Cystinosis Research Foundation SCIENCE REPORT

PUBLISHED BY THE CYSTINOSIS RESEARCH FOUNDATION FEBRUARY 2009

Welcome to the third edition of the *Cystinosis Research Foundation Science Report*

This is our third edition of the Cystinosis Research Foundation Science Report, which is designed to provide research updates on studies underway and list the Abstracts of the newly awarded funded studies.

Less than six years ago, research in the area of cystinosis was minimal and multi-year scientific studies were only a dream. Today we have significantly changed the course of cystinosis research and we are moving closer to our mission: to find better treatments and a cure for cystinosis. Our funding efforts have allowed talented doctors and researchers to initiate novel research studies and advance their research efforts. CRF is the largest non-profit fund provider of cystinosis research in the world.

In 2008 alone, the Cystinosis Research Foundation raised more than $2.7 million. Since its inception, the Foundation has raised more than $9.3 million and has committed and funded $7.4 million for cystinosis research, with $1.2 million available for the upcoming research call. CRF is now funding 25 research studies including 7 research fellows. Our researchers are working in six countries around the world and have formed collaborations to ensure the sharing of ideas and energy.

2009 Call for Funding Proposals

The Cystinosis Research Foundation will announce *A Call for Research and Fellowship Proposals* in the spring and autumn of 2009. CRF is prepared to fund proposals to improve the immediate care of children and young adults with cystinosis and to develop new understanding and treatment of cystinosis to help these children in the future. In addition, CRF is focused on funding translational research that will result in human clinical trials. The Foundation has over $1.2 million available for funding grants awarded in the spring. The number of awards and their value will depend on the number of outstanding proposals received and the funds available in 2009.

Cystinosis Research Foundation Fellowship Program

The Cystinosis Research Foundation has established a postdoctoral research fellowship program to attract qualified, promising investigators to establish careers in cystinosis research. Fellows will be funded for 2–3 years to a maximum of $75,000 per year. Applications will be available in conjunction with the spring and autumn *Call for Funding Proposals.*

Visit www.cystinosisresearch.org for details.

The Second International Cystinosis Research Foundation Symposium for scientists and researchers will be held April 8 and 9, 2010 at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering.

SCIENTIFIC REVIEW BOARD

The Scientific Review Board is composed of leading cystinosis scientists and experts from around the world. The members are actively involved in the grant review process and advise the Cystinosis Research Foundation on the merit of each proposal. We are indebted to the Scientific Review Board members for their guidance and commitment to helping our children.

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Dean for Academic Affairs Emeritus
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We are pleased to announce our newest member:

Elena Levtchenko, MD, PHD
Associate Professor
University Hospital Leuven, Leuven, Belgium

CYSTINOSIS RESEARCH FOUNDATION
18802 Bardeen Avenue, Irvine, CA 92612
949.223.7610 • www.cystinosisresearch.org

2008 RESEARCH STUDIES FUNDED AND COMMITTED $ 1,741,889

SPRING

Corinne Antignac MD, PHD
Hospital Necker, Paris, France
" Characterization of the Interaction of Cystinosin with Galectin-3 and Vacuolar H+ - Atpase"
$230,000 – 2-year study

Stephanie Cherqui, PHD
Daniel Salomon, MD
The Scripps Research Institute, La Jolla, California
"Treatment of Cystinosis Nephropathy Using Ureteral Injection of Adeno-Associated Virus Expressing *CTNS*"
$249,128 – 1-year study

Francesco Emma, MD
Anna Taranta, PHD
Bambino Gesù Children's Hospital and Research Institute, Rome, Italy
"Identification and Analysis of Cis- and Trans-acting Elements that Activate the *CTNS* Gene"
$125,140 – 2-year study

Bruno Gasnier, PHD, Mentor
Xiong Chen, PHD, Fellow
Institut de Biologie Physico-Chimique, Paris, France
"Molecular Anatomy and Physiology of Human Cystinosin"
$223,200 – 3-year study

Vasiliki Kalatzis, PHD
Eric J. Kremer, PHD
Institut Génétique Moléculaire Montpellier, Montpellier, France
"Gene Transfer Studies for Cystinosis"
$66,900 – 1-year study

Vasiliki Kalatzis, PHD, Mentor
Claire Hippert, Fellow
Institut Génétique Moléculaire Montpellier, Montpellier, France
"Gene Transfer Studies for Cystinosis"
$63,300 – 1-year study

Elena Levtchenko, MD, PHD
Lambertus van den Heuvel, PHD
Francesco Emma, MD, Mentors
Wilmer Martijn, Fellow
University Hospital Leuven, Belgium
"Pathogenesis of Renal Disease in Nephropathic Cystinosis"
$165,000 – 2-year study

Jennifer Simpson, MD
James Jester, PHD
University of California, Irvine
"Evaluation of Novel Corneal Imaging and Therapeutics in the *CTNS* Knockout (Cystinosis) Mouse Model"
$89,399 – 2-year study

Mary L. Taub, PHD
State University of New York at Buffalo
"Mechanisms Underlying the Fanconi Syndrome in Cystinosis"
$104,294 – 1-year study

AUTUMN

Betty Cabrera, BS, MPH
University of California at San Diego
"Bench and Clinical Research Assistant"
$111,045 – 3 years

Elena Levtchenko, MD, PHD
University Hospital Leuven, Belgium
"Unraveling the Mechanisms of Cysteamine Toxicity in Patients with Cystinosis"
$150,000 – 2-year study

Minnie M. Sarwal, MD
Renee A. Reijo Pera, PHD
Stanford University School of Medicine
"Characterization of Novel Lysosomal Genes for Immune Regulation and Spermatogenesis in Nephropathic Cystinosis"
$164,483 – 1-year study

For information about our 2009 Call for Funding Proposals or the Cystinosis Research Foundation Fellowship Program, visit www.cystinosisresearch.org.

