Final Report to the Cystinosis Research Foundation:

Molecular and Pathogenesis Study of Cystinosis (Period: Jul. 07 - Jun. 09)

Taosheng Huang, MD, PhD – Mentor; Sha Tang, PhD – Research Fellow

Hypothesis:

We hypothesized that a cystine transporter defect in the lysosome of cystinotic patients results in a cysteine shortage in the cytosol, where the three major thio/disulfides, glutathione (GSH), thioredoxin and cysteine, are coupled. Cysteine is also one of the precursors for glutathione biosynthesis. Therefore, the glutathione and thioredoxin levels are decreased in the cytosol and mitochondria in patients with cystinosis. This affects mitochondrial function and other nuclear and cytoplasmic redox processes.

Accomplished Work:

We observed a slower growth rate of cystinotic cells when compared to normal cells. Accordingly, augmented programmed cell death in cystinotic cells was recorded even without apoptotic stimuli, suggesting that apoptosis does play an important role in pathogenesis of cystinosis. Cystinotic cells generally displayed reduced ATP content and total GSH level, as well as an increase in GSSG/total GSH ratio, indicating perturbed redox balance due to cystine trap in lysosomes and resultant defective energy production capability of the mitochondria. In addition, we performed the first comprehensive gene expression analysis of human cystinotic cells and had identified four differentially expressed genes in cystinotic cells that are involved in cell proliferation and development.

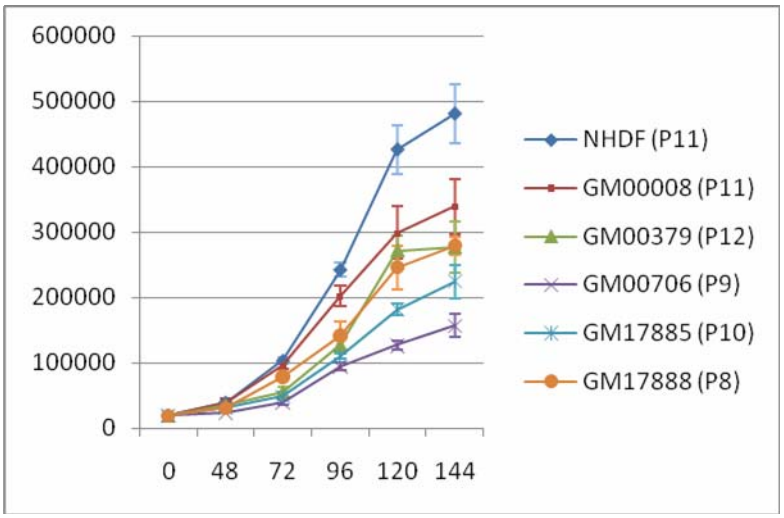
1. Cell cultures and intracellular cystine levels:

