Research Update: August 2023

Project overviews and status reports on the projects now underway

Our portfolio of therapies funds research across multiple research strategies

Research Strategy

1 Correct the Defect

Correcting the source of dysfunction in ATP1A3 could eliminate patient symptoms

2 Reduce Symptoms Indirectly

Indirect targets can be addressed to reduce the effect of neuronal dysfunction, improving patient symptoms

3 Enhance Understanding of AHC (Basic Science)

Multidisciplinary efforts to elucidate mechanism, pathways, and biochemistry can uncover new lines of investigation, identify new intervention/ treatment opportunities

4 Build Research Infrastructure

A set of research tools, including animal and cell models, patient data, and diagnostic markers, assists researchers across multiple projects

Projects fall into each of these four categories

Strategies to "Correct the Defect"

1	Edit	*Edit out the incorrect DNA and replace it with corrected sequence*	Gene Editing
2	Over-compensate	*Provide additional functional genes to supplement normal function and dilute dysfunction*	AAV Mediated Gene Therapy
3	Silence	*Silence a mutated gene to eliminate toxic effects*	Antisense Oligonucleotides (ASOs)

We are using three different strategies to impact the genetic mechanism of AHC

Correct the Defect

Gene Editing (David Liu, CZI Grant)

Project Overview

Therapeutic Strategy: How Does it Work?

The Liu Lab is using prime editing and base editing to make precise "edits" to genetic mutations in DNA– replacing a mutated pathogenic nucleotide with the correct one.

- A guide RNA (gRNA) and a prime editing guide RNA (pegRNA) containing genetic instructions are designed and delivered.
- Inside the cell, Cas9 binds to gRNA and pegRNA, forming a complex. The gRNA guides the complex to the target site in the genome, while the pegRNA carries genetic instructions.
- The prime editing complex performs a series of biochemical reactions at the target site: this generates a nick in one of the DNA strands. The pegRNA template encodes instructions for an insertion, deletion, or substitution.
- The cell's natural DNA repair mechanisms finalize modifications: genetic changes are incorporated.
- ***Gene editing would permanently edit a patient's DNA.***

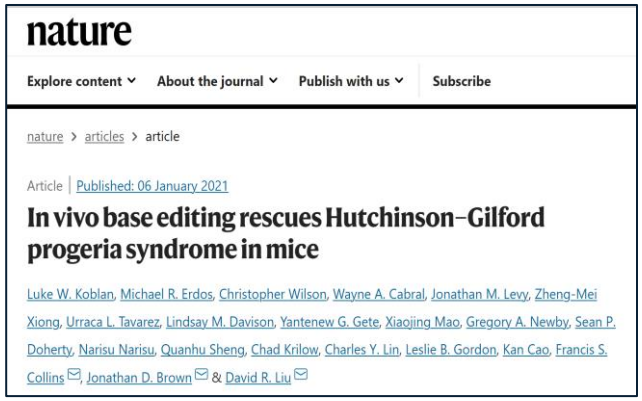
What Would a Treatment for Patients Look Like?

- ✓ *One time treatment*
- ✓ *Mutation specific*
- ✓ *Four ATP1A3 mutations are currently being targeted: D801N, E815K, G947R (both G->A and G→C mutations), and L839P*
- ✓ *Therapeutic window is unknown*

Correct the Defect

Gene Editing (David Liu, CZI Grant)

The Liu lab's pioneering work in genome editing rescued mouse model of Progeria ("I am Sam")



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In vivo base editing rescues Hutchinson–Gilford progeria syndrome in mice

Luke W. Koblan, Michael R. Erdos, Christopher Wilson, Wayne A. Cabral, Jonathan M. Levy, Zheng-Mei Xiong, Urraca L. Tavarez, Lindsay M. Davison, Yantenew G. Gete, Xiaojing Mao, Gregory A. Newby, Sean P. Doherty, Narisu Narisu, Quanhu Sheng, Chad Krilow, Charles Y. Lin, Leslie B. Gordon, Kan Cao, Francis S. Collins ✉, Jonathan D. Brown ✉ & David R. Liu ✉