CORINNE ANTIGNAC, MD, PHD, PRINCIPAL INVESTIGATOR

Necker Children's Hospital, Paris, France
"Characterization of the Interaction of Cystinosin with Galectin-3 and Vacuolar H+ - ATPase"
Total Gift Award: $230,000 – 2-year study

ABSTRACT/SUMMARY

Cystinosis is an inherited lysosomal storage disorder characterized by a defective lysosomal efflux of cystine. The causative gene, *CTNS,* encodes a glysosylated lysosomal membrane protein, cystinosin, that contains 7 transmembrane (TM) domains and is targeted to the lysosome by two lysosomal sorting signals, a classical tyrosine-based GYDQL lysosomal sorting motif in its C-terminal tail, and a novel conformational lysosomal sorting motif localized to the 5th inter-TM loop, both of which are oriented toward the cytoplasm. Experiments in which cystinosin-GFP constructs (wild-type or deleted for each of the lysosomal targeting signals) were overexpressed in different cell lines suggest that this novel lysosomal targeting signal might play a role in lysosomal fusion. By immunoprecipitation of cystinosin in cells overexpressing cystinosin-GFP, followed by electrophoretic separation of immunoprecipitated proteins and mass spectrometry, we searched for cystinosin-interacting proteins and showed that cystinosin consistently interacts with several subunits of the proton-pumping vacuolar ATPase (V-ATPase) as well as with galectin-3, a beta-galactoside binding lectin recently shown to be required for apical sorting. We confirmed the interaction with galectin-3 by immunoprecipitation of endogenous galectin-3 in MDCK cells stably transfected with cystinosin-GFP, and showed that the interaction is abolished when incubating the cells with lactose or thiogalactoside, proving that the interaction proceeds through a carbohydrate-dependent mechanism.

Our project or the next two years is to understand the role of these interactions in the pathophysiology of cystinosis, especially in the light of the recently demonstrated roles of both V-ATPase and galectin-3 in cellular trafficking.

BETTY CABRERA, BS, MPH

University of California, San Diego
"Bench and Clinical Research Assistant"
Total Gift Award: $111,045 – 3 years

STEPHANIE CHERQUI, PHD, PRINCIPAL INVESTIGATOR
DANIEL SALOMON, MD, CO-PRINCIPAL INVESTIGATOR

The Scripps Research Institute, San Diego, California
"Treatment of Cystinosis Nephropathy Using Ureteral Injection of Adeno-Associated Virus Expressing *CTNS*"
Total Gift Award: $249,128 – 1-year study

ABSTRACT/SUMMARY

The objective of this proposal is to test the hypothesis that retrograde ureteral injection of self-complementary adeno-associated virus (scAAV) expressing a functional *CTNS* gene will treat or prevent the proximal tubulopathy and progression of renal defects in cystinosis when delivered very early in the disease and ameliorate the renal disease if administered to older patients. Retrograde ureteral adminstration would be a minimally invasive procedure and would complement systemic cysteamine therapy. Indeed, AAV vectors presently represent the safest vehicles for gene therapy because of their low immunogenicity, low toxicity, and absence of integration into the host genome and there are now a large number of ongoing human clinical trials underway including studies of genetic diseases in children.

FRANCESCO EMMA, MD, PRINCIPAL INVESTIGATOR
ANNA TARANTA, PHD, CO-PRINCIPAL INVESTIGATOR

Bambino Gesù Children's Hospital and Research Institue, Rome, Italy
"Identification and Analysis of Cis-and Trans-Acting Elements that Activate the *CTNS* gene"
Total Gift Award: $125,140 – 2-year study

ABSTRACT/SUMMARY

As part of our previous and still ongoing Cystinosis Research Foundation grant, we have tested if the *CTNS* promoter was actively regulated. Our results show that *CTNS* mRNA is markedly up-regulated when cell are shifted from a standard DMEM/F12 medium to a cystine-free DMEM medium. Furthermore, we have shown that this up-regulation is mediated simultaneously by increased activity of the *CTNS* promoter and stabilization of its mRNA.

Based on these results, we are herein submitting a new proposal as an extension of our previous grant, which is aimed at identifying with a proteomic approach transcription factors that activate *CTNS* gene expression. In the proposed experiments, the *CTNS* promoter will be tested by electrophoresis mobility shift assays as a probe to isolate transcription factors that activate the gene in cystine-free media. The identified candidate trans-activating elements will thereafter be tested directly in cell culture systems. In addition, we propose as an ancillary specific aim, to begin analyzing the mechanisms that regulate *CTNS* mRNA stabilization using a reporter gene system containing the *CTNS* 5' and 3' untranslated regions.

BRUNO GASNIER, PHD, RESEARCH MENTOR
M. XIONG CHEN, PHD, RESEARCH FELLOW

Centre National de la Recherche Scientifique, Paris, France
"Molecular Anatomy and Physiology of Human Cystinosin"
Total Gift Award: $223,200 – 3-year Fellowship

ABSTRACT/SUMMARY

This project aims to characterize in some detail the mechanism by which cystinosin transports cystine across membranes as well as its molecular architecture. It takes advantage of the existence of a functional sorting mutant mislocalized to the plasma membrane and thus directly accessible to biochemical and biophysical techniques under conditions which mimic the lysosomal environment (acidic extracellular medium).

