Cystinosis Research Foundation Progress Report

Report: #3
Date: August 31, 2023
Project: Developing a therapeutic strategy for nephropathic cystinosis with iPS cells
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Major goals of the project: The goal of this proposal is to reveal mechanisms underlying nephropathic cystinosis and develop therapeutic approaches by applying a suite of powerful next-generation research tools, including human induced pluripotent stem (iPS) cell lines and CRISPR gene editing. The work will be performed in two Specific Aims. In our first aim, we will recapitulate complex pathophysiological hallmarks of nephropathic cystinosis *in vitro*, and rescue phenotypes with a gene editing technique. In our second aim, we will assess the potential of kidney grafts derived from cystinosis patient iPS cells to functionally integrate in a small animal model of cystinosis. These Aims have not changed.

Progress towards key milestones: In our third reporting period, we have made important progress on this project. Major activities included initial assessments of gene expression stability and phenotypic rescue in human *CTNS*$^{-/-}$ iPS cells and kidney organoids containing a transgene knock-in of an inducible *CTNS* cassette in iPS cells, better characterization of grafts in immunodeficient mouse hosts, and continuing efforts to generate an immunodeficient *Ctns-/-* mouse host colony. These are summarized below on an individual basis.

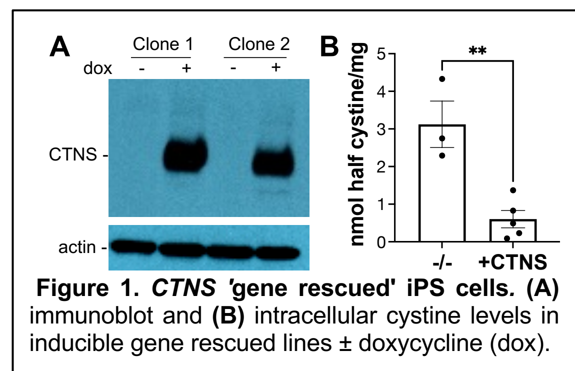

Figure 1. *CTNS* 'gene rescued' iPS cells. (A) immunoblot and **(B)** intracellular cystine levels in inducible gene rescued lines ± doxycycline (dox).

1. Toxicity assessments in human kidney organoids with cystinosis (related to Aim 1). In the previous period, we conducted genomic knock-in of *CTNS* in one of our cystinosis iPS cell lines. The knock-in was confirmed to be present in the proper locus and orientation in multiple clonal cell lines. These will be a new resource for our 'CRF collection' of iPS cells. This new copy of *CTNS* was designed to be turned on or off on-demand, like a remote control. We successfully established multiple clones that respond in this way, turning on the artificial *CTNS* gene on-demand. We successfully established multiple clones that respond in this way, turning on the artificial *CTNS* gene on-demand (**Figure 1A**). We have furthermore found that turning on the transgene dramatically reduces cystine accumulation in these cells (**Figure 1B**). Thus the gene therapy approach appears to be working overall. We are currently differentiating these cells into kidney organoids and plan to test whether gene induction rescues nephron injury phenotypes resulting from cystine accumulation.

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EDITED SAMPLE 217 TO 282 BP

CONTROL SAMPLE 216 TO 281 BP (G1 UNDERLINED)

EDITED SAMPLE 176 TO 241 BP

CONTROL SAMPLE 175 TO 240 BP (G2 UNDERLINED)

Mutation	Genotype (Short)	Protein
-8bp	c.91_94del;96_98del;136del	p.T31CfsX26
-43bp	c.93_135del	p.V31LfsX5
-17bp	c.90_94del;124_135del	p.T30EfsX23
-43bp	c.93_135del	p.V31LfsX5
-42bp	c.91_132del	p.32V_45Tdel
42splice	g.6521_6544del;6580_6597del	N/A

This is the Sanger sequence view showing edited and wild-type (control) sequences in the region around the guide sequence. This shows sequence base calls from both the control and the experimental sample .ab1 files, which will contain mixed base calls. The horizontal black underlined region represents the guide sequence. The horizontal red underline is the PAM site. The vertical black dotted line represents the actual cut site. Cutting and error-prone repair usually results in mixed sequencing bases after the cut.

