs and therapeutics which could suppress the stops. Here we present a versatile tool that allows monitoring eukaryotic termination. The tool is based on a light-making protein that becomes active only upon release from the ribosome. Using the tool, we were able to show inhibition of termination by a competitive inhibitor and a small drug. Currently, we are expanding the tool so it can sense both termination and readthrough of premature stop – an act of decoding of the stop by aminoacid tRNA.

Enrichment of miRNA:mRNA chimeras to deeply profile miRNA targets using miR-eCLIP

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Identification of direct microRNA (miRNA) targets in a transcriptome-wide manner remains a challenge for the field. Here, we have applied enhanced crosslinking and immunoprecipitation (eCLIP) technology and probe-based enrichment methods to develop a robust specialized alternative, miR-eCLIP, to unambiguously identify miRNA targets transcriptome wide. miRNAs function as guides directing Argonaute proteins (AGO) to complementary target sites in the 3′ untranslated region (UTR) of target mRNAs. Several studies have shown that at a low frequency (~1%), chimeric RNA molecules can be generated in which miRNA and mRNA fragments are directly ligated together while bound in AGO. Once the miRNA is linked to the corresponding mRNA, direct targets of individual miRNAs are easily identifiable from high-throughput sequencing data using miRNA:mRNA chimeric reads. miR-eCLIP is based on AGO2 eCLIP and includes an additional ligation step to enhance formation of chimeric RNA molecules that can subsequently be sequenced. While global target identification for all miRNAs in a single experiment is appealing, miRNA expression levels vary greatly, making it difficult to deeply profile all individual miRNAs in a whole-miRNAome approach. Thus, we have developed probe-based enrichment technologies that can be utilized to enrich chimeric libraries for miRNAs or genes of interest. The miRNA enrichment strategies employed here result on average in 500-fold more chimeric reads for each miRNA of interest, facilitating identification of >30-fold additional mRNA targets. Similarly, we have developed a probe-based gene enrichment strategy to deeply profile the repertoire of miRNAs binding a target of interest, resulting in significantly more chimeric reads and identification of 10 to 20-fold more targeting miRNAs. Each of the enrichment strategies can be multiplexed to examine several genes or miRNAs in a single experiment, allowing for substantially increased throughput. We have utilized miR-eCLIP to examine individual miRNA binding sites transcriptome wide and have validated that miR-eCLIP identifies high-confidence, functional mRNA targets that are in agreement with previous approaches. Furthermore, miR-eCLIP can be used to validate the specificity of therapeutic siRNAs by directly mapping both on-target and off-target binding.

Noise to Signal: exploring the landscape of noisy splicing

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Splicing is likely a major contributor to overall error in mRNA regulation, which reduces efficiency of transcription and wastes transcriptional output. Noisy splicing, which differs from aberrant splicing caused by genetic mutations, is the use of non-canonical or non-productive splice sites leading to transcripts that are more likely to be targeted for decay. Splicing noise has been previously observed in homeostatic cell conditions, but the extent to which it occurs is likely underappreciated due to the challenges of identifying noisy splice site usage in mature mRNA data. Blocking or redirecting the use of noisy splice sites towards the usage of productive splice sites may provide a novel strategy for up-regulating gene expression in healthy or disease contexts with high-levels of splicing noise (e.g., cancer). However, the underlying mechanisms promoting noisy splicing and sites at which it occurs have not been characterized. Here, we compare RNA enrichment protocols and find that nascent RNA sequencing greatly increases the detection of noisy splice sites relative to polyA pull down or nuclear enrichment methods. We use these methods to identify noisy splicing events in erythroleukemia and glioblastoma cell lines and characterize genomic features, sequence elements, and gene properties associated with the occurrence of noisy splice sites across and between cell types. Our findings will inform our understanding of stochasticity in gene regulation and aid the development of novel disease therapeutics to inhibit noisy splicing.

Kinetic barcoding: stream-lined multi temporal estimates of RNA biogenesis kinetics

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P.80

The rate of mRNA biogenesis is determined by the time required to transcribe and process pre-mRNA molecules. While recent advances in RNA sequencing techniques allow for high-resolution interrogation of transcriptional and processing steps, there are still limitations towards understanding the mature mRNA production rates. Existing methods for genome-wide kinetic profiling of RNA maturation involve isolation and high-throughput sequencing of RNA intermediates along a temporal progression towards maturation, often after culturing cells with a modified nucleotide that is incorporated into nascent RNA and differentiates newly transcribed RNA molecules from existing populations. However, current strategies are limited in temporal resolution, presenting challenges for measuring very fast biogenesis rates (<2 minutes) and an inability to assess when the labeling clock was stopped due to experimental handling steps. Finally, multiple labeling experiments are needed to measure nascent RNA across a short time course, with each additional time point introducing additional technical biases and added sequencing expenses. To address these issues, we developed a labeling strategy, which we call time-resolved "kinetic barcoding", that uses step-wise labeling of nascent RNA with multiple nucleosides to enable the measurement of multiple time points within the same sequencing library. Specifically, we sequentially add, in order, 5-ethynyl-uridine (5eU), 6-thio-guanosine (6sG), and 4-thio-uridine (4sU) at distinct time points separated by 2-3 minutes over a 10 minute time course to interrogate multiple time scales of nascent intermediates within a single experiment. We isolate all nascent RNA with CLICK chemistry targeting the 5eU labeled RNA, followed by alkylation of the 6sU and 4sU thiol groups to induced G>A and T>C substitutions, respectively, where these nucleotides were incorporated. After short-read Illumina sequencing of this pool of labeled nascent RNAs, we can computationally distinguish molecules transcribed during the first, second, and final labeling windows by their substitution patterns. As proof of principle and utility, we apply our novel kinetic barcoding approach to measure transcription elongation rates in human cells. We show that sequential labeling of RNA with multiple nucleosides introduces no nucleotide-specific biases and provides increased temporal resolution for measuring the variation in elongation rates between genes.

Targeting translation of KRAS in pancreatic cancer

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Pancreatic adenocarcinoma (PDAC) epitomizes a deadly cancer driven by abnormal KRAS signalling. Here we show that the eIF4A RNA helicase is required for translation of key KRAS signaling molecules and that pharmacological inhibition of eIF4A has single-agent activity against murine and human PDAC models at safe dose levels. EIF4A was uniquely required for the translation of mRNAs with long and highly structured 5'UTRs including those with multiple G-quadruplex (GQ) elements. Computational analyses identified these features in mRNAs encoding KRAS and key downstream molecules. Transcriptome-scale ribosome footprinting accurately identified eIF4A-dependent mRNAs in PDAC including critical KRAS signaling molecules such as PI3K, RALA, RAC2, MET, MYC, and YAP1. These findings contrast with a recent study that relied on an older method, polysome fractionation, and implicated redox-related genes as eIF4A clients. Together, our findings highlight the power of ribosome footprinting in conjunction with deep RNA sequencing in accurately decoding translational control mechanisms and define the therapeutic mechanism of eIF4A inhibitors in PDAC.