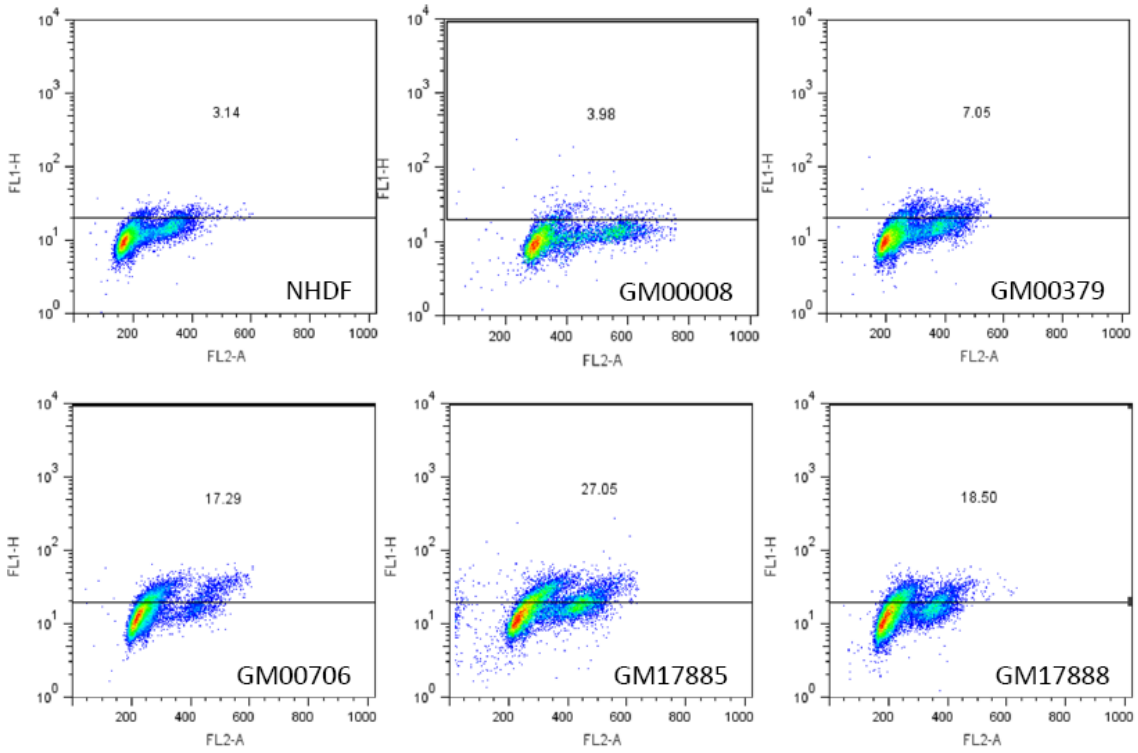
Cultures of normal and cystinotic human fibroblasts were obtained from the Coriell Cell Repositories and are maintained in the laboratory. The cystinotic lines represent different genotypes and total intracellular cystine levels of these fibroblasts were determined in Dr. Schneider's laboratory at UCSD.

Cell line	Type of Cystinosis	Age (Yrs)	Sex	Genotype	Mutation in Allele 1	Mutation in Allele 2	Cystine Levels (n mol/mg)
NHDF (control)	------	0	M	------	------	------	0.06
GM00008	nephropathic	5	F	homozygous	57 kb deletion	57 kb deletion	N/A
GM00379	late-onset	4	M	heterozygous	753G>A	IVS11+2T>C	18.52
GM00706	nephropathic	1	M	homozygous	57 kb deletion	57 kb deletion	12.06
GM02894	nephropathic	9	F	homozygous	57 kb deletion	57 kb deletion	3.38
GM17885	nephropathic	9	F	heterozygous	57 kb deletion	Gly308Arg (G308R)	12.29
GM17888	non-nephropathic	26	M	heterozygous	IVS10-3C>G	545delTCCTT	3.46

2. Apoptosis /cell cycle analysis:

We noticed that cystinotic cells did not grow as fast as the NHDF cells, as shown in the cell growth curve. Depletion of intracellular cystine by cysteamine (incubation for 20 hr) did not enhance cell growth (data not shown). Retarded cell growth may indicate slow proliferation, elevated cell death, or both. Increased apoptosis had been reported in cystinotic cells and proposed to be an important factor in the pathogenesis of cystinosis. We used TUNEL assay to study the apoptosis rate and cell cycle properties of cystinotic cells.





Cell Line	Apoptosis Rate (%)	G1 (%)	S (%)	G2 (%)
NHDF	3.14	53.79	36.56	7.51
GM00008	3.98	47.16	41.84	8.11
GM00379	7.05	55.87	33.21	6.28
GM00706	17.29	62.83	33.21	3.39
GM17885	27.05	48.21	44.52	5.22
GM17888	18.50	49.92	41.71	5.64

From our data, even under basal conditions (without apoptotic stimuli) we saw enhanced apoptosis rates of cystinotic cells, some of which were unreasonably high. The milder forms of cystinosis could display higher basal cell death rate than the more severe nephropathic types. We also used UV (60 mj) radiation to trigger apoptosis and 1 hr incubation of 1 mM cysteamine-HCl to remove cystine before UV treatment. However, we did not see decreases in apoptosis rate due to lysosomal cystine depletion (except for GM17888) as previously reported.

