Cystinosis Research Foundation Progress Report

Report: #3
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Project: Developing a therapeutic strategy for cystinotic nephropathy with iPS cells
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Major goals of the project: The goal of this proposal is to use human induced pluripotent stem (iPS) cells and derived kidney organoids to screen and develop therapeutics for cystinotic nephropathy. Specifically, we will use kidney organoids as a surrogate for human tissue to explore the potential of renal regeneration, gene therapy, and compound screening for the development of cystinosis therapies. The work will be performed in three Specific Aims. In our first aim, we will reprogram a cohort of cystinosis patient cells into iPS cells that could be further developed into *bona fide* regenerative therapeutics, and transplant these into animals. In our second aim, we will develop techniques to restore *CTNS* function in kidney cell types, by applying cutting-edge gene therapy techniques in differentiating organoids. In our third aim, we will re-create the cellular pathophysiology of cystinosis in a petri dish to gain specific insight into why tubular cells die and how this can be prevented chemically. These Aims have not changed.

Progress towards key milestones: In our third reporting period, we presented at the Cystinosis Research Symposium, which was very beneficial, and have continued to grow our collaborations with other groups in the cystinosis community including Dr. Bruce Barshop and Dr. Elena Levtchenko. Dr. Freedman published a commentary paper with fellow cystinosis researcher Dr. Alessandro Luciani (Luciani and Freedman, *Kidney International* 98, 54-57), which discusses the use of induced pluripotent stem cells for modeling endocytic disorders and Fanconi syndrome. For our milestones, we have made progress in establishing and characterizing our new cohort of cystinotic iPS cell lines and derived organoids, which are not yet published. We are conducting a careful phenotyping of these cells, using several different cell lines and controls. Our work implanting the cells into animals has provided the first glimpse of *in vivo* grafts from cystinosis patients. We have also improved our ability to conduct genome editing in organoids as a model of gene therapy for cystinosis. Each of these is described in further detail below.

1. Determining the disease phenotype of a cohort of cystinotic organoids derived from iPS cells. An important goal is to re-create the phenotype of nephropathic cystinosis *in vitro.* We have found that our mutant iPS cell lines accumulate cystine, based on cystine assays performed by Dr. Barshop in San Diego. To confirm this result, we have repeated the experiment and sent Dr. Barshop a second batch of samples, including both iPS cells and derived organoids, with defined total protein levels. This will help us verify that cystinosin is not functional in these cells. To complete characterization of our stem cell lines, we have implanted these into an animal model, to form teratoma tumors. We have also initiated a collaboration to sequence the genetic mutation in our patient cell lines, which will increase their value for the community.

Ultimately, we seek to identify tissue-scale phenotypes that would provide insight into the mechanisms of nephropathic cystinosis. The importance of identifying such phenotypes was also emphasized during conversations with experts in the field during the recent CRF Research Symposium. We initially observed that differentiation of cystinosis lines into kidney organoids and particularly podocytes appeared to be impaired, particularly in our patient-derived cell lines. In more recent follow up experiments, however, we have found that cell lines with cystinosis mutations can differentiate efficiently into mature kidney organoids with well-defined tubules and podocytes. We are currently modifying culture conditions to identify tissue-scale phenotypes. We are also investigating whether cysteamine can rescue these cystine accumulation and its effects on the organoids.

2. Isolating and implantation of nephron progenitor cells (NPC). An important aspect of our plan is to implant human kidney progenitor cells into mouse kidneys to determine their ability to engraft and form structures that could be safe and functional. When we previously implanted mature organoids beneath the kidney capsule of mice, these engrafted and became vascularized from the host. However, maturation remained incomplete, and non-kidney cells became enriched in the graft over time (*Experimental and Molecular Medicine*, 2019). Having established this as a baseline, we are currently implanting earlier-stage NPC, with the hypothesis that these may show improved engraftment. We are conducting these experiments with iPS cells from patients with cystinosis, to determine whether mutations affect the grafts. Our experiments to date indicate that NPC derived from patients with cystinosis can successfully grow in the animal model. One week post-implantation, human NPC structures (renal vesicles) were visible in the graft. At later time points, podocytes, proximal tubules, and distal tubules appeared in nephron-like structures (Figure 1). This is interesting proof of principle that supports the possibility of growing new kidney tissue *in vivo* from patients with cystinosis. We are currently quantifying and analyzing these findings, to determine whether the grafts show signs of being functional or express cystinosis-specific phenotypes. We are also expanding these experiments to include NPC from additional patients.