The transport mechanism of human cystinosin will be dissected out by a hypothesis-driven approach using site-directed mutagenesis and electrophysiological

recording of mutants' transport current at the plasma membrane. The advantages of this technique over classical biochemical assays are its much higher time resolution and the possibility to identify substeps of the transport cycle using voltage clamp or transient kinetics analysis.

In a second part of the project, we will characterize the actual topology of cystinosin and the spatial relationship between transmembrane domains (TMDs) using cysteine-scanning mutagenesis and thiol reagents. This approach is based on preliminary experiments which showed that cystinosin remains fully active after replacing its 5 endogenous cysteines by alanines. This approach will also be used to identify conformational changes associated with transport steps by studying how environmental factors (cystine, extracellular pH, voltage) or site-directed mutations alter the accessibility of single cysteine residues.

Besides its academic interest in the field of transport biology, we believe that such a deep knowledge of the molecular architecture and inner workings of cystinosin should benefit cystinosis research by providing a framework to cell biological or pathophysiological studies carried out by diverse groups. For instance, a more accurate structural model should help interpreting the mechanism and consequences of cystinosin interactions with other proteins or may help designing a rational search of exposed epitopes to raise efficient anti-cystinosin antibodies (a tool which is still lacking thus far).

Within the scope of this project, we will use the framework and tools generated by our structural and electrophysiological studies to investigate whether pathogenic mutants with residual activity (in particular, G197R) or the recently described splice variant of cystinosin exhibit peculiar functional properties.

VASILIKI KALATZIS, PHD, PRINCIPAL INVESTIGATOR
ERIC J. KREMER, PHD, CO-PRINCIPAL INVESTIGATOR

Institut Génétique Moléculaire Montpellier, Montpellier, France

"Gene Transfer Studies for Cystinosis"
Total Gift Award: $66,900 – 1-year study

VASILIKI KALATZIS, PHD, RESEARCH MENTOR
CLAIRE HIPPERT, PHD, RESEARCH FELLOW

Institut Génétique Moléculaire Montpellier, Montpellier, France

"Gene Transfer Studies for Cystinosis"
Total Gift Award: $63,300 – 1-year fellowship

ABSTRACT/SUMMARY

Our work is focused on using viral-mediated *CTNS* gene transfer to reduce the lysosomal cystine storage that characterises cystinosis. We recently demonstrated the feasibility of this strategy in vivo by delivering E1-E3-deleted (E1 E3) adenovirus (Ad) vectors expressing *CTNS* to the liver of *Ctns-/-* mice. This organ was chosen for the proof-of-concept because of its accessibility, however we will now concentrate on tissues that are more clinically relevant for cystinosis. Rather than a multisystemic approach, we are initially concentrating on the corneal and central nervous system (CNS) anomalies that we have characterised in the *Ctns-/-* mice and that mimic those of patients. We propose to compare the efficiency (in terms of titre, tropism and transduction) of two different viral vectors: a helper-dependent (HD) canine Ad (CAV-2) and an adeno-associated virus vector (AAV-8). These vectors effectively transduce both the cornea and the CNS and, due to their low immunogenicity, are capable of long-term gene expression in the absence of toxicity. We will perform targeted gene transfer studies using both these vectors and compare the efficiency of cystine clearance in parallel with that of cysteamine. This work is aimed at further understanding the pathogenesis of cystinosis, in particular in terms of the poorly understood CNS anomalies, and developing a complementary treatment that addresses the cause of the disease rather than the symptoms.

ELENA LEVTCHENKO, MD, PHD
LAMBERTUS VAN DEN HEUVEL, PHD
FRANCESCO EMMA, MD, RESEARCH MENTORS
WILMER MARTIJN, RESEARCH FELLOW

University Hospital Leuven, Belgium

"Pathogenesis of Renal Disease in Nephropathic Cystinosis"
Total Gift Award: $165,000 – 2-year study

ABSTRACT/SUMMARY

Cystinosis is a multi-organ disease caused by lysosomal cystine accumulation due to defects in lysosomal cystine carrier cystinosin encoded by the *CTNS* gene [Gahl et al. 2002]. Renal disease in cystinosis is characterized by various degrees of proximal tubular dysfunction, proteinuria and progressive decline of glomerular filtration rate (GFR). Treatment with amino thiol cysteamine ameliorates renal dysfunction in cystinosis, but does not have a curative effect in the majority of the patients. Histological examination of cystinotic kidneys demonstrates pronounced tubular atrophy (so-called swan-neck deformity), tubulo-interstitial fibrosis and glomerular lesions resembling focal segmental glomerulosclerosis [Gubler et al. 1999, Mahoney et al. 2000, Servais et al. 2007]. The pathogenesis of renal disease in cystinosis remains largely unknown, limiting therapeutic interventions downstream cystine accumulation. The following recent findings make the rational background for this research proposal, which is aimed to unravel the mechanisms of cystinotic renal damage.

Although cystinosis is generally considered to be a tubular disorder with secondary damage of glomeruli, our recent data suggest also a primary glomerular defect in cystinosis [Wilmer et al. 2008].

The most common mutation in patients with cystinosis from North-European origin is a 57kb deletion, affecting both the *CTNS* and the *CARKL* genes. The function of *CARKL* has recently been described to be involved in the phosphorylation of sedoheptulose, an intermediate of the pentose phosphate pathway (PPP) and sedoheptulose was demonstrated to be elevated in urine of cystinotic patients with the common 57kb deletion [Wamelink et al. 2008]. One of the functions of PPP is delivering NADPH+ for maintaining cellular redox status. Whether the absence of the *CARKL* gene is involved in the pathogenesis of cystinosis in patients carrying 57kb deletion is unknown so far.