- Our gene editing project has been underway for 18 months: successful genetic edits in the first phase (V1) showed significant promise: further optimization has followed in V2
- The project is funded with a **$2,000,000 grant from the Chan Zuckerberg Initiative**
- The project focuses on 4 AHC mutations **D801N**, **G947R, E815K**, and **L839P**: the Liu lab designed and evaluated hundreds of correction strategies for each
- **Testing in D801N mice** of the V1 gene editing strategy has begun
    - Mice were injected at birth; at 4 weeks tissue will be harvested for DNA analysis; behavioral tests will follow
- The next phase, testing in **primary patient cells**, will begin this summer at Northwestern University

Results from in vitro experiments are near "best case" for the Liu Lab, with a high rate of correction and a minimal error rate

Correct the Defect

AAV Mediated Gene Therapy (Jackson Labs)

Project Overview

Therapeutic Strategy: How Does it Work?

The concept of the AAV mediated gene therapy project is to deliver functional copies of the ATP1A3 gene to a patient's neurons. The objective to improve the functionality of neurons by increasing the quantity of functional pumps in the cell.

- An ATP1A3 gene with AHC-associated mutations codes for a dysfunctional ATP1A3 protein. These mutated proteins do not function properly and impair the function of the critical sodium potassium pump.
- Adding additional functional copies of the ATP1A3 gene could dilute the deleterious effect of the mutant gene copy.
- A viral vector containing ATP1A3 cDNA has been designed to deliver its payload to neurons. ATP1A3 is then transcribed and translated by the cellular machinery, producing a functional protein.
- ***A one-time treatment is designed to deliver gene copies that remain in neurons for years, and produce functional copies of the ATP1A3 protein.***

What Would a Treatment for Patients Look Like?

- ✓ *One time treatment*
- ✓ *Route of administration still tbd*
- ✓ *All AHC patients with ATP1A3 mutations may benefit from current vector*
- ✓ *Therapeutic window is unknown*

Correct the Defect

AAV Mediated Gene Therapy (Jackson Labs)

- After problems with the mouse model delayed the project, **the concept was proven in vitro in iPSC neurons** (L839P) in Dr. Al George's lab at Northwestern
- Preparations for the next phase of the AAV project have been underway
- Jax has spent the last 8 months moving the D801N AHC mutation mouse model to a **new, more robust genetic background** and characterizing it
    - Phenotypes have remained penetrant and the mice are no longer extremely vulnerable to sudden death
- **The new study will begin this summer**
    - 140 mice will be enrolled in the study
    - Jax's phenotyping plan includes body weight, survival, HID, rotarod and open field
    - Initial data on a potential rescue is expected beginning in December/January

Jax has conducted extensive characterization work on the new mouse model– at no cost to the AHC foundations; the AAV study is also provided for free

Correct the Defect

Antisense Oligonucleotides (ASOs)

Project Overview

Therapeutic Strategy: How Does it Work?

An ASO is designed to improve a patient's symptoms by minimizing the harmful effects of a mutant allele.

- AHC patients (with ATP1A3 mutations) have one good copy and one bad copy ("alleles") of ATP1A3.
- Preliminary data suggests that silencing the bad copy (or mutant allele) and leaving the good copy intact will provide therapeutic benefit in AHC.
- ASOs are designed to target mutant mRNA, bind to it, and mark it for degradation, "knocking down" the levels of a mutated mRNA and protein.
- ASOs for AHC can be designed to target either the disease-causing mutation or non-pathogenic SNPs (or variants) in a patient's ATP1A3 mRNA sequence.
- An ASO designed against a specific mutation can be used for all patients with that specific mutation; an ASO designed against non-pathogenic SNPs may also work in multiple patients.

What Would a Treatment for Patients Look Like?