Estimating the Kinetics of mRNA 3' End Cleavage

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P.82

Alternative mRNA processing is increasingly appreciated to play a large role in driving transcriptome variability, disease etiology, cellular identity, and the molecular response to diverse environmental stresses. Although we have extensive insights into the regulatory factors and sequence elements that influence alternative isoform usage, less is known about the temporal dynamics and co-regulation of RNA processing decisions. Intermediate processing events—splicing and 3' end cleavage—often occur co-transcriptionally, with the interplay between transcriptional elongation rates and rates of each processing event often impacting choices that lead to alternative isoform production. Consequently, measuring the kinetics of these processes may shed light on how early gene regulatory decisions are made. Recent development of high-throughput sequencing techniques that capture nascent RNA over defined temporal intervals has made genome-wide kinetic profiling of RNA maturation possible. Though rates of mRNA splicing have been estimated globally, the rate at which an mRNA is cleaved and polyadenylated to complete the maturation process has never been investigated. Here, we present a novel computational method to estimate genome-wide kinetic parameters for mRNA cleavage rates. This method capitalizes on short-read sequencing data from nascent mRNAs isolated after a time-course of 4sU metabolic labeling to model the rate of mRNA maturation over time. To specifically measure cleavage rates, we first use patterns of read coverage from our sequencing data to approximate the position at which cleavage occurs. We then estimate the fraction of reads derived from cleaved or uncleaved molecules at that site across time to model the rate of 3' end cleavage. We applied this method to nascent RNA-seq data from Drosophila melanogaster S2 cells to estimate polyadenylation-site (PAS) specific rates of mRNA cleavage. Our findings shed light on the timing of decisions involved in alternative PAS usage within genes and the variable efficiency of 3' end cleavage and polyadenylation across genes.

Evidence for functional specialization of ribosomal proteins isoforms

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Ribosomes are ubiquitous ribonucleoprotein complexes required for protein synthesis in all living organisms. They are usually considered as monolithic machines. However, as with alternative transcription and splicing, emerging evidence suggests that differential translation is an important mechanism of gene regulation. Yeast genomes have duplicated ribosomal protein genes (RPGs) and there has been some debate about whether paralogs have specialized functions or module ribosome dose. We used gene conversion between two gene copies to investigate the differences in function of ribosomal protein paralogs. Using growth curve analysis, translation profiling, and mass spectrometry, we find important difference in function for uL30/RPL7, where by the minor and major paralogs have opposite effects on the translation of a key set of stress response genes with unusually long ORFs. Intriguingly, the stress-inducing drug staurosporine induces a feedback loop involving the minor paralog that obviates the normal effects of the drug and leads to resistance. Our observations suggest that alternative ribosomal proteins regulate translational activity. In humans and mice, RPGs contain 3-9 introns and have many annotated alternative splicing isoforms, leading to the potential to produce numerous protein-coding alternative splicing isoforms (PC-ASIs). Alteration of RP expression through regulated RPG alternative splicing may provide cells with enormous plasticity across a range of growth and differentiation signals by modulating ribosome composition and function. During my postdoctoral studies, I will systematically identify, functionally analyze, and probe the impact of RPG PC-ASIs on developmental and disease progression to test the hypothesis that alternative splicing of RPs play major role in regulating protein synthesis in humans and other mammals.

Exon-Dependent Effect on DNA Methylation and Alternative Splicing by Repeated Depolarization

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P.84

Cells change their gene expression programs in response to long term repeated stimulations, for which the underlying mechanisms remain to be fully understood, particularly regarding the relationship between epigenetic and splicing changes. Here we repeatedly depolarized GH3 pituitary cells with high concentrations of KCl and studied how the cells adapt by examining their DNA methylation level and exon usage by whole genome bisulfite sequencing of the nuclear DNA and RNA-Seq of the cytoplasmic RNA. Overall, the levels of exon methylation are inversely correlated with the fold changes of exon usage at the extremes; however, for most of the exons their relationships are more complex, in an exon-dependent way. We analysed more specifically 5 exons of different genes for their methylation and splicing changes along the KCl treatment process. The methylation and splicing changes of these exons can be divided into three categories: same direction of changes; opposite direction of changes or no corresponding changes of methylation versus splicing. Moreover, for the same exon, the relationship between methylation and splicing change could vary dependent on the number of KCl treatments. Therefore, our observation suggests that methylation may have a determining effect on splicing only when at hypermethylation states and its modulatory effect is exon-dependent.

Detection of Pseudouridine Modifications in mRNAs Using Direct, Long-read Sequencing

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P.86

Pseudouridine is one of the most abundant and dynamic RNA modifications in mammalian cells and plays an important role in post transcriptional gene regulation. However, we still don't know the precise sequence coordinates of all pseudouridylated sites or the exact biological function. Previous studies have identified putative sites of modification, however these methods are limited by a critical, chemical-labeling step with 1-cyclohexyl-(2-morpholinoethyl)carbodiimide metho-p-toluene sulfonate (CMC). We developed a direct-RNA nanopore sequencing pipeline and algorithm to independently-validate previously identified pseudouridylated sites and identified previously unknown pseudouridylated sites in HeLa cells. Using this data, we model three types of hyper-modification: 1. Single pseudouridylated sites that are frequently modified; 2. Individual mRNAs with more than one site of pseudouridylation; and 3. Individual mRNAs that are targets for more than one type of RNA modification on the same strand.

Mutant Huntingtin mRNA Forms Nuclear Clusters at Transcriptional Sites in vivo Associated with Overexpression of the Aberrant Isoform HTT1a

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P.87

Mutant mRNA and protein both contribute to the clinical manifestation of many repeat-associated neurodegenerative and neuromuscular disorders. The presence of nuclear RNA clusters is a feature shared amongst these diseases, such as C9ORF72/ALS and myotonic dystrophy 1/2 (DM1/2); however, this pathological hallmark has not been conclusively demonstrated in Huntington's disease (HD) in vivo. Investigations into HD – caused by a CAG repeat expansion in exon 1 of the huntingtin (HTT) gene – have largely focused on toxic protein gain-of-function as a disease-causing feature, with fewer studies investigating the role of mutant HTT mRNA in pathology or pathogenesis.

Here we report that in two HD mouse models, YAC128 and BACHD-97Q-ΔN17, mutant HTT mRNA is preferentially retained in the nucleus in vivo with the widespread formation of large mRNA clusters (approximately 0.6 to 5 μm3 in size) present in over 50-75% of striatal and cortical neurons. These clusters associate with chromosomal transcriptional sites and quantitatively co-localize with the expression of the aberrantly spliced N-terminal exon 1-intron 1 isoform, HTT1a. Cluster formation is driven by repeat expansion, is not age-dependent, and is not observed with wild-type huntingtin or housekeeping mRNAs. HTT1a mRNA clusters are observed in ~1% of neurons in human HD postmortem brains and are likely driven by somatic expansion and mosaicism. In YAC128 mice, clusters, but not individual HTT mRNA foci, are resistant to treatment with antisense oligonucleotides. Our findings identify mutant HTT/HTT1a mRNA clustering as an early, robust molecular signature of HD, further supporting HD as a repeat expansion disease with suspected mRNA involvement.

Intercellular mRNA transfer via macrophages in human to mouse xenograft models

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P.89

Intercellular transfer of mRNA molecules in mammals has been subject to several decades of research, producing a few studies that indirectly support this phenomenon, but no direct evidence of in vivo mRNA transfer. The sequence identity between transcripts in donor and acceptor cells contributes to the challenge of detecting intercellular mRNA transfer. Thus, mRNA transfer between cells of different species would enable the different mRNA sequences to act as donor-receptor specific tags. We have analyzed RNA-seq data from xenotransplanted human cells cultured in and recovered from mice for the presence of mouse transcripts in human cells.

To investigate the transfer of mouse transcripts into engrafted human cells, we re-analyzed RNA-seq data from three studies with appropriate experimental conditions. Since contamination of acceptor cells with ambient RNA from damaged donor cells is a persistent concern for detection of RNA transfer, a mouse and human barnyard study was used to define contamination signatures. As standard mapping software could not provide complete separation between mouse and human-specific sequencing reads, all low complexity sequencing reads were removed and the species identity of all remaining reads were confirmed by blastn.