Recently, a new cystinosin isoform (cystinosin-LKG), generated by an alternative splicing of exon 12, removing the carboxy-terminal lysosomal targeting GYDQL motif of the protein, was identified [Taranta et al. 2008]. Consequently, this isoform is not restricted to the lysosomal compartment, suggesting alternative localized cystine transport by cystinosin.

In this research proposal we aim to extend our ongoing studies towards understanding the pathogenesis of renal disease in cystinosis in order to provide a rational basis for new therapeutic interventions for preventing the deterioration of the renal function. In particular we would like to examine the mechanisms of interstitial fibrosis in cystinosis and the role of cystinosin, cystinosin-LKG and CARKL proteins in this process. In addition to the established proximal tubular cell model of cystinosis, we will study recently obtained cystinotic podocyte cell lines to investigate the glomerular effects observed in cystinosis. For accomplishing this project we will bundle the efforts of three European Institutions (Radboud University Nijmegen, Catholic University of Leuven, and Gesù Bambini Children Hospital in Rome). To realize our goals we formulate the following key-objectives:

A. Studies in cystinotic ciPTEC

To study albumin-mediated production of pro-inflammatory and pro-fibrotic substances by cystinotic conditionally immortalized proximal tubular cells (ciPTEC) compared to control ciPTEC before and after incubation with cysteamine.

The role of cystinosin, cystinosin-LKG and CARKL will be studied by transfection of these proteins in cystinotic ciPTEC carrying homozygous 57 kb deletion and comparing their phenotype with non-transfected cystinotic and control ciPTEC. The role of oxidative stress will be evaluated by measuring of reactive oxygen species (ROS) in the above mentioned ciPTEC models.

B. Studies in cystinotic podocytes

To examine glomerular podocyte cell function in cystinosis. A newly developed ciPodocyte cell line of control and cystinosis will be evaluated morphologically and for metabolic changes.

ELENA LEVTCHENKO, MD, PHD

University Hospital Leuven, Belgium

"Unraveling the Mechanisms of Cysteamine Toxicity in Patients with Cystinosis"
Total Gift Award: $150,000 – 2-year study

ABSTRACT/SUMMARY

Background

Nephropathic cystinosis is an autosomal recessive metabolic disorder caused by cystine accumulation within lysosomes. An amino thiol cysteamine is currently the only available treatment, depleting lysosomal cystine and postponing renal and extra-renal organ damage in cystinosis. During recent years the strategy to administer the highest tolerable doses of cysteamine was challenged by the development of serious adverse events consisted of skin lesions (striae, vascular tumors on the elbows), bone pain, muscular weakness and vascular complications in 8 patients, treated with high cysteamine doses. One patient deceased from cerebral ischemia. Histological analysis of the skin in one patient demonstrated irregularities of collagen fiber caliber, resembling lesions described in Ehlers-Danlos syndrome and endothelial proliferation disrupting underlying extracellular matrix.

Specific aims

Because only a small proportion of cystinosis patients, treated with high cysteamine doses, developed the above-mentioned symptoms, we hypothesized that these patients might have a genetic susceptibility to cysteamine. In this project *we aim* to compile a risk profile, using the combination of genetic make-up and molecular expression patterns of relevant markers using genomic DNA and cultured skin fibroblasts of cystinosis patients suffering from cysteamine adverse events, compared to the patients treated with high cysteamine doses without the above-mentioned symptoms and those of healthy subjects. The availability of biomarkers for cysteamine endurance would allow: a) to identify patients at risk, requiring extra medical attention and eventually cysteamine dose reduction and b) to monitor early phases of cysteamine hyperresponsiveness.

Key-objectives/work plan

To collect full information on patients reported with the above-mentioned symptoms. This information will include the exact Cystagon® dose, duration of treatment and WBC cystine levels. Next, differences in pharmacokinetics and pharmacodynamics of cysteamine, which might cause high intracellular cysteamine levels in these patients, will be evaluated by measuring plasma and granulocyte cysteamine levels after the administration of the equal doses of cysteamine.

To analyze genetic variations in genes encoding extracellular matrix proteins, angiogenesis-related factors and proteins involved in the regulation of intracellular redox status by using SNP analysis on genomic DNA. Genes involved in classical EDS (COL5A1, COL5A2, COL1A1, COL1A2, TNXB) will be directly sequenced.

To perform expression analysis (mRNA expression arrays and quantitative proteomics) in cultured skin fibroblasts exposed to the concentration range of cysteamine and derived from cystinosis patients with and without the above-mentioned symptoms and those from healthy subjects. Furthermore, we will evaluate a direct influence of cysteamine on a production of angiogenesis-related factors by control endothelial cells *in vitro* using a similar technical approach.

Conclusion:

This research proposal will provide new insights into the mechanisms of cysteamine – induced Ehlers-Danlos like syndrome and may allow identifying patients at risk. This would be of paramount importance for preventing the development of potentially life-threatening adverse effects of cysteamine treatment in cystinosis.