- ✓ *Administration estimated every ~6 months after initial ramp up*
- ✓ *Spinal tap route of administration*
- ✓ *Mutation-specific or patient-specific*
- ✓ *Therapeutic window is unknown*

Correct the Defect

Antisense Oligonucleotides (ASOs)

FOR HENRY AHC

For Henry AHC and the AHC Foundations: A Collaboration for ASOs

- For Henry AHC has been pursuing an ASO therapy for Henry Saladino, with support and input from the AHC foundations
- In previous months, Charles River designed ASOs to target Henry's SNPs and ranked them in terms of off target binding risks and annealing strength (allele specificity)
    - The top candidate ASOs were synthesized commercially and Dr. Al George is testing them on Henry's iPSC neurons to determine efficacy of each
- Last month, we received word that Dr. Tim Yu who specializes in ASOs at Boston Children's Hospital, has accepted our ASO project and will be leading preclinical work for us moving forward
- The project will start with Henry Saladino's genotype, but the goal is for it to be expanded to include other patients who could be treated by the ASO, expanding as broadly as possible while bearing risk, cost, timeline and reward in mind
- Over the next month, we will be working to develop and approve a contract to define the specifics: we will be working to find a good balance between a "For Henry AHC" project and a "community" effort, in order to benefit all parties

Our hope is that the project with Tim Yu will lead to a methodology for precision medicine at scale for AHC

Correct the Defect

Antisense Oligonucleotides (ASOs)

ASO Rationale Project

- Dr. George is continuing work on the ASO Rationale project
- We are awaiting data from Dr. George from E815K and L839P iPSC neurons on the compensatory mechanism he saw in D801N ("knock out" of the mutant allele resulted in restored "normal" ATP1A3 protein levels in the cell): these findings would help determine whether ASOs were a therapeutic option for other mutation
- Dr. George has also been trialing ASOs designed against the pathogenic mutation in D801, and we are awaiting these results as well
- For Henry AHC is co-funding this effort along with AHCF, Cure AHC and Hope for Annabel

Mouse P.O.C.

- We are collaborating with Cat Lutz (Jax) and ASO scientist Brian Bettencourt to demonstrate therapeutic efficacy of an "optimal" ASO in our mouse model
- ASOs have been designed to knock down the mutant allele in D801N B6/CAST mouse model; they will be synthesized and screened *in vitro* before *in vivo* testing at Jax

We are discussing partnerships with ASO scientists to move the effort forward, including Tim Yu at Harvard/Boston Children's

Strategies to "Reduce Symptoms Indirectly"

1	Inhibit Other Inhibitors	*Eliminating other inhibitory compounds could boost pump function*	Endogenous Ouabain Inhibitors
2	New Strategy	*Investigating a strategy that eliminated cellular defects and could be therapeutic for patients*	New Project in Development
3	New Strategy	*Confirming a mechanism present in other conditions in AHC could lead to new strategies for intervention*	New Project in Development

The project exploring Endogenous Ouabain Inhibitors is underway; we are currently developing research in the other areas

Reduce Symptoms Indirectly

Endogenous Ouabain Antagonists

Project Overview

Therapeutic Strategy: How Does it Work?

The premise behind this effort is "inhibiting an inhibitor" of the sodium potassium pump.

- The sodium potassium pump is typically impaired in AHC.
- Other naturally-occurring compounds also interact with this pump and can impact or reduce pump function.
- One of these is endogenous ouabain, a cardiotonic steroid.
- Overstimulation or over-exposure to stressors is known to cause the release of endogenous ouabain, which then blocks normal NKA pump function.
- The hypothesis has clinical relevance: stress or "triggers" can lead to episodes in patients
- Inhibiting endogenous ouabain could be beneficial to pump function.
- Tests in mice will evaluate whether reducing the inhibitory effects of endogenous ouabain has therapeutic benefit.

What Would a Treatment for Patients Look Like?

