Moser Center for Leukodystrophies
at Kennedy Krieger Institute

PROGRESS AND CURRENT STATE OF RESEARCH – 2021 SUMMARY

Over the past year, we have made great progress in our cell studies and have established a number of different ways in which to study LBSL cells and how they are different from control cells.

1. Cell Studies

As a reminder, a few years ago we collaborated with a stem cell expert, Dr. Mingyao Ying, here at Kennedy Krieger, to turn blood cells from LBSL patients into induced pluripotent stem cells (iPSCs). These iPSCs were then treated with different factors to become nerve cells that we continue to maintain in a dish. In summary, we established a nerve cell culture model for several LBSL patients and are comparing those to several control cells. Over the last few years, we have been optimizing our techniques and determining the conditions necessary to best handle these fragile and valuable cells. This year we have established cell lines from seven LBSL patients, and have successfully turned each of these iPSC lines into nerve cells that we can study.

Identifying and characterizing mitochondrial dysfunction in LBSL
While LBSL is a disorder of mitochondria, the cell's energy factory, little has been studied about how severely mitochondria are affected. Answering this question is critical, since one can then attempt to correct the mitochondrial dysfunction, resulting in an improvement of energy status. Studying mitochondria is difficult in blood cells, but by studying patient-stem-cell-derived neurons, one can answer this question. We previously reported that we see dramatic changes in proteins found within the mitochondria, which affect many aspects of their function. We also observed that the main job of

Mitochondria are found within every cell and the function of these unique organelles can be studied with a Seahorse Analyzer. Seahorse data from our labs shows consistent differences in oxygen consumption between healthy and LBSL neurons.
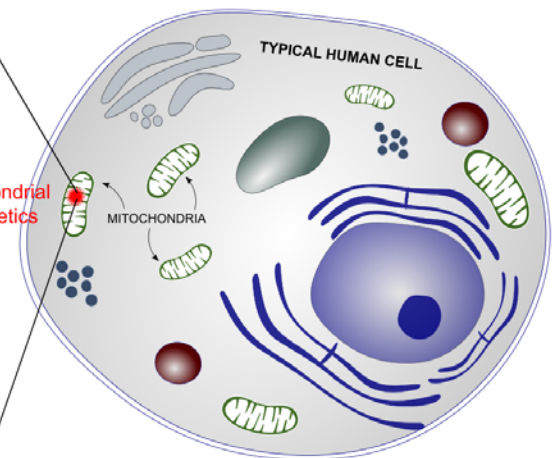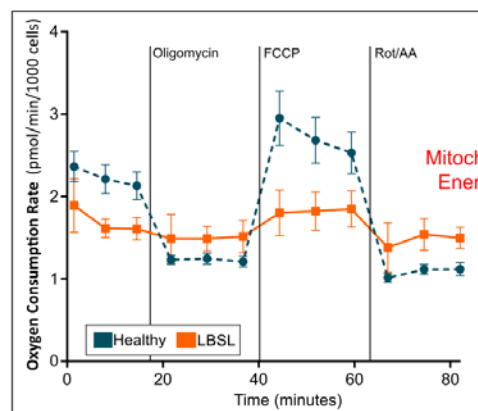

DARS2—to help the mitochondria *produce* proteins—is largely unaffected. Over the last year we have completed additional studies to examine mitochondrial function.

We are fortunate to have recently recruited Dr. Joseph Scafidi, a practicing pediatric neurologist with many years' research experience of the developing brain and mitochondrial energetics. Dr. Scafidi, upon arrival to Kennedy Krieger, purchased a brand-new Agilent Seahorse Analyzer, a specialized piece of equipment that can assess mitochondrial function in living cells or tissue in a multitude of ways. In addition to sharing a physical lab space with the LBSL team, Dr. Scafidi is an avid educator and collaborator, and has helped our team to accurately assess mitochondrial function within LBSL cells. Importantly, we have completed several experiments to find consistent dysfunction of mitochondria within LBSL patient cells and now have a quantitative measure of the level of dysfunction. These studies are instrumental to understanding the effect of DARS2 on cells, and these assays will be important when testing the effects of future therapeutics on cell mitochondrial function. In addition, we continue to interact with Dr. Jose Abdenur and his team at Children's Hospital at Orange County, who have received patient cell lines from us and are planning to conduct mitochondrial therapy studies.