Several to hundreds of mouse transcripts were detected for a considerable portion of transplanted human cells, however, cells from only one study did not demonstrate contamination signatures. Deconvolution analysis of sets of transferred transcripts indicate macrophages as the most probable mRNA donors. These data identify mouse macrophage expressed Ftl1 as one of the frequently detected transcripts in the sequenced human cells. We have confirmed in vitro transfer of mouse Ftl1 transcript from both immortalized and primary peritoneal macrophages to human HeLa cells.

In conclusion, our data provide direct evidence of mRNA transfer between mammalian cells. This has implications for both the autonomy of cells and cell types in animals, particularly in xenotransplants, both experimental and therapeutic, and suggests that macrophages may serve as mRNA donors for gene therapy.

Spurious activities of T7 RNA polymerase during in vitro transcription.

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P.90

The ability to transcribe genes of interest in vitro has led to the dissection of key biological steps that are involved in gene expression. In addition to their use as tools for the biochemical understanding of physiological processes, synthetic RNAs are being used in biopharmaceuticals, including RNA-based therapeutics, vaccines, and diagnostics. These applications have necessitated the development of enzymes for robust and efficient synthesis of homogeneous RNA populations that can be used both in vitro and in vivo. The technology used for the synthesis of these in vitro-transcribed (IVT) RNAs utilize phage-encoded single-subunit RNA polymerases (ssRNAPs). The most widely used ssRNAP is T7-RNAP because of its high processivity, specificity for small promoter sequence, and extensive characterization. However, T7-RNAP has spurious activities resulting in formation of by-products such as abortive transcripts during transcription initiation, heterogeneous 3' ends due to non-templated additions, and 3'-extended RNAs due to self-extension of the run-off product. In some cases, the spurious products can result in formation of dsRNA regions that have been identified as a trigger of cellular immune responses. It is critical to either eliminate these by-products from the mRNA preparations or minimize their formation. Thus, the discovery and characterization of ssRNAPs that can overcome the limitations of T7-RNAP and can generate IVT RNA devoid of unwanted by-products is highly desired.

Here, we discuss our multi-pronged approach to characterize the nature and source of dsRNA by-products generated by 3'-extension of the run-off product or by non-template strand transcription (antisense dsRNAs). These two classes of dsRNA are formed through distinct mechanisms. The 3'-extended RNA, which constitutes the bulk of dsRNA by-products, is formed in an RNA-dependent manner, whereas antisense dsRNA is formed by template switching in a DNA-template-dependent manner by T7 RNAP. We demonstrate that certain sequences at the 3' end of the DNA template have a higher propensity to promote non-template strand switching by T7 RNAP. We also present data on the propensity of other ssRNAPs that are similar and distant to T7 RNAP to form 3' extension of the run-off transcript.

We envision that understanding the mechanism of by-product formation in multiple ssRNAPs will provide insights into altering these activities in the RNAPs and generating more-homogeneous IVT RNA preparations.

Delivery of functional mRNA throughout the conducting airway and alveolar regions including Type I and Type II pneumocytes

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P.91

In 2017, even prior to COVID-19, the World Health Organization stated that 'respiratory diseases are the leading causes of death and disability in the world'.1 Whether these respiratory diseases are acute or chronic, affecting young or old, there is a high unmet need for effective treatments. Translate Bio has a focus on developing therapeutics for respiratory diseases using an mRNA-based platform. mRNAs, encapsulated in lipid nanoparticles (LNPs), are delivered directly into the lungs via an inhaled route of administration. To characterize these LNPs and their delivery, we employed the TdTomato Ai14 mouse model, which is transgenic for TdTomato with a loxP-flanked STOP cassette that prevents transcription and expression of the TdTomato protein. The transgene is located in the Gt(ROSA)26Sor locus and can serve as a reporter throughout the body. Upon Cre expression, the STOP cassette is excised and TdTomato is continually expressed. TdTomato Ai14 mice were exposed to LNP-encapsulated Cre mRNA, which had been nebulized via an Aeroneb nebulizer connected to an inhalation tower. Where effective delivery occurred, the Cre protein was expressed and resulted in TdTomato expression. Approximately 48 hours post-exposure, mice were euthanized and cryofluorescent tomography (CFT) was used to understand whole body biodistribution. We observed TdTomato expression throughout the respiratory tract, from the nose through the lungs, with no significant test article-driven systemic expression. Additional mice were used to further characterize spatial TdTomato expression throughout the lungs by immunohistochemistry. In the Cre-LNP treated mice, we observed TdTomato expression throughout the conducting airway and into the alveolar regions in the Type I and Type II pneumocytes. No signal was observed in the saline-treated or control-treated mice. These data demonstrate that nebulization of mRNA-encapsulated LNPs offer direct delivery into the lung with minimal systemic exposure. This approach is currently being investigated in cystic fibrosis patients, as a first-in-human study to explore the use of a wild-type CFTR mRNA-based therapy to treat a chronic genetic disease. Many potential applications exist for an inhaled mRNA therapy to treat both acute and chronic disorders, which are currently being explored.

1. Forum of International Respiratory Societies. The Global Impact of Respiratory Disease – Second Edition. Sheffield, European Respiratory Society, 2017.

Targeted Augmentation of Nuclear Gene Output (TANGO) Technology for Protein Upregulation in Dravet Syndrome

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P.92

TANGO is a novel technology which exploits antisense oligonucleotide (ASO)-mediated modulation of pre-mRNA splicing to increase protein expression. TANGO ASOs prevent naturally occurring non-productive events that lead to either transcript degradation by nonsense-mediated mRNA decay (NMD) or nuclear retention. This results in increased productive mRNA and full-length, fully functional protein. Using bioinformatic analyses of RNA sequencing datasets, we identified non-productive events in more than 50% of protein-coding genes, of which approximately 1,246 are disease and NMD-associated. To prove the TANGO concept, we selected targets representing various types of NMD-inducing, non-productive alternatively spliced (AS) events (cassette exons, alternative splice sites, and alternative introns). TANGO ASOs showed dose-dependent reductions in non-productive mRNA and increases in both productive mRNA and protein levels from genes of diverse size, type and function. TANGO ASOs designed to target the three types of non-productive AS events showed a dose-dependent increase of productive mRNA and protein in vitro. The effectiveness of this approach for treatment of genetic epilepsy due to haploinsufficiency was demonstrated for Dravet Syndrome (DS) using the Scn1atm1Kea, F1:129S-Scn1a+/- x C57BL/6J mouse model (DS mouse). This model has been shown to recapitulate features of DS including seizures and sudden unexpected death. DS mice were administered a single dose of STK-001 via intracerebroventricular (ICV) injection. We show STK-001 increases productive Scn1a mRNA and restoration of NaV1.1 protein from 50% to near normal levels in the brain for up to 14 weeks. The restoration of NaV1.1 resulted in 76% of STK-001-treated mice being seizure-free vs 48% of placebo-treated mice with 80% reduction in the average number of spontaneous seizures in the STK-001 group measured by electroencephalography (EEG). Two dose levels of STK-001 were also evaluated in cynomolgus monkeys via a single intrathecal lumbar bolus injection. Twenty-eight days post-injection, NaV1.1 was increased up to 3-fold in the high-dose group in regions of the cerebral cortex which are thought to be involved in disease pathology. STK-001 was well-tolerated with no observed adverse events at the highest dose tested, no changes in platelet counts or hepatic function, and no adverse histopathology in the brain, liver or kidney. Collectively, these data suggest that STK-001 has potential to provide a gene-specific, disease-modifying treatment to restore NaV1.1 to physiological levels to provide therapeutic benefits for patients with DS. STK-001 is currently being evaluated in a Phase 1/2a clinical trial in patients with DS.