JENNIFER SIMPSON, MD, PRINCIPAL INVESTIGATOR
JAMES JESTER, PHD, CO-PRINCIPAL INVESTIGATOR

University of California, Irvine

"Evaluation of Novel Corneal Imaging and Therapeutics in the *CTNS* Knockout (Cystinosis) Mouse Model"
Total Gift Award: $89,399 – 2-year study

ABSTRACT/SUMMARY

The lysosomal storage abnormality that underlies cystinosis results in the accumulation of cystine crystals in various tissues, including the kidney, thyroid, brain and eye. Progressive photophobia and visual deterioration are hallmarks of cystinosis and are associated with increasing concentrations of corneal crystals. While oral administration of cysteamine, along with replacement of thyroxine and renal losses has significantly improved overall

prognosis, no improvement in ocular manifestations of the disease have been demonstrated with these measures.

The current mainstay of ocular therapy is topical cysteamine, which, though modestly effective in reducing corneal crystals, requires an impractical, every one hour dosing regimen. Thus, ocular disease effects and therapies have become an increasingly significant burden for cystinosis patients. Alternative ocular treatment strategies have been tried, but so far have required costly and time consuming clinical trials, which are further hampered by the very low prevalence of the disease.

The identification ten years ago of mutations in the *CTNS* gene, which codes for the cystine lysozomal membrane transporter has recently led to an animal model, the mixed background *Ctns* knockout *(Ctns-/-)* mouse. The development of ocular cystinosis has been studied in this model using both standard photographic and retinal functional tests, with findings that appear to mimic those seen in cystinosis patients.

The proposed project will further adapt this model system for use in identifying potential therapeutic interventions for corneal cystinosis. Our initial goal will be the addition of imaging techniques to the *Ctns*-/- mouse model, including the use of second harmonic and confocal microscopy, which can allow quantitative, in vivo imaging of corneal crystals.

Once in place, the adapted model will be used to screen a variety of novel treatment alternatives that have the potential for increased effectiveness and/or reduced burden to patients and their families. Such treatments include nanotechnology based, sustained release drug delivery systems, as well as surgical approaches using either corneal transplantation techniques or tissue electrolysis to reduce or eliminate crystal formation and offer longer term relief from symptoms.

Diagnostic and therapeutic modalities that demonstrate promise in the mouse model for corneal cystinosis will form the basis for human testing that can be pursued in future clinical proposals to appropriate funding sources, including government agencies, private foundations and industry.

MARY TAUB, PHD, PRINCIPAL INVESTIGATOR

State University of New York at Buffalo
"Mechanisms Underlying the Fanconi Syndrome in Cystinosis"
Total Gift Award: $104,294 – 1-year study

ABSTRACT/SUMMARY

The proposed project is concerned with the mechanisms responsible for emergence of the Fanconi Syndrome in renal proximal tubule cells of individuals with cystinosis. The hypothesis is to be examined that the Fanconi Syndrome emerges as a consequence of reduced intracellular phosphate (Pi) levels, as a consequence of a reduced level of the Na+/Pi cotransporter (Npt2) in the membrane, ultimately resulting in the Fanconi syndrome, as well as apoptosis. A well-characterized rabbit renal proximal tubule (RPT) cell culture system in serum free medium will be studied both in monolayer and matrigel cultures using siRNA against cystinosin to induce cystinosis. Primary cells have a genotype and phenotype that closely resembles normal renal cells in vivo.

MINNIE M. SARWAL, MD, PRINCIPAL INVESTIGATOR
RENEE REIJO PERA, PHD, CO-PRINCIPAL INVESTIGATOR

Stanford University School of Medicine, Stanford, California
"Characterization of Novel Lysosomal Genes for Immune Regulation and Spermatogenesis in Nephropathic Cystinosis"
Total Gift Award: $164,483 – 1-year study

ABSTRACT/SUMMARY

Cystinosis is an autosomal recessive disorder with free cystine accumulates in lysosomes, eventually causing damage to most body tissues. Elevated cystine crystals, ocular abnormalities, severity of cystine tissue accumulation and the proximal tubulopathy and renal failure have been seen in affected children with nephropathic cystinosis. In an ongoing collaboration with the Antignac and Gahl laboratories with funding from the Cystinosis Foundation of Ireland, we have generated microarray data set from *Ctns* (+/+) and *Ctns* (-/-) mice as well as from human samples (peripheral blood, fibroblasts and kidney cells) from patients with and without nephropathic cystinosis. The aim of these studies was to study global changes in differentially expressed genes and biological pathways specific to cystinosis in mouse and human [Sarwal, unpublished]. A number of key biological pathways and candidate genes were identified relating to apoptosis, Nrf2-mediated oxidative stress, ATP metabolism, and lysosome dysfunction, which are currently under study in the Sarwal Lab.

Previous reports have shown that cystinosis patients have low incidence of rejection and better graft survival after renal transplantation, without an increased incidence of bacterial, viral or fungal infections, either before or after transplantation. Male cystinotic patients are also known to have a high incidence of male infertility after pubertal development, the cause of which is currently unknown, and does not appear to be related to the extent of tissue cystine accumulation.

Recent human microarray data from adolescent cystinosis transplant recipients in the Sarwal Lab, has identified 2 *key regulatory molecules, found in lysosomes, involved in immune regulation (LP1 and LP2) and spermatic development (LP2)*. We hypothesize that further analysis of these two lysosomal candidate genes, may offer critical insights into differential immune regulation in cystinotic patients after organ transplantation, and also uncover critical pathways that may dysregulate sperm development in adolescent males with nephropathic cystinosis.