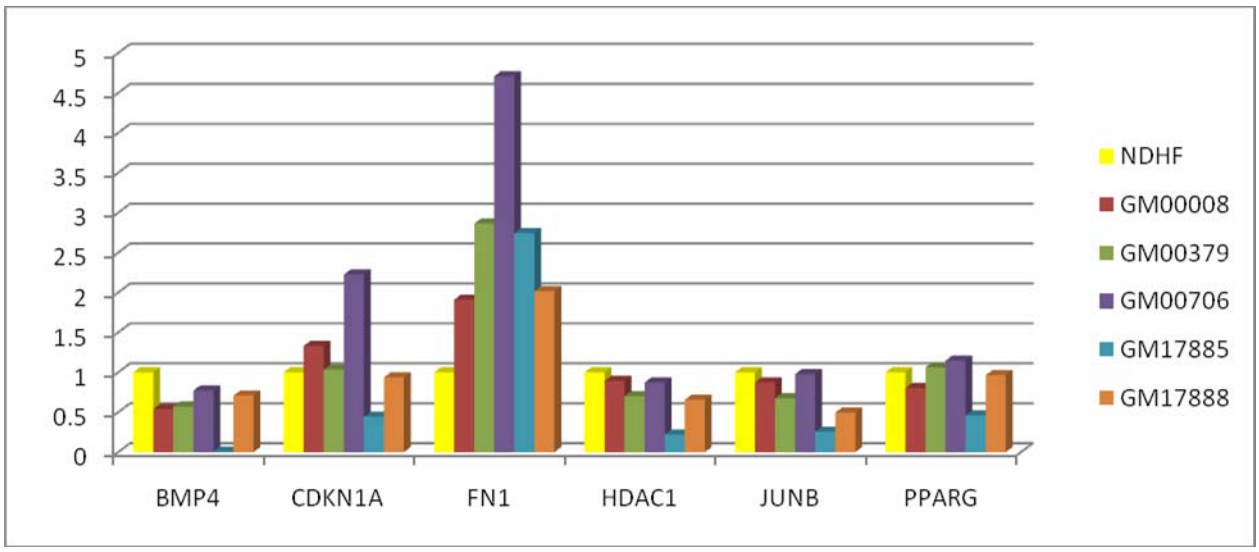
3. Gene expression analysis:

To investigate if cystine accumulation in the lysosomes affects gene expression levels in cystinosis, we used Affymetrix Human Genome U133 Plus 2.0 Array to identify differentially expressed genes in cystinotic cell. We used cystinotic GM00706 (homozygous for 57 kb deletion) and NHDF as control. These two cell lines were sex, age, race, and passage-matched and the microarray expression analysis were implemented in triplicates for each. Combined analysis of two algorithms (LIMMA and Cyber-T) as well as straight fold change yielded 990 protein IDs that were significantly differentially expressed in GM00706 versus NHDF. Functional annotation of the 990 genes found some enriched biological themes that may be involved in the pathogenesis of cystinosis.

Category	**Gene number**
Apoptosis	39
Cell Cycle	64
Cell Proliferation	52
Transcription regulator	100
Development	204
Signal Transduction	187
Cell-Cell signaling	50
Ion Transport	18
Glutathione Metabolism	7
Oxidoreductase	44
Carboxylic Acid Transport	11

Next, Pathway Studio was used to identify the genes that played central roles in the differentially expressed functional groups for cell proliferation and apoptosis, transcriptional regulation, development and signal transduction (listed in Table). Quantitative RT-PCR was used to verify the expression profiles of these genes generated by Affymetrix arrays. Then, the expression levels of these six genes were studied in the other four cystinotic cell lines.

Tag	Gene	Function	Expression in GM00706
BMP4	bone morphogenetic protein 4	development, differentiation	Down
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	suppress cell growth, senescence marker	Up
FN1	fibronectin 1	cell adhesion, morphology, senescence marker	Up
HDAC1	histone deacetylase 1	transcriptional regulation	Down
JUNB	jun B proto-oncogene	cell proliferation	Down
PPARG	peroxisome proliferative activated receptor, gamma	anti-inflammatory, repress tumor	Down



When compared to normal control, expression of FN1 was up-regulated while BMP4, HDAC1 and JUNB were down-regulated in all the five cystinotic fibroblasts investigated.