Probing the Mechanism of Action of Small Molecule Therapeutics in Myotonic Dystrophy Type 1

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The expansion of microsatellite repeats within the human genome has been identified as the cause of numerous hereditary diseases, including Myotonic dystrophy (DM). DM is an autosomal dominant disease with multisystemic symptoms such as myotonia, cardiac conduction defects, muscle wasting and weakness, insulin resistance, and cataracts. There are two genetically distinct, yet clinically similar types of DM: DM type 1 (DM1) is caused by CTG repeat expansions in the 3' UTR of the dystrophia myotonica protein kinase (DMPK) gene, while DM type 2 (DM2) results from CCTG repeat expansions in the 1st intron of the CCHC-type zinc finger nucleic acid binding protein (CNBP) gene. When transcribed, these repeat expansions produce toxic expansion RNAs that sequester RNA binding proteins and lead to abnormal cell function. There is currently no cure for DM, but different approaches are being explored to develop therapeutics for the disease. Our lab has focused on small molecules approaches, such as diamidines and microtubule inhibitors, which have been shown to reduce the toxicity of the expanded repeats. To further develop this approach and potentially improve their efficacy, we want to understand how these small molecules are reducing toxicity. Previous work using a 5-ethynyl uridine (EU) pulse labeling assay indicates that colchicine is inhibiting transcription of the toxic repeats. Our aim is to use EU pulse labeling to isolate nascent RNA in DM cell models to assess effects on CTG transcription and CUG mRNA stability. To focus on the effects the small molecules have on the RNA itself, we will transfect cells with CUG RNA, treat them with small molecules and monitor the effects. Taken together these series of assays will allow us to determine at what molecular level- transcription vs RNA turnover- these small molecules are acting upon. Publicly available RNA-seq data of small molecule treatment on various DM1 models (mouse and cell) will be re-analyzed to look for effects on the transcriptome and on specific genes of interest in DM1, which could provide hints into the MOA of our small molecules of interest. Information on MOA can be used for selection of more efficient small molecules and for the guided design of combinatorial small molecule treatments.

RNAi as an Approach to Alleviate Ischemia-Reperfusion Injury During Liver Preservation

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Liver diseases claim approximately 2 million lives worldwide each year. Transplantation is the curative treatment for acute end-stage and chronic liver diseases, yet the waitlist mortality is estimated at 10-20%. A major factor perpetuating the donor shortage is the susceptibility of organs to ischemia-reperfusion injury (IRI), a process resulting in oxidative damage, inflammation, and hepatocyte death during procurement, ex vivo preservation, and surgery. In fact, 20-30% of procured liver grafts are discarded prior to transplant due to IRI damage. Current strategies of ex vivo organ preservation including static cold storage (SCS) and machine perfusion (MP) do not adequately protect the organ from IRI, and clinical therapeutic strategies have yet to be developed due to lack of efficacious targeting and delivery strategies. Thus, developing targeted therapies that mitigate the molecular pathways underlying IRI would increase the number of viable organs thereby reducing the number of waiting list candidates and improving patient survival.

Preliminary studies from our lab demonstrate that ex vivo MP supports the delivery of unmodified small interfering RNA (siRNA) targeting cell death receptor Fas to the liver, but the downstream effect on IRI and transplant success remains unexplored. Thus, the goal of this project is to use RNA interference (RNAi) technology to silence liver IRI gene expression and improve the quality of liver grafts during ex vivo preservation. Chemically-modified N-acetylgalactosamine (GalNAc) siRNA modulates liver gene expression in a sustained and sequence-specific manner. Thus, is it possible to silence liver IRI pathways using a GalNAc-siRNA approach, and how do we optimally deliver siRNAs during ex vivo liver preservation to reduce IRI and improve post-transplantation outcomes?

A panel of chemically-modified siRNAs targeting Tp53, a known mediator of IRI-induced apoptosis, was designed and synthesized consisting of a 2'-O-methyl and 2'-fluoro backbone of modified ribose sugars with conjugation to cholesterol for initial in vitro screening. In vitro mRNA silencing efficacy and potency were tested in rat hepatocytes (ATCC, RH-7777) using a top siRNA dose of 1.5 uM. mRNA expression was quantified using the QuantiGene 2.0 Assay. The screen revealed 2 potent lead siRNA compounds targeting Tp53 mRNA with a maximal silencing of >75%. In vivo studies to determine the efficacy of the lead siRNA compounds in a rat liver IRI model are ongoing. Once confirmed, ex vivo MP will be implemented to assess its compatibility for GalNAc-siRNA delivery and silencing efficacy in a rat liver transplant model.

Design and Evaluation of VE-PTP siRNA For the Treatment of Diabetic Retinopathy

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Diabetes is a disease that affects around 422 million people worldwide, which one of its main complications is diabetic retinopathy (DR) that lead to blindness, within the mechanisms involved in this pathology are angiopoietins pathway and especially the vascular endothelial protein tyrosine phosphatase (VE-PTP), which is associated with the pathological neovascularization. For this reason, the aim of this work is to develop a siRNA against VE-PTP to decrease neovascularization in diabetic retinopathy. The siRNA was designed using the siRNA Wizard v3.1, RNAfold web server and EMBOSS softwares and the sequences synthesized using a MERMADE 8 equipment. Subsequently, male Wistar rats were administered with streptozotocin (STZ) to induce diabetes and after 2 weeks were administrated with the siRNA, the biochemical parameters, the neovascularization (junctions and lacunarity) in the retina and the expression of the VE-PTP mRNA were evaluated after 4 weeks. The results showed a significant increase in the neovascularization and VE-PTP expression in the diabetic group compared with the nondiabetic group, while the administration of VE-PTP-siRNA produced a significantly decreased in the neovascularization and expression of the VE-PTP mRNA. In conclusion, our results suggest that the administration of a VE-PTP siRNA could be a promising diabetic retinopathy treatment.

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LinearDesign: Efficient Algorithms for Optimized mRNA Sequence Design

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A messenger RNA (mRNA) vaccine has emerged as a promising direction to combat the current COVID-19 pandemic. This requires an mRNA sequence that is stable and highly productive in protein expression, features which have been shown to benefit from greater mRNA secondary structure folding stability and optimal codon usage. However, sequence design remains a hard problem due to the exponentially many synonymous mRNA sequences that encode the same protein, e.g., for the spike protein of SARS-CoV-2, which contains 1,273 amino acids (plus the stop codon which is part of the mRNA but not part of a protein), there are about 10^632 mRNA candidates. We show that this design problem can be reduced to a classical problem in formal language theory and computational linguistics that can be solved in O(n^3) time, where n is the mRNA sequence length. Based on this framework, we develop a cubic-time algorithm that can optimize mRNA folding stability, i.e., find the mRNA sequence that has the minimum folding free energy among all synonymous mRNA sequences. This algorithm could still be too slow for large n (e.g., n = 3, 822 nucleotides for the spike protein of SARS-CoV2), so we further developed a linear-time approximate version, LinearDesign. This algorithm, LinearDesign, can compute the approximate minimum free energy mRNA sequence for this spike protein in just 11 minutes using beam size b = 1, 000, with only 0.6% loss in free energy change compared to exact search (i.e., b = +∞, which costs 1 hour). On the other hand, codon usage is also important for mRNA functional half-life. Thus, we extend the algorithm to jointly optimize folding stability and codon usage. We observe that the sequences designed by our algorithm has much lower folding free energy (i.e., much stable) and higher codon adaptation index (CAI) than the wildtype sequence. For example, one of our design on the spike protein genome has the folding free energy change of -2,414.6 kcal/mol and CAI of 0.823, while the wildtype has the folding free energy change of -967.80 kcal/mol, and CAI of 0.655. Our work provides efficient computational tools to speed up and improve mRNA vaccine development.