PROGRESS REPORT

CORINNE ANTIGNAC, MD, PHD, PRINCIPAL INVESTIGATOR

"Characterization of Cystinosin Intracellular Trafficking"

Date: 1/10/09

Persons working on the project :
Dr Véronique Chauvet (funded by the EU grant Eunefron)
Nathalie Nevo (technician, Inserm funded)

Background and objectives

The global aim of the research project is to characterize intracellular trafficking of cystinosin. The specific aims of the projects are:

1. To characterize how cystinosin is sorted to the lysosome
2. To identify proteins interacting with cystinosin

Update on the progress of research plan

We have shown that cells transiently or stably overexpressing a cystinosin-GFP fusion protein display striking aggregation of lysosomes into a few large juxtanuclear structures, and a diminution of the usual pattern of small discrete intracytoplasmic vesicles characteristic of lysosomes and that the number of these large structures is drastically decreased when the 5th inter-TM loop of cystinosin, and/or its C-terminal tail were altered. These large juxtanuclear structures are reminiscent of what is observed in cells overexpressing hVam6p, a protein of the Vamp (Vesicle associated membrane protein) family, which has been identified as a mammalian tethering/docking factor with an intrinsic ability to promote lysosome fusion in vivo, or the lysosomal proteins Oa1 (Ocular albinism type 1) and LGP85. Along the same line, mutations in the VPS33B gene encoding a lysosomal protein involved in vesicle membrane fusion have been identified in patients with ARC syndrome, characterized by arthrogryposis, cholestasis and renal tubular dysfunction. Moreover, the endolysosomal CLN3 protein, which contains a domain that resembles of the 5th inter-TM loop of cystinosin, has been recently shown to be involved in membrane fusion. Mutations of the CLN3 gene are responsible for the Batten disease, a severe neurodegenerative disorder.

Altogether, data from the literature and our preliminary observations led us to explore the possibility that cystinosin has additional functions apart from its cystine transport activity, especially a role in membrane fusion. We hypothezised that the 5th inter-TM loop and the C-terminal tail are probably important for cystinosin function and trafficking.

Characterization of the composition and function of these enlarged structures

The precise composition of these structures was assessed using different antibodies directed against markers of intracellular compartments, such as Rab5 (early endosome), Rab11 (recycling endosomes), Lamp-1/Lamp-2 (lysosomes), LC3 (autolysosomes), Rab7 (late endosomes). These structures were only positive for late endosome and lysosome markers such as Lamp-1, Lamp-2 and Rab7 suggesting they are not recruiting membrane from other intracellular compartments.

We also looked for a possible role of the V-H+ATPase on the biogenesis of these compartments. After pharmacologic inhibition of the pump by bafilomycin A, these structures were still present, suggesting their formation is not dependent of intravesicular pH.

In order to get insight into the possible implication of the 5th inter-TM loop of cystinosin and its C-terminal tail, we conducted a yeast two hybrid screen using these domains as bait. The 5th inter-TM loop of cystinosin was used for a large screen against a mouse kidney cDNA library (collaboration with Hybrigenics). We identified different putative partners and will focus on two of them: Vps39 and Snf8 (the murine homolog of Vps22). Both of them are involved in traffic and membrane fusion. After these interactions are confirmed, their physiological relevance will be studied.

Given the importance of the small GTPases from the Rab family in intracellular trafficking, we also looked for possible interactions between the cystinosin bait (5th inter-TM loop) and the Rab proteins using a new tool developed by our collaborators in Bruno Goud's laboratory (UMR144, Curie Institute, Paris). This screen was done using a yeast living array that contains the majority of the mammalian Rab genes in their wild type or mutant forms (either dominant active or negative forms). An interaction with the GTP-bound form of Rab24 was identified but needs to be confirmed. We will also perform the "Rab screen" using the the C-terminal tail of cystinosin.

Cystinosin trafficking

Cystinosin is a lysosomal protein but the way it is targeted to this organelle is still unknown. Although cystinosin is mainly located on lysosomes when transiently or stably expressed in LLC-PK1 (proximal tubular cell line), Cos-7 and HeLa cell lines, small amounts of the protein are also found at the cell surface. Moreover, a staining with a Rab5 antibody (early endosomal marker) showed a colocalization with the cystinosin-GFP. Altogether, these observations suggest that cystinosin might reach lysosomes via an indirect route. In order to confirm this hypothesis, we are currently performing a direct two hybrid screen using the C-terminal tail of the protein as a bait with different sub-unit of the AP complexes (AP-1 to AP-4) which are known to interact with a tyrosine-based motif.

We are also studying the effect of endocytosis inhibition by Eps15 mutants and a Rab5 dominant negative mutant on cystinosin localization. We have made stable cell lines over-expressing a cystinosin construct fused at its C-terminus to a red fluorescent protein (cherry). These cell lines will be used for cell surface biotinylation experiments in order to compare the the amount of cystinosin at the cell surface before and after a transient co-transfection with the various mutants mentioned above that have been shown to impair endocytosis.

Work in progress

The importance of the 5th inter-TM loop and the tyrosine-based motif on cystinosin trafficking and membrane fusion events will be studied by generating a chimeric protein: the lysosomal CD63 tetraspanin on which we will replace its cytoplasmic loop and C-terminal tail by those of cystinosin. The impact of the enlarged structures seen when the wild type cystinosin is overexpressed on vesicular traffic and lysosome degradative functions will be assessed. We are studying the uptake and the subcellular localization of well-used labeled markers, such as fluorescent transferrin and albumin, that normally traffic through endolysosomal compartments. We will also used a chimeric protein: the Tac protein (interleukin 2 receptor) which is artificially addressed to these compartments after being fused to a lysosome targeting motif, in order to define its intralysosomal degradation rate.