✓*Repeated/chronic administration*

✓*Route of administration: likely systemic*

✓*All mutations in ATP1A3 likely to benefit*

✓*Unknown benefit to non-ATP1A3*

✓ *May benefit all age groups but therapeutic window unconfirmed*

Reduce Symptoms Indirectly

Endogenous Ouabain Antagonists

- Dr. Steve Clapcote is using mice to investigate the use of endogenous cardiotonic steroid antagonists as a therapeutic strategy for AHC
- Dr. Clapcote's lab is testing four different compounds in mice: three small molecules (BD-15, rostafuroxin and compound 16) and one antibody (DigiFab)
    - Rostafuroxin and DigiFab could be repurposed for AHC on an expedited path: both have been assessed or approved by the FDA
- Behavioral tests have begun in WT and het mice who have been treated with BD-15
- Dr. Clapcote collaborates with the Jax team: Experiments are discussed and findings shared between projects
- Results are anticipated in the fall and winter
- ***Evaluation of efficacy in the mouse model aims to determine whether endogenous ouabain inhibitors could be a potential therapeutic strategy for AHC patients.***

Building a Research Infrastructure

Investing tools for researchers supports efforts across current projects, enables future projects, and helps us prepare early for future clinical trials

Build Research Infrastructure

Animal and Cellular Models

C. elegans

- Dr. Anne Hart (Brown University) will be designing a worm (C. elegans) model for 3 different AHC mutations: D801N, L839P, and E815K
- A worm model of AHC would provide a simpler, widely studied in vivo model to enable future drug and small molecule screens; current mice models are not well-suited for this application
- This model enables comparisons between genotypes, both at baseline and after “therapeutic” intervention

Humanized Mouse)

- Jackson labs is creating a “humanized” mouse model: mouse DNA will be exchanged for a human DNA sequence matching a patient’s
- The humanized mouse will be used for testing multiple therapies and will provide a more clinically relevant data to predict treatment efficacy
- Jax is generating this model at no charge

Neurons and Cell Models

- iPSC-derived neurons have been studied and tested by Dr. Al George (Northwestern) to identify a baseline phenotype
- Therapeutic strategies are being evaluated in these: Dr. George has demonstrated efficacy of the AAV-mediated gene therapy strategy in iPSC neurons and will begin testing gene editing efficacy
- Cellular models of multiple AHC mutations have been evaluated in Dr. Kathleen Sweadner’s lab; new applications for these are being considered

Established, characterized models of AHC genotypes are designed to motivate scientists to use them: we want AHC to be an “easy target”

Build Research Infrastructure

WGS, Patient Data, and Biomarkers

Whole Genome Sequencing

- We are building a WGS coalition with Illumina and Gene DX with two core objectives:
  - Funding WGS through Medicare for ***all*** rare diseases
  - Gathering and centralizing data as foundation for genotype, -omics, phenotype, clinical and PRO data correlation.
- This foundational data is critical for identifying therapeutic targets for for all rare diseases; background genetics can provide clues in AHC

Natural History

- The objective is to follow patients longitudinally, assessing symptoms and collecting biosamples, to track disease progression / variability over time, while incorporating patient input, AHC expert opinions, and best practices from past and parallel AHC natural history studies
- Other parallel natural history studies are underway, but access to data may be limited

Biomarker Investigation

- We are also looking to identify biomarkers to correlate with AHC
- Biomarkers are a valuable objective measure of therapy efficacy in clinical trials; they could also help clinicians diagnose new AHC patients and chart disease variability or progression
- Biomarker investigation can be costly: our approach will likely be hypothesis-based, using findings from other related diseases as clues

Patient data can be tremendously valuable, particularly for clinical trial readiness, if it is multifaceted, layered, and correlated

New Projects in Development

We are currently developing 4 new pilot projects to investigate new therapeutic strategies for AHC. These are based on the following concepts:

1	Use expanded understanding of AHC cellular phenotypes and mechanisms to identify therapeutic targets and approaches
2	Leverage advances in adjacent fields
2	Use existing models in new ways

Exploring new therapeutic strategies is feasible because costs are low for early-stage pilots; multiple approaches maximize probability of success