4. Redox status and energy metabolism analysis:

Total GSH and oxidized GSH levels of cystinotic cells were determined by enzymatic cycling assays using both a commercial kit (Cayman Chemical) and the method developed in Dr. Luderer's laboratory at UCI. Total intracellular ATP contents were measured with the ATP Bioluminescence Assay Kit HS II (Roche).

	cystine	apoptosis rate	ATP	GSH	GSSG	GSSG/GSH
NHDF	0.06	3.14	24.06	103.5	2.1	4.00%
GM00008	N/A	3.98	21.59	N/A	N/A	N/A
GM00379	18.51	7.05	22.17	120.6	10.4	8.60%
GM00706	12.11	17.29	25.61	30.9	0.9	6.11%
GM17885	12.31	27.05	31.22	146.9	2.6	3.50%
GM17888	3.52	18.5	19.45	86.27	1.88	4.37%

Generally, cystinotic cells had reduced ATP content and total GSH level, and increased oxidized GSH ratio when compared to normal cells. Nevertheless, there were always exceptions (see GM17885). There was no clear correlation between severity of the disease and level of the biochemical indicators characterized here.

5. Problems and updates:

Despite intensive efforts, there were always some inconsistencies in our data and some of the cell lines (e.g. GM00706) we used grew extremely slow and displayed dramatically high apoptosis rate even at under basal conditions. We presented our results in the First CRF International Research Symposium and discussed our problem with the experts in the field. We also consulted Drs. Thoene and Schneider for the discrepancies we encountered. Dr. Schneider thought the Coriell cells we used are all very old and have gone through too many doublings to be useful for our purposes. Therefore, we aim to derive cystinotic and normal skin fibroblast cells in house, which requires the recruitment of age, sex, ethnicity-matched cystinotic patients and normal subjects. This process takes time and thus we did some other characterizations, not necessarily directly related to the proposed work but very important, in parallel.

Molecular Analysis of CTNS Mutations from an Indian Cystinotic Boy

We performed the first molecular characterization of cystinotic patients from India. The proband was born to consanguineous parents and has an older brother sibling dying with a diagnosis of cystinosis. The proband also presents with certain phenotypes not associated with cystinosis previously. The boy displays skin and hair hypopigmentation and has ichthyotic skin on the legs and arms. Sequencing analysis of the *CTNS* exons revealed that the proband is homozygous for a 3-bp in-frame deletion in exon 10, resulting in the loss of a conserved Ser270 within the fifth transmembrane domain of CTNS. Interestingly, identical amino acid change in CTNS was reported in a European cystinotic patient. Therefore, the S270del mutation most likely arose independently in the two different continents. A case report describing the proband and the mutation analysis has been accepted by Genetic Testing. We acknowledged CRF in the manuscript.

Heterozygous Mutation of Opa1 in Drosophila Shortens Lifespan Mediated through Increased Reactive Oxygen Species Production

We demonstrated that heterozygous mutation in Optic atrophy 1 (OPA1), a mitochondrial protein, resulted in shortened lifespan, increased susceptibility to oxidative stress and elevated production of Reactive Oxygen Species (ROS) in *Drosophila*. Heterozygous *dOpa*1 mutation also caused an impairment of respiratory chain complex activities, especially complexes II and III, and

reversible decreased aconitase activity. A manuscript summarizing the findings has been accepted by PloS ONE (http://www.plosone.org/article/info:doi/10.1371/journal.pone.0004492) and CRF is acknowledged for supporting the work. Although these data are not directly related to cystinosis research, through the OPA1 work we now have the expertise in the techniques necessary for our proposed mitochondrial dysfunction-cystinosis correlation study. These important methods include mitochondrial respiration analysis, mitochondrial complex enzymatic activity measurements, ROS generation characterization and mitochondrial aconitase activity assays.

The Effects of Cysteamine on the Eye Phenotype of Drosophila OPA1 Optic Atrophy Model

OPA1 is a dynamin-like GTPase located in the inner mitochondrial membrane and mutations in *OPA1* are associated with autosomal dominant optic atrophy (DOA). In our *Drosophila* model, homozygous mutation of *dOpa*1 in the eyes (large clone) caused rough (mispatterning) and glossy (decreased lens and pigment deposition) eye phenotype. Cysteamine, the FDA-approved drug for standard treatment of cystinosis, has neuroprotective effects and is also in clinical trials for therapy of Huntington's disease. Cysteamine has been shown to increase the level of BDNF in treated striatal nerve cells and prevent mitochondrial depolarization. We tested the effects of cysteamine to rescue the eye phenotype of large clone progenies. As shown in the figure, treatment of cysteamine (1 mM) did not result in significant improvements in either the glossy or rough eye phenotypes.