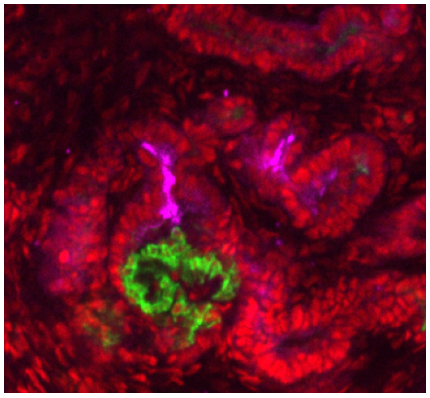

Figure 1. Nephron-like structures from cystinosis patients. Small portion of an NPC graft three weeks post-implantation. Red, human cells; green, podocytes; magenta, proximal tubules.

3. Progress towards gene therapy. We are developing an off-the shelf methodology to perform gene therapy for cystinosis in the kidneys, using organoids as a surrogate for patient tissue. Our greatest interest lies in developing the use of CRISPR-Cas9 ribonucleoprotein (RNP) complexes to integrate a wild-type copy of *CTNS* into the genome. We had previously succeeded in obtaining a 3 % editing rate using GFP as a readout. By refining the timing of treatment with the RNP, we have now improved this to 20 % editing. We have also completed the design of our *CTNS* rescue

vector, which will be inserted into genomic safe harbor loci using CRISPR-Cas9 and will be inducible with doxycycline so we can control the levels of cystinosin.

In addition to CRISPR RNP, we are exploring the utility of more established platforms for gene transfer including adeno-associated virus (AAV) and lentivirus in organoids. Here too, timing of virus addition proved to be important. We obtained substantial infection of organoids with three different lentiviral promoters, in collaboration with Dr. Elena Levtchenko (Leuven University, Belgium). One of Dr. Levtchenko's students obtained a travel award to visit our laboratory in September and further assist with these experiments. We have also obtained successful infection of organoids with two different AAV serotypes, which may represent a safer option *in vivo* compared to lentivirus.

Logistical, personnel, and manuscripts in preparation. Cystinosis has grown into a major focus of our lab. The COVID-19 pandemic has raised some challenges. Only critical staff have been allowed to come into lab, and the month of April we had limited staff and operations. During this time it was unclear what the guidance was or how severe the outbreak would be. Since that time, the first wave of cases has resolved, and it became clear that we were permitted to continue work in the laboratory, in accordance with safety guidelines. This is because research related to human disease and therapeutics development is considered essential work in the state of Washington. We have therefore been able to continue to make progress on this important project. Unfortunately, during this vulnerable time, Ivan Gomez, who was helping to engineer cell lines and transplant these into animals, was recruited by a pharmaceutical company, and resigned his position in our lab. Ivan's effort on the project has, however, been replaced by Thomas Vincent, a second year Bioengineering PhD student who is highly motivated about kidney regeneration. Thomas has completed all of his animal training, is helping maintain our mouse colony, and has learned to perform implantation surgeries and analyze them. Other personnel remain unchanged. We are completing work on our manuscript about iPS cell derivation from urinary cells, and strategizing for funding applications to continue the cystinosis project.

Future work: In the coming funding period, we will complete (1) establishment of our cohort of cystinosis patient lines, including recently recruited patients (the study is ongoing); (2) phenotyping of cystinotic organoids and attempt to rescue these with treatments such as cysteamine; (3) characterization of grafts resulting from NPC injection into mouse kidneys from multiple patients with or without cystinosis; and (4) development of our gene therapy methodology to re-introduce *CTNS* into kidney organoid cultures. We also hope to submit a manuscript describing derivation of our cell lines and demonstrating their use, as well as apply for additional funding to support these projects. This will be a busy season for cystinosis work and we are looking forward to it.

We are very grateful for the support and opportunities the Cystinosis Research Foundation provides and hope that you and the community are doing well during these turbulent times.