Effect Of Hiv-1 Infection On The Distribution Of The Transmembrane Nucleoporin Pom121

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Over the last decade it became evident that nucleoporins are integrally linked to the life-cycle of the HIV-1 genome. Recently an N-terminal truncated form of the nucleoporin POM121C was reported to inhibit HIV-1 replication and earlier it was shown that the knockdown of POM121 inhibited HIV-1 replication at the nuclear import step. Our group had data from a HeLa cell line with an integrated HIV-1 test genome, which carried the BGlg RNA aptamer binding site, that showed how POM121 (labeled with TdTomato) relocates within the nuclear envelope and that a sub-fraction of POM121 co-localizes with the viral full length RNA. Conducting multi-channel imaging experiments with our home-built state of the art microscope, this time using Jurkat, and primary human T-cells infected with pNL4-3-GFP HIV-1 virus (NIH AIDS reagent 11100), we observed progressive changes in the distribution of POM121. Starting from its normal abundance within well-spaced nuclear pores at time zero, POM121 separated into aggregates later in the infection cycle. We showed that cells with this phenotype are not apoptotic, using an AnnexinV-Alexa647 co-stain (Biolegend 640912). Our data point toward a new mechanism for at least a sub-class of HIV-1 full length genomes during cell nuclear export that provide further insight into how the HIV virus manipulates the host cells transport machinery, possibly to arm itself for next round of infection.

Time-resolved Cryo-EM Visualizes Ribosomal Translocation with EF-G and GTP

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Accurate and efficient translation requires a GTPase elongation factor (EF-G in bacteria; eEF2 in eukaryotes) to translocate tRNA and mRNA through the ribosome. EF-G has been proposed to act as a flexible motor that propels tRNA and mRNA movement, as a rigid pawl that biases unidirectional translocation caused by ribosome rearrangements, or as various combinations of motor- and pawl-like mechanisms. Structural visualization of how EF-G facilitates translocation has remained a challenge.

Using time-resolved cryo-EM, we have visualized GTP-dependent translocation without inhibitors, capturing elusive structures of ribosome•EF-G intermediates at near-atomic resolution. Visualization of the translocation trajectory from pre-translocation to late translocation states answers long-standing questions and elucidates the structural roles of GTP hydrolysis and ribosome rearrangements in the universally conserved process of translocation.

Understanding small DNA guide generation for Thermus thermophilus Argonaute (TtAgo)

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Argonaute proteins (pAGOs) are present in thousands of bacterial and archaeal genomes, including clinical isolates of some commonly antibiotic-resistant pathogens. pAGOs use small RNA or DNA guides to defend bacteria and archaea against invading viruses or plasmids. Yet little is known about the molecular mechanisms underlying pAGO guide acquisition or function.

TtAgo, the pAGO of the eubacterium Thermus thermophilus, is one of the few pAGOs whose function has been examined in vivo. Endogenous TtAgo participates in DNA replication and can decatenate circular chromosomes when gyrase is inhibited with ciprofloxacin (Jolly et al., Cell 2020). TtAgo is guided by ~16 nt DNAs derived from the terminus of replication on the chromosome and megaplasmid. How these guides are generated and what role other endogenous proteins may play in this process remains unclear.

In vivo studies of TtAgo-associated guides and proteins suggest a model for guide acquisition in which TtAgo binds DNA nicks generated by stalled replication forks. The model envisions that TtAgo then cleaves the DNA strand opposite the nicked DNA. Endo- or exonucleases then trim the guide to its mature length. We have developed an in vitro assay using recombinant TtAgo and a model double-stranded DNA substrate bearing a single nick. Our preliminary experiments show that TtAgo can bind DNA nicks and cleave the opposite DNA strand as predicted by the model. Notably, the rate of TtAgo-catalyzed cleavage differs only slightly between nicked DNA bearing a 5′ phosphate and a 5′ hydroxyl, even though mature guides in vivo have 5′ monophosphorylated ends. We are currently examining the turnover rate of TtAgo at DNA nicks to determine if TtAgo remains bound after target strand cleavage. Finally, to understand the role of other endogenous proteins in TtAgo guide biogenesis, we will examine TtAgo cleavage rates and guide lengths in the presence of T. thermophilus cell lysate. Future work will examine the importance of endogenous DNA nucleases in guide generation and the conservation of this pathway in other eubacteria, including human pathogens. Our goal is to decipher the molecular mechanism and partner proteins necessary for TtAgo function, a prerequisite for exploring pAGOs as a target for novel antibiotic therapies.

RiPCA: A cellular assay for the detection of RNA-protein interactions

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P.103

The physiochemical characteristics of RNA makes it a very challenging target for small-molecule drug discovery, highlighting the necessity for alternative approaches to targeting RNA biology. With the development of technologies for genome-wide query of RNA molecules and RNA-binding proteins (RBPs) enabling the discovery of RNA-protein interactions (RPIs), many of which have since been linked to human diseases, there are now countless RPIs that have been presented as potential for novel therapeutics. Despite increasing interest in developing strategies for targeting therapeutically relevant RNAs and RPIs, there remains a lack of enabling technologies for the discovery and development of RPI inhibitors. To fill in this technological gap, I have developed an assay for the dynamic cellular monitoring of RPIs. To address this technological gap, I have developed an assay for the dynamic cellular monitoring of RPIs. In the assay, termed RNA interaction with Protein-mediated Complementation Assay (RiPCA), cellular RPIs are monitored via protein complementation of Promega's NanoLuc® Binary Technology (NanoBiT). We recently reported our proof-of-concept development of a RiPCA protocol capable of detecting the interaction between pre-microRNA, pre-let-7, and its RBP, Lin28. Current efforts to continue developing this technology include (1) optimizing RiPCA as a high-throughput platform to enable screening of pre-let-7/Lin28 inhibitors and (2) adapting RiPCA to detect additional RPIs, including additional pre-miRNA-RBP interactions, but also RPIs involving mRNAs, lncRNAs, and expanded repeats.

Superparamagnetic Iron Oxide Nanoparticle RNA-antagomiR targeting miR-24-3p for Molecular Therapy of Multiple Endocrine Neoplasia type 1

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Multiple endocrine neoplasia of type 1 (MEN1) is a rare (1 / 30,000) endocrine tumor syndrome characterized by the presence of at least two neuroendocrine tumors in a single patient. Most MEN1 tumor tissues present LOH at the level of the 11q13 region or, alternatively, a second inactivating somatic mutation on the second copy of the MEN1 gene. However, the loss of both wild type copies of the gene for loss of heterozygosity (LOH) at the somatic level in the neuroendocrine tissues (according to Knudson's "two hits" model for tumor suppressor genes) has been established as a fundamental step for tumorigenesis and neoplastic progression.

This mechanism, however, occurs only in specific neuroendocrine tissues and not in all the tissues of the body. The main candidates responsible for this tissue-specificity of MEN1 tumorigenesis are miRNAs.