PROGRESS REPORT

ANGELA O. BALLANTYNE, PHD, PRINCIPAL INVESTIGATOR
AMY M. SPILKIN, PHD, CO-PRINCIPAL INVESTIGATOR
DORIS A. TRAUNER, MD, CO-INVESTIGATOR

"Academic Functioning in Cystinosis: A Comprehensive Study of the Process of Achievement"

Eighteen-Month Progress Report: Cystinosis Research Foundation
9/1/06 – 3/1/08

A. SPECIFIC AIMS

The specific aims for this project are:

1) To be the *first study to comprehensively examine academic functioning in children with cystinosis,* compared to children with another chronic illness (cystic fibrosis) and typically developing controls, using a comprehensive academic achievement battery. Our goal is to delineate the academic profile of school-age children with cystinosis to provide a more thorough understanding for parents and school personnel. Moreover, these results may elucidate a potential non-verbal learning disability profile in children with cystinosis.
2) To use academic testing scores *within a context* as opposed to viewing the scores in isolation to allow for a more complete picture of the academic functional level of the child. This will be done by assessing academic competence through questionnaires from the perspective of the child's teacher, as well as the child, in multiple domains (e.g., academic skills, interpersonal skills, academic motivation, study skills, classroom engagement).
3) To examine processes underlying potential areas of deficit, by pinpointing *where* breakdowns in academic competence occur. The assessment measures we are proposing are designed not only to assess *the amount* of academic knowledge a child has, but also how the information is utilized (i.e., the process). Understanding the process will allow for the future application of precise intervention strategies that pinpoint the specific areas of difficulty for children with cystinosis.

B. PROGRESS TO DATE: 9/1/06 – 3/1/08

Over the past 18 months, we have recruited, inducted, and tested 23 individuals with cystinosis, 12 control subjects, and 5 cystic fibrosis subjects. Despite the best efforts of our consultant Dr. Mark Pian, the pediatric pulmonologist at Children's Hospital, we continue to have difficulty recruiting and inducting cystic fibrosis (CF) patients due to their hectic schedules and medical status. Given this significant difficulty with recruiting CF subjects, we went through the lengthy process of applying for Joint Institutional Review Board (Joint IRB) approval between UCSD and Rady Children's Hospital of San Diego. Our application was successful and we are now able to test the CF patients when they are admitted into the hospital for medical care, as long as they are stable enough medically to complete the testing. We have recently been able to test four CF patients in this way, although there are more sessions (and hence time) involved due to the routine hospital interruptions and procedures that the CF patients are undergoing. Our CF recruitment advertisements in Orange, Los Angeles, and Riverside counties have not yielded subjects from these areas; again, it appears that the CF group has constraints on their availability due to their medical condition and related aspects of their health.

C. RESULTS

On all 40 participants tested to date, we have collected WJ-IIIs, Wechsler IQs, and the age-appropriate academic functioning questionnaires (ACES and/or SMALSI). We are also in the process of scoring, reliability checking, and data entering all of the test and questionnaire data collected so far. Since we are still in the process of collecting data for this research study, we have not yet analyzed the data.

D. FUTURE PLANS

In the next 6-month period, we plan to continue to recruit, induct, and test individuals with cystinosis, cystic fibrosis, and control participants, as we work toward the total projected participants. We are arranging for cystinosis families to travel here to San Diego, as well as traveling to cystinosis families in various parts of the country. We will continue in our efforts to recruit and test cystic fibrosis subjects by testing them while they are staying at Rady Children's Hospital in San Diego. We are also willing to travel to adjacent counties to test cystic fibrosis patients if they are interested in participating in the study. Our efforts to recruit and test control participants are also ongoing.

PROGRESS REPORT

RITA CEPONIENE, MD, PHD, PRINCIPAL INVESTIGATOR
DORIS TRAUNER, MD, CO-PRINCIPAL INVESTIGATOR
AMY SPILKIN, PHD, CO-INVESTIGATOR

"Neural Functioning in Auditory and Visual Systems in Cystinosis: Linking Brain to Behavior "

Period: 8/16/07– 3/5/08

The main goal of this project is to investigate the neural functioning of auditory and visual systems in children with cystinosis. By employing a non-invasive, children-friendly technique of event-related brain potentials (ERPs) we are examining auditory and visual perception, the strength and the breadth of auditory and visual spatial attention, and attentional orienting elicited by unexpected, novel events. All of the above-mentioned functions are important for the successful day-by-day functioning as well as academic peformance in children and adults.

Our second main goal is to determine how, if at all, the observed neuro-functional abnormalities change with age. This information will help to understand the neural mechanisms of adult-onset cystinotic encephalopathy as well as will speak to the issue of early neuro-developmental insult vs. progressive brain injury as the pathogenesis of the disorder.

Our third main goal is to bridge the brain function and structure with cognitive and academic performance. We will correlate the ERP indices of brain function obtained in this project with structural brain measures obtained through an NIH grant awareded to Dr. Trauner, as well as cognitive measures obtained in the project "Academic Functioning in Cystinosis" funded by the Cystinosis Research Foundation.

The completed study will include 40 individuals diagnosed with infantile nephropathic cystinosis and 40 healthy age, gender, and SES matched control participants, age 6 years and above.

During the funding period 3/16/07 – 3/5/08, the following progress has been made.