Single Cell RNA-sequencing to understand disease process in mini-brains
In parallel to the neuron culture work, this year we also completed a very lengthy project to study gene expression within our LBSL cerebral organoids or "mini-brains".
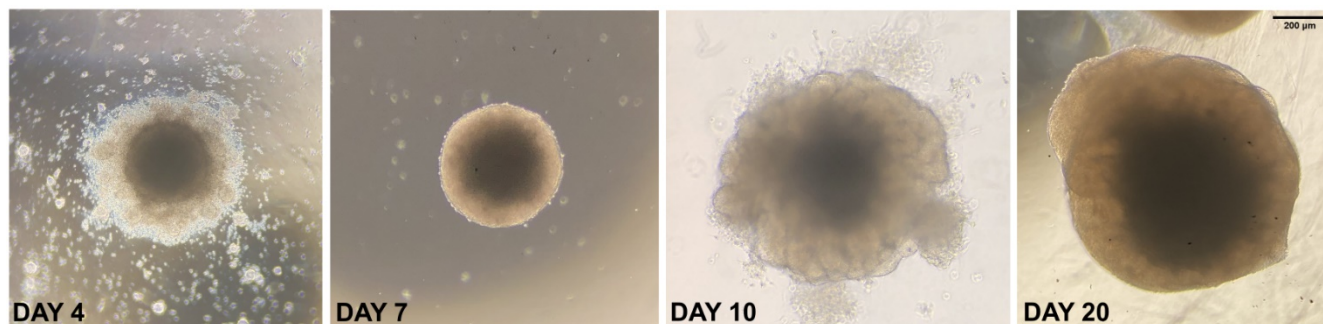
Individual patient mini-brains grow suspended in a plastic dish. These cell clusters show developmental changes over time, including "rosettes" or areas of budding and development, observable by the eye beginning around Day 10. These mini brains can grow for several months; however, the bigger they grow, the more difficult it becomes for nutrients to reach the center core, and thus we examine cells around Day 70 of growth.

We introduced the concept of this study in last year's update and are happy to report that it has been successfully completed. Using our own in-house cerebral organoid protocol, these mini-brains are grown in a dish from iPSCs that naturally form spherical structures and grow to develop various cell types, as a real brain would do. Shiqi Guang, a very talented medical student from Xiangya Hospital of Central South University in China, who has been studying in our lab for the last two years, has grown mini-brains from each of our patient lines, then after reaching a state of maturity, has separated each mini-brain to single cells and had them sequenced to be able to study the gene expression and composition of every different cell type within these structures. These studies allow us to understand how a nerve cell might be differentially affected by DARS2 than a cell less essential to brain function and development. Shiqi's work is novel to the field of LBSL and even novel to the field of mitochondrial tRNA synthetases, and she is in the process of preparing a manuscript of these data for publication. We look forward to sharing these data with you at the next research summit.

Antisense Oligonucleotide Therapy
As of last year's update, we had begun closely examining antisense oligonucleotides (ASO)—short pieces of RNA that can be used to "hide" the DARS2 mutation in cells. ASO therapy is now the standard of care in Spinal Muscular Atrophy and is being tested for a series of other diseases.

In order to find the specific sequence that works best, we tested dozens of iPSCs and neurons to find two that increased the proper splicing, or proper production, of the DARS2 transcript needed to make healthy protein. How well the ASO was able to improve splicing varied, based on how we delivered the ASO to the cells, which would not work well as a therapeutic. Since discovering this, we partnered up with the Center for Nanomedicine at Johns Hopkins and had them design for us a novel nanoparticle which can be attached to the ASO to standardize delivery. This custom structure took a few months to generate, validate, and test, but is now in our hands ready for testing in LBSL cells. The construct also has a small tag attached which glows red under a microscope, allowing us to see exactly where these constructs end up, ensuring that the ASO is delivered within the cell where it can have the greatest impact on function. We are currently in the process of testing these ASO-nanoparticles with patient iPSCs and neurons, and look forward to reporting the results of our work.

Summary of previous therapeutic attempts
In the past we attempted the following strategies, none (so far) with any success:
1) We treated the DARS2 conditional knock-out mouse with a potent antioxidant using a nanodendrimer platform for selective delivery of the drug into the brain. Despite several months of treatment with the dendrimer-N-Acetylcysteine compound, we did not improve the animals' behavior or disease pathology.
2) We also have treated both animals and patient cell lines with a drug that blocks the Integrated Stress Response (ISR) with no positive results. In addition, treating patient neurons with an ISR activator drug resulted in further deterioration of mitochondria.