Results of studies carried out by our research group have clearly identified a direct link between miR-24-3p and MEN1 tumorigenesis, suggesting this molecule as a possible target against which to develop "preventive" gene therapies with RNA-antagomir, to restore the correct expression of menin in the cells of MEN1 patients and for the prevention of tumor progression before the advent of irreversible gene LOH. Transfection of an antisense 2'-O-methyl RNA (antagomir) against miR-24-3p/ conjugated with magnetic nanoparticles in pancreatic human BON1 cells causes silencing of endogenous miR-24-3p with consequent increase in expression of the protein of onc-suppressor menin (encoded by the tumor suppressor gene MEN1), highlighting a correlation between miR-24-3p / MEN1 / menin and a direct role of miR-24-1 in the negative regulation of menin wild type and consequently in the onco-genesis of the MEN1 syndrome. Cross-sectional imaging by TEM was further used to qualitatively analyse the cell internalization and the intracellular location of the 2'-O-methyl RNA (antagomir) against miR-24-3p/ conjugated with magnetic nanoparticles.

Acting on miR-24-3p in the phases preceding the development of tumors may therefore prove to be a possible molecular therapy in this syndrome.

Similar mechanisms can also be hypothesized for other tumor types, for which, in the future, targeted molecular therapies could be used using "antagomirs", complementary to the oncogenic miRNA to be silenced, conjugated with magnetic nanoparticles in order to be recognized in alive, by insertion in high-field MR tomographs, directly at the level in the organs and tissues of interest and also used as diagnostic tracers that bind to biomarker miRNAs of interest.

3D Human Ocular Tissue Model as a Potential Tool for Gene Screening for RNA Therapeutics

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Glauconix Biosciences

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This study aims at developing and validating a human 3D tissue model of the anterior segment of the eye that is amenable for gene screening of intraocular pressure lowering RNA therapeutics for treatment of glaucoma. Primary human trabecular meshwork cells isolated from cadaveric donors were cultured on highly porous microfabricated scaffolds that facilitated the rearrangement of the cells, forming a multilayer 3D tissue construct. Hypertension (glaucoma) was induced by steroid induction, and subsequent fibrotic deposition of ECM and myocilin proteins was confirmed using confocal microscopy. Constructs were transfected with 200 nM FITC-siRNA and imaged via confocal microscopy to evaluate transfection efficiency. Constructs were also transfected with siRhoA, and siSPARC at various concentrations (50, 200 and 400 nM) with transfection confirmed via qPCR analysis. The effects on intraocular pressure were evaluated using a microfluidic perfusion system which allows constant monitoring of pressure and flow rates. 3D tissue constructs revealed nearly 90% transfection efficiency following transfection with 200 nM FITC-siRNA while cells cultured on glass achieved nearly 20% transfection efficiency. qPCR analysis demonstrated that siRhoA and siSPARC exhibited a dose dependent down-regulation profile. siRHOA inhibited of F-actin expression and siSPARC down-regulated collagen I & IV, and fibronectin (*$P<0.001$, Mean ± SD with N= 9). Pressure across the tissue constructs was lowered by siRHOA treatment indicative of an increase in outflow facility. Overall, this study demonstrated the potential for bioengineered 3D tissue constructs as a useful tool for gene screening.

Tissue Specific Modulation of APOE for AD

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Among many putative genetic risk variations reported to date, the APOE4 allele remains the most common genetic risk factor for late-onset AD, and is associated with both an increase in incidence and a decrease in age of clinical onset. The majority of APOE is produced in the: 1) central nervous system (CNS) by astrocytes to transport lipids between cells and modulate the inflammatory response; and 2) liver, where it facilitates lipid uptake into peripheral tissues via low-density lipoprotein (LDL) receptors. Consistent with its dual roles, genetic knockout of APOE increases the risk for atherosclerosis, but it also dramatically improves AD phenotypes in mouse models.

Antisense oligonucleotide (ASO) based modulation of CNS APOE has only marginal effects on AD phenotypes, leading to the conclusion that post-embryonic silencing of APOE is not a viable therapeutic strategy. However, the recent development of novel CNS siRNA chemical structures enables widespread distribution and potent target silencing throughout the brain. Using this technology, we demonstrate that liver and brain APOE pools are spatially and functionally distinct, and that complete silencing of brain, not liver, APOE results in robust reduction of amyloid plaque formation, without impacting systemic cholesterol. Furthermore, RNAseq analysis shows minimal off target effects of the siRNAs and identifies immune modulation and metabolic alterations as potential mechanisms behind APOE's role in plaque formation and clearance.

Moving forward, these results build upon the rationale to modulate APOE expression and provide the technology necessary to further evaluate the impact APOE silencing in AD and other neurodegenerative diseases.

RNAi-based modulation of C9ORF72 variants as a potential therapy for ALS

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Amyotrophic Lateral Sclerosis (ALS), a progressive and fatal disease, is characterized by loss of motor neurons in the motor cortex, brainstem, and spinal cord. An expansion mapped to chromosome 9 (C9ORF72) presents as the leading cause of familial ALS (~40%), and accounts for some sporadic cases (~6%). Using a proprietary algorithm, we designed 20-mer siRNA sequences targeting different regions within the human C9ORF72 gene. To this end, the genomic sequence, containing both introns and exons, from position 1 to 12,320 was analysed and scored based on previously published criteria. The 20-mer siRNA sequences were synthesised onto a fully chemically modified scaffold and screened using a commercially available vector, into which we cloned regions of interest from the C9ORF72 gene. We identified sequences within the first exon, first intron, and second exon which produced mRNA knockdown similar to a clinical candidate siRNA used as a positive control. All hits were evaluated through a concentration response to further select lead compounds. Lead compounds were also validated using primary cortical neurons derived from a C9-ALS mouse model. Finally, compounds targeting intron one or targeting exon two were injected in to a C9-ALS mouse model, and efficacy analysed through mRNA and protein knockdown. We observe promising in vivo results suggesting the promising potential of RNAi to target disease specific mRNA transcript variants to reduce toxic RNA.

Understanding RNA Self-Encoded Primer Extension by T7 RNA Polymerase

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P.109

RNA-Seq of in vitro transcription products have demonstrated that the synthesis of self-encoded, primer extended products by T7 RNA polymerase involves re-binding by the enzyme of nascent transcripts in the form of (heterogeneously distributed) hairpin-like structures poised for extension. The current work supports a model in which stem-loop structures bind to the elongation configuration of the enzyme, mimicking the normal RNA-DNA hybrid that exists during transcription elongation and placing the 3' end of the RNA in the active site, poised for extension. During normal transcription elongation, the polymerase active site accommodates (only) an ≈8 bp RNA-DNA hybrid. The results reported here are generally consistent with overall sizing constraints (including loop) for the hairpin structures. Interestingly, just as the polymerase stabilizes otherwise unstable (too short for solution stability) RNA-DNA hybrids during elongation, it also stabilizes RNA priming structures that are not expected to be stable in solution. Thus algorithms that predict RNA structures in solution are not useful in predicting susceptibility to primer extension. The results of the current work provide design guidance for those who aim to reduce primer extension by appropriate sequence re-design of the target RNA.

Co-tethered and Flow Synthesis of RNA by T7 RNA Polymerase Substantially Reduces Primer Extended Impurities

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Traditional batch synthesis of RNA by T7 RNA polymerase allows accumulating RNA to rebind the enzyme, in (Le Chatelier-driven) competition with promoter-initiated transcription. RNA re-binding leads to primer extension of the RNA, converting DNA-encoded RNA to double-stranded impurities. These double stranded impurities are recognized by the innate immune system and can trigger an undesired immune response in both mRNA and CRISPR therapeutics. To favor promoter-initiated transcription, we have tethered T7 RNA polymerase to promoter DNA, mimicking earlier work from our lab. The resulting binding enhancement also allows increasing the salt concentration of the reaction, further disfavoring RNA re-binding. The result is both a dramatic reduction in double stranded impurities and an increase in yield of the encoded length RNA. Tethering of both the polymerase and promoter DNA to an immobilized support further allows the implementation of a continuous flow reactor, in which product RNA is continuously removed from the reaction. This ensures that the transcription dynamics is shifted towards formation of encoded RNA and thus increases the yield of encoded RNA per promoter DNA or polymerase. Optimization of this system will allow maximizing both yield and purity of RNAs.