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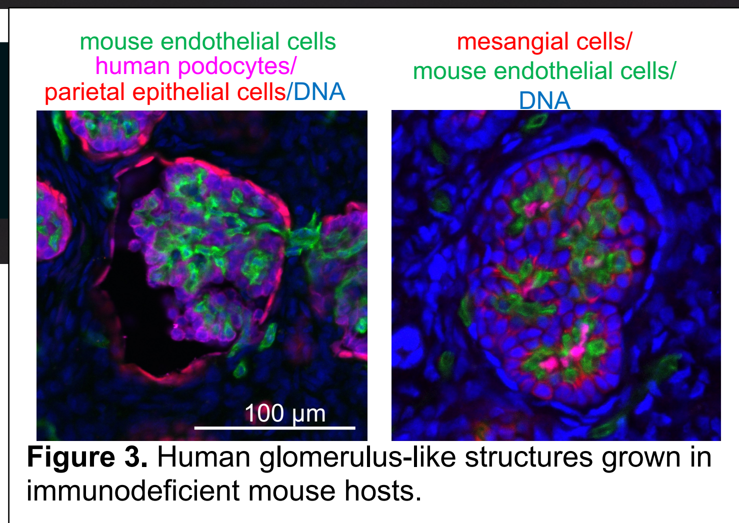

Figure 3. Human glomerulus-like structures grown in immunodeficient mouse hosts.

is thought to be required for its proper formation. Our data showed the presence of human cells expressing mesangial cell markers in these grafts, which are localized internal to the endothelial cell bouquets (**Figure 3**). This suggests that our grafts contain not only epithelial progenitors, but also stromal progentiors, which may explain their ability to generate more sophisticated structures.

Logistical, personnel, and manuscripts in preparation. Dr. Raghava Reddy, a senior scientist in the lab, is driving the work modeling cystinosis phenotypes *in vitro.* Raghava

was assisted in the development of the gene therapy approach by Dr. Nicole Vo, an Acting Instructor (junior faculty) with expertise in knock-in strategy, but is now steering the effort to phenotype the cells on his own. Drs. Julie Mathieu and Bob Hunter are helping develop the CRISPR knock-out mouse in the Transgenics Core (UW Comparative Medicine). As the colony is growing, we are strategizing to transfer it in its entirety to our own lab. Thomas Vincent, a third-year graduate student, is performing graft experiments, using techniques he has previously developed. Thomas is also helping genotype the mice, along with Ramila Gulieva, a research scientist in our lab, and Samera Nademi, a first-year postdoctoral fellow. Thus there is a well qualified team in place with specific roles. We are working on two papers, one regarding the formation of grafts in vivo, and one about modeling *CTNS* phenotypes in vitro. We are also working on a paper about the biobank of cystinosis cells from patients.

Future work: A major goal is to complete our initial studies of cystinosis phenotype in iPS cells and organoids, including characterizing our new inducible *CTNS* knock-in iPS cells. We are currently collecting the data for quantitative analysis of the cystinotic phenotype with multiple isogenic pairs and ± cysteamine. This work will be synthesized into final figures for the paper described above, which we hope to submit for publication by the end of the funding period.

Another major goal is to stabilize and expand our new *Ctns*$^{-/-}$ NOD-SCID mouse colony. We are not yet sure whether the homozygotes can breed. We have encountered some general difficulties in breeding these animals, and they are susceptible to infections (due to the immunodeficiency) which are causing problems with the colony. Genotyping has also been a challenge, as this is a relatively new area for us. We will troubleshoot these issues and establish the colony, either as heterozygotes or homozygotes. Any homozygote animals we obtain will be phenotyped for any indications of cystinosis, including cystine accumulation. They may be used for implantation experiments if time permits.

These experiments will establish new tools that we can use for the remainder of the project, and which will be of general use to the cystinosis research field. Dr. Freedman plans to present an update on this work at the upcoming CRF Cystinosis Research Symposium in September. He is also receiving the Young Investigator Award at the American Society of Nephrology Annual Meeting, and will deliver a plenary address. This is one of the greatest honors in the nephrology field, and we are grateful for CRF support which has helped us reach this milestone. We also hope to submit a renewal application for funding from CRF in the coming funding period.