Plan for the upcoming year in the lab:
1) Now that we have developed a clear method in the laboratory to study mice as well as cells and their mitochondria, we plan to conduct extensive studies using the ASO-nanoparticles.
2) We have also begun a new collaboration with the University of Maryland and hope to build a gene therapy platform using AAV9 viruses that we will then test in the next two years.
3) Finally, we are in active discussion with the Johns Hopkins Drug Discovery program to develop a high-throughput methodology to test in the patient-derived neurons and hopefully identify other drugs that may be beneficial.

2. Human Studies
While we are hopeful that we will identify a working therapy in animals and cells, it is critical that we better characterize the progression of LBSL clinically. In order to test any drug in a patient, we first need to understand at what pace the disease gets worse and how variable this disease is. Importantly, this is not just a scientific question but a vital issue that needs to be fully addressed before the Food and Drug Administration (FDA) will permit human trials.

Accordingly, with the help of our LBSL patient community, we have made great progress in collecting outcome variables and studying LBSL. Despite COVID-19, our remote assessments research study has continued without interruption with excellent compliance of participants. We have recruited a new subject since the last update, and are happy to report that as our youngest natural history study participants have grown older, three more now have the maturity to follow the complex instructions required to complete walking, balance and clinical ataxia score tests.

Dr. Amena Smith Fine is now in her second year as an Assistant Professor of Neurology at Kennedy Krieger and retains her primary focus on seeing patients at the Moser Center and focusing on LBSL research. In June 2021, she successfully competed for a two-year grant award from the NIH that supports a large portion of her salary. Her award provides additional mentoring from Dr. Adeline Vanderver, a leukodystrophies expert at Children's Hospital of Philadelphia, as well as support of the

GLIA-CTN provider network. There continue to be other institutional resources that we can utilize for this project, including the expertise in the Motion Analysis Laboratory at Kennedy Krieger headed by our Chief Science Officer, Dr. Amy Bastian; the neuropsychology core services; the highly advanced FM Kirby Research Center for Functional Brain MRI; and our clinical trials unit.

Importantly, we have partnered with Dr. Marjo van der Knaap and Dr. Marc Engelen at VUMC Amsterdam to begin a parallel study to evaluate sensory motor outcome measures in LBSL using the wearable OPAL system that Dr. Smith has been using. Our collaboration began in summer 2019, and their evaluation of the European LBSL cohort is ongoing.

We have resumed neuroimaging research at the Institute using the recently developed advanced white matter imaging protocols of the brain and spinal cord, which is critical to advance our neuromotor outcomes research. We have performed scans of six healthy volunteers to date. This has provided important data as well as allowing us to optimize the protocol and decrease the scan time duration, which is ideal for our youngest participants to complete their studies.

Due to postponement of the Fall 2021 LBSL conference, we are taking the approach of obtaining imaging data from LBSL participants on an individual basis as families are willing to travel to KKI.

Planned Upcoming Clinical Exercise study
We are working with our research physical therapist, Jennifer Keller, to develop an exercise intervention program for balance in LBSL, which will involve in-person PT training while participants are visiting us for their imaging study. Please stay tuned!

Machine learning
In addition to the wearable technology, we have developed new MRI techniques that we would like to apply to LBSL. We have spent a lot of time developing machine learning tools to generate a neural network that can automatically analyze imaging data from the spinal cord. This has been in collaboration with Dr. Unberath. Dr. Unberath has trained our postdoc, Dr. Bela Turk, in machine learning and Python, and has also provided us with a free master's degree student for this year to help with the effort. Dr. Turk has trained Dr. Fine in these machine learning methods as well.

We are also planning to approach the Food and Drug Administration for a Critical Path Innovation Meeting once our initial data is published. We anticipate this manuscript will be accepted within the next few weeks, after a favorable initial review with minor revisions required.

Summary:
We have made several new discoveries and believe that we are on the right path toward identifying therapeutics that we can then push forward toward clinical trials. Meanwhile, we need to continue our human studies to identify the right set of outcome measures for the conduction of clinical trials. We have established a network of collaborators, including national and international partners now conducting both basic and clinical work in LBSL. While there is still a long way to go, we are optimistic that our work will lead to fruition of new therapies and clinical trials for LBSL.

Please contact Leslie Marsiglia at marsiglia@kennedykrieger.org to further support this work by making a donation.