Do Fragile X Syndrome and Other Intellectual Disabilities Converge on Aberrant pre-mRNA Splicing?

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P.112

Fragile X Syndrome is a neuro-developmental disorder caused by the silencing of the FMR1 gene, resulting in the loss of its protein product, FMRP. FMRP binds mRNA and represses general translation in the brain. Transcriptome analysis of the Fmr1-deficient mouse hippocampus reveals widespread dysregulation of alternative splicing of pre-mRNAs. Many of these aberrant splicing changes coincide with those found in post-mortem brain tissue from individuals with autism spectrum disorders (ASDs) as well as in mouse models of intellectual disability such as PTEN hamartoma syndrome (PTEN) and Rett Syndrome (MeCP2). These splicing changes could result from chromatin modifications (e.g., in Fmr1, MeCP2) and splicing factor alterations (e.g., PTEN, autism). Based on the identities of the RNAs that are mis-spliced in these disorders, it may be that they are at least partly responsible for some shared pathophysiological conditions. The convergence of splicing aberrations among these autism spectrum disorders might be crucial to understanding their underlying cognitive impairments.

The Perlman syndrome DIS3L2 exoribonuclease safeguards endoplasmic reticulum-targeted mRNA translation and calcium ion homeostasis

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DIS3L2-Mediated Decay (DMD) is a surveillance pathway for certain non-coding RNA (ncRNAs) including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), and RMRP. While mutations in DIS3L2 are associated with Perlman syndrome, the biological significance of impaired DMD is obscure and pathological RNAs have not been identified. Here, by ribosome profiling (Ribo-seq) we find specific dysregulation of endoplasmic reticulum (ER)-targeted mRNA translation in DIS3L2-deficient cells. Mechanistically, DMD functions in the quality control of the 7SL ncRNA component of the signal recognition particle (SRP) required for ER-targeted translation. Upon DIS3L2 loss, sustained 3'-end uridylation of aberrant 7SL RNA impacts ER-targeted translation and causes ER calcium leakage. Consequently, elevated intracellular calcium in DIS3L2-deficient cells activates calcium signaling response genes and perturbs ESC differentiation. Thus, DMD is required to safeguard ER-targeted mRNA translation, intracellular calcium homeostasis, and stem cell differentiation.

ASO as specific DNA damage response inhibitors: efficacy in aging-related diseases and oncology

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P.115

The DNA damage response (DDR) is a highly regulated set of events that plays important roles in aging and cancer. In aging, DDR controls cells entry into cellular senescence, a form of cell aging and a demonstrated contributor to several aging-related diseases. In cancer, perturbation of DDR is exploited for therapeutic purposes. So far, RNA has received limited attention as a target to modulate or inhibit DDR.

Our group has reported that DNA double-strand breaks (DSBs) trigger the synthesis by RNA polymerase II of damage-induced long non-coding RNA (dilncRNA) that can be processed into shorter DNA damage response RNAs (DDRNAs). Such transcripts are generated locally starting from broken DNA ends and are essential for full DDR activation by favouring molecular crowding of DDR proteins into liquid–liquid phase-separation (LLPS) condensates. DilncRNA/DDRNA inhibition by antisense oligonucleotides (ASO) allows site-specific inhibition of DNA damage signalling and repair (Francia Nature 2012, Michelini Nature Cell Biology 2017, D'Alessandro Nature Communications 2018, Pessina Nature Cell Biology 2019, Sharma Cell Reports 2021).
Only targeting RNA generated at DNA damage sites allows site-specific DDR selectively, while targeting proteins does not. We explored the potential of ASO targeting dilncRNA/DDRNA for therapeutic purposes.

In physiological and pathological aging, telomeres, the ends of linear chromosomes, progressively shorten and accumulate DNA damage, activate DDR and drive cells into cellular senescence. Telomeric DDR and cellular senescence depend on telomeric dilncRNA/DDRNA (Rossiello et al. Nature Communications 2017). In independent animal models of accelerated aging and of age-related human conditions, telomeric ASO improve detrimental phenotypes and extend lifespan (Aguado Nature Communications 2019 and unpublished).

Neurodegeneration is often associated with DNA damage accumulation. By mapping neurodegeneration-associated preferential DSB and consequent DDR events, we designed ASO that allow DDR reduction in relevant neurodegenerative cellular models.

In oncology, targeting with ASO the DNA repair events associated with cancer cells intrinsic genome instability, allows to selectively inhibit DNA repair and impair cell survival in specific subsets of cancer cell lines.

Novel miRNA-binding sites that recruit miR-652 and miR-223 in AAV vector designs boost transgene levels and synergistically suppress cell-mediated immunity

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Recombinant adeno-associated virus (AAV) vector gene therapy offers tremendous promise for the treatment for a variety of genetic diseases. Regulatory approvals for the treatment of two genetic diseases have already been received and clinical development for many more are on the horizon. One of the challenges for long-term success of gene therapy is the development of immune response to the transgene product. This effect is attributed to the undesirable transduction of antigen presenting cells (APCs), which in turn triggers host immunity towards rAAV-expressed transgene products. miRNA-mediated regulation to detarget transgene expression from APCs has shown promise for reducing immunogenicity. Skeletal muscle has been considered a viable target for AAV vector-mediated gene transfer to achieve sustained production of secreted therapeutic proteins. We have previously shown that miR-142 mediated detargeting allows continued expression of transgene in myofibers, represses cytotoxic T cell response and blunts the activation of co-stimulatory signals. However, the combinatorial effect of more than one miRNA binding site in the 3'-UTR of the transgene on anti-transgene immunity has not been reported previously. In this study, we performed in vitro screening of binding sites for 26 miRNAs that were selected based on their high expression levels in APCs, such as dendritic cells (DCs) and macrophage cell lines, but low in myoblasts. We identified two novel miRNA binding sites, miR-652BS and miR-223BS, that are efficient at APC detargeting in vitro, either individually or in combination with miR-142BS. Intramuscular administration of rAAV1 vectors containing either miR-142+652BS or miR-142+223BS demonstrate higher transgene expression in skeletal myoblasts as compared to previously published detargeting constructs, with negligible anti-OVA IgG production. Immunophenotyping of cells isolated from liver, spleen and muscle tissues revealed suppression of DC and co-stimulatory signals, and macrophage activation. In addition, there was a marked reduction in OVA specific CD8+ T cell response in those tissues accompanied by a reduction in the production of inflammatory cytokines, TNF? and IFN?. Moreover, we present evidence that miR-142-, miR-652-, and miR-223-mediated detargeting also leads to significant repression of Th17 response in vivo. Transgene detargeting mediated by the combination of miR-142BS and miR-652BS within the same vector cassette proves to be the most efficient at muting transgene specific immunity. Our approach, thus, advances the efficiency of miRNA-mediated detargeting to achieving synergistic reduction of transgene-specific immune responses and the development of safer and more efficient delivery vehicles for gene therapy.

Rapid detection of SARS-CoV-2 RNA by DNA nanoswitches

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P.117

Rapid detection is critical to slow down the spread of COVID19 pandemic caused by SARS-CoV-2. Here, we developed an assay to detect SARS-CoV-2 RNA using DNA nanoswitches and isothermal nucleic acid amplification. As shown in our preliminary tests, this method can detect as low as 50 copies of the targeted SARS-CoV-2 RNA within one hour. Besides, we reprogrammed our DNA nanoswitches so that they can detect influenza A, influenza B, and SARS-CoV-2 in a single reaction. Each detection cost is less than three dollars, and the operation is simple, making it practical for point-of-care tests. We are also investigating and optimizing our method's detection ability by using nasopharyngeal swab samples from collaborators. We envision that this detection assay can be developed into a marketable test kit, assisting the fight of COVID19 and other emerging pandemics.

Chemical targeting of SARS-CoV-2 programmed ribosomal frameshifting

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P.118

Translation of open reading frame 1b (ORF1b) in severe acute respiratory syndrome-associated coronavirus 2 (SARS-CoV-2) requires programmed −1 ribosomal frameshifting (PRF) promoted by an RNA pseudoknot. To identify chemical modulators of −1 PRF, we generated fluorescent protein-based PRF reporters and applied them in a high-throughput microscopy-based compound screen. From a library of 4,434 approved drugs and drug candidates, we identified a known antibacterial compound that specifically inhibited −1 PRF of SARS-CoV-2 and other coronaviruses. Frameshift inhibition impeded SARS-CoV-2 replication in Vero cells, suggesting that targeting −1 PRF is a plausible and effective antiviral strategy for SARS-CoV-2 and other RNA viruses. In this talk, I will discuss our ongoing effort to understand the mechanism of action and structure-activity relationship of this novel frameshifting inhibitor.

The Democratization of RNA Therapeutics

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The development of mRNA vaccines against COVID-19 brought worldwide attention to the transformative potential of RNA-based therapeutics. Integral to this potential, mRNA therapeutics are not solely the province of big biopharma. RNA therapies are a disruptive technology precisely because small biotech startups and academic researchers can rapidly develop innovative and personalized mRNA constructs. However, most of these small groups lack the key competencies to translate their transformational mRNA therapeutics into the clinic. To facilitate the democratization of mRNA therapeutics, we have built the crucial infrastructure to assist smaller groups as they bring their novel ideas to the clinic. In the Texas Medical Center, our fully integrated hospital-based RNA therapeutics program provides a single-entry point with consultation to ensure the seamless development of RNA therapy candidates into transformative drugs. Our RNA Biology and Bioinformatics faculty assist with construct design; our cGMP-trained personnel and clean rooms support the synthesis, purification, and validation of RNA drugs; plus our Nanomedicine colleagues formulate suitable lipid nanoparticles for local or systemic delivery. We have a Comparative Medicine Program with expertise in GLP preclinical studies; a first-in-human clinical trials unit for Phase 1 and 2a studies; and a large hospital system with a clinical research infrastructure that supports Phase 2 and 3 clinical trials. Furthermore, our industry partner manufactures large batches needed for Phase 2, 3 and/or commercialization. To our knowledge, we are the sole academic center with a fully integrated and operational infrastructure to support both academic groups and startups. We intend to support the translational efforts of small groups of scientists as they attain the near limitless potential of RNA Therapeutics.

LncRNA NIHCOLE promotes DNA damage repair in hepatocellular carcinoma cells

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P.123

Long noncoding RNAs (lncRNAs) are emerging as key players in cancer by enabling poorly understood molecular mechanisms. Here, we identified NIHCOLE, a novel lncRNA induced in hepatocellular carcinoma (HCC) with oncogenic potential and a role in ligation efficiency of DNA double-stranded breaks (DSBs). NIHCOLE expression associates with bad prognosis and poor survival of HCC patients. Depletion of NIHCOLE from HCC cells leads to impairment in proliferation, G2/M arrest, apoptosis, accumulation of DNA damage, and decreased non-homologous end-joining (NHEJ) activity. Biochemistry and, electron and atomic force microscopy, reveal that NIHCOLE recruits several molecules of the Ku70/Ku80 heterodimer. Further, NIHCOLE putative structural domains support stable multimeric complexes formed by several NHEJ factors including Ku70/80, APLF, XRCC4, and DNA Ligase IV. NHEJ reconstitution assays show that NIHCOLE promotes the ligation efficiency of blunt-ended DSBs. Collectively, we show that NIHCOLE serves as a scaffold and facilitator of the NHEJ machinery, conferring an advantage to hepatocellular carcinoma cells.

RNA modification in Filamin A pre-mRNA and its role in colon inflammation

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P.124

Adenosine to inosine deamination by ADARs is the most abundant type of RNA-editing in metazoans. A-to-I RNA editing has been shown to play an essential role in mammalian embryonic development and tissue homeostasis (1). A-to-I editing can lead to the recoding of mRNAs as inosine is primarily interpreted as guanosine during translation leading to the formation of novel proteins that are not encoded in the genome (2). Filamin A pre-mRNA editing is a prominent recoding event that leads to a Q-to-R amino acid exchange in repeat 22 of the Filamin A protein (3). Filamin A (FLNA) is an essential actin crosslinking protein that leads to the formation of orthogonal actin networks, primarily at the cellular cortex. The site of the editing-induced amino acid exchange servers as a platform for the interaction with many proteins involved in cell migration, cell contraction, and sell signaling. Editing of filamin A¬ pre-mRNA primarily occurs in the vasculature and the gastrointestinal tract in wild-type mice (4). We previously noted that mice unable to edit FLNA display increased vascular contraction, left ventricular wall thickening, which ultimately drives to left-ventricular hypertrophy and cardiac remodeling (5). Here we investigate the role of filamin A pre-mRNA editing in the large intestine and its function in tissue homeostasis and microbiome dysbiosis. Interestingly, mice only expressing unedited FLNAQ are more susceptible to DSS-induced colitis than mice expressing pre-edited FLNAR mice which are almost resistant towards induced colitis. Currently we are testing the underlying mechanisms to understand if colon barrier function, dysbiosis of intestinal microbiota or mucosal immunity in the colon explain the different responses to chemically induced colitis in mice with different Filamin A editing status.

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Evolution and Structural Analysis of Ionizable Lipid in RNA Delivery

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In the last decade, the development of nanoparticles (NPs) for RNA therapeutics facilitates clinical trial recruitment, which improves the efficacy of treatment modalities to a large extent. Following FDA approval for siRNA drugs containing ionizable lipid (IL) NPs, like MC3 from 2018, and with the occurrence of COVID-19 pandemic in the last year, at least two other ILs have been approved in RNA translational discovery through mRNA delivery like SM-102 and ALC-0315. IL is the most essential NPs component to provide successful delivery of RNA. Its properties are critical to the self-assembly process of the particle itself, the ability of the NPs to be taken up into cells, and the escape of the RNA from the endosome. In our study, we reviewed the time line synthesis, structures and chemistry rational of ILs, which encapsulated RNA to deliver all types of NPs in vitro and in vivo. Following backbone analysis of ILs, synthesis protocols and characterization were assessed, and then the challenges were discussed in depth. Furthermore, the critical roles of ILs like morphology, uptake, encapsulation, endosomal escape toxicity, and immunogenicity in the NPs were evaluated in detail. Finally, we summarized the whole characterization of ionizable lipids, which encapsulated siRNA or mRNA with lipid or polymer-based NPs in the research and clinical trial. These results, including carrier and RNA type, pka, zeta-potential, PDI, size, and case study, have not been announced so far. This research clears new synthesis strategies and manipulates functional chemistry of ILs for delivery of ASOs, RNAi, CRISPR/Cas 9, and other approaches based RNA like molecular biology and immunotherapy.

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