1. **Subject recruitment**
   - We continue serious recruiting efforts of individuals with Cystinosis. To this end, a new collaboration regarding participant sharing has been established with Dr. R. Dohil's laboratory. We continue recruiting through the Cystinosis Research Network website as well as Dr. Trauner's laboratory. As a result, we are keeping with the projected timeline.
2. **Data collection**
   - A group of young healthy adults comprising our normative data set has been collected and analyzed. The results show that our experimental design produces reliable indices of auditory and visual sensory, attentional, and executive functions.
   - To date, we have collected data on auditory and visual ERP experiments from 14 individuals with Cystinosis, and by the end of March we will have tested 19 participants. This is roughly half of the proposed sample. This is 38 testing sessions.
   - Data collection from typically developing control children is ongoing on an individual case-control matching basis. The controls are being matched on the basis of gender, age, and SES. To date, we have tested 12 typical children and adults matched in this manner. This is 24 testing sessions. Five more, for the total of 17, will be tested by the end of March 2008.
3. **Experimental Procedure and Data analysis**

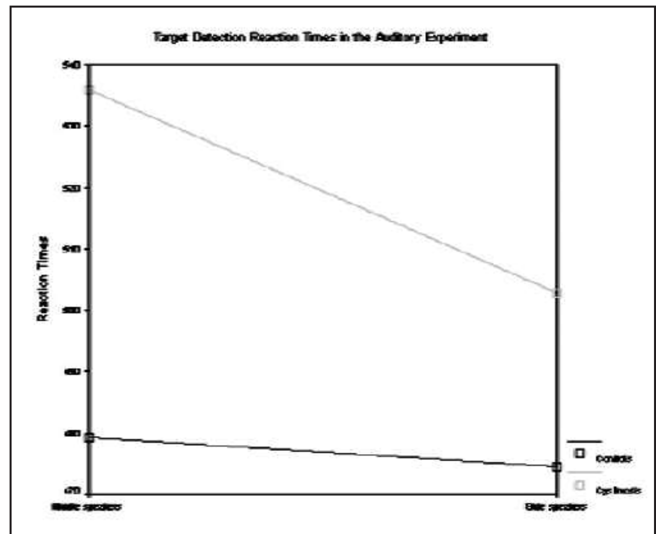
The study participants sat in a comfortable armchair while the EEG/ERP were recorded. Meanwhile, three types of stimuli were presented randomly at four different locations. In the visual experiment, those were four boxes drawn on the computer screen and in the auditory experiment, they were four speakers evenly spread out in front of a participant. The three stimulus types were frequent, "standard" stimuli, infrequent target stimuli, and varied, salient novel stimuli. The auditory stimuli were complex tones and the visual stimuli were shapes. Novels were more complex. The stimuli were presented in blocks of 400 stimuli. The participants had to attend to one pre-determined location during the duration of a block and to press a button in response to the target stimulus. They had to look directly in front of them at the fixation mark at all times. Attended location changed from block to block, for a total of 4 blocks per location. The EEG was cleaned from blink, motion, and electrical artifacts, segmented, and averaged by stimulus type. Behavioral response times to target stimuli and accuracy were also determined.

All behavioral and electrophysiological data that has been collected has been analyzed. The first preliminary results are illustrated in the figures below.

4. **Results**

Behavior

No group differences in Target Detection accuracy were found in the visual task. However, in the auditory task, the Cystinosis group performed significantly poorer than their controls ($p<.05$). Almost half of participants with Cystinosis had severe difficulty localizing sounds. Interestingly, in both modalities, Reaction Times were longer for the central than peripheral targets, and there was a strong tendency for this to be more pronounced in the Cystinosis group (Group x Location interaction at the level of $p<.1$; see Chart).



Visual ERPs

Overall, ERP amplitudes were larger in the Cystinosis group than in the controls. This was entirely unexpected. However, this finding seems to be related to general changes in neural and extra-cerebral tissues rather than to be specific to brain function.

- At the level of visual sensory processing, we found enhanced P2 peak in the Cystinosis group (Figure 1, top panel). This finding is frequent in neuro-developmental populations. However, the Cystinosis group showed an appropriate enhancement of sensory response upon spatial attention as shown by the enhancement of the N1 peak at the attended locations (Figure 1, bottom panel).

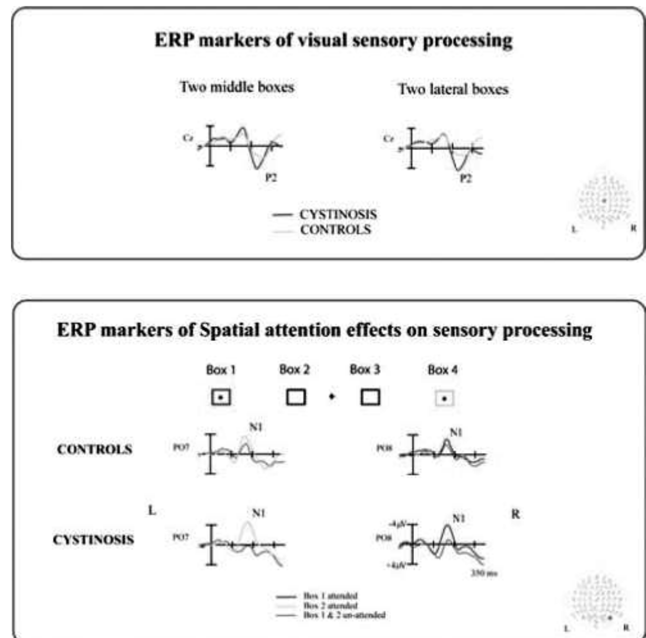


Figure 1

- At the level of target detection, the Cystinosis group showed a broader spread of visuo-spatial attentional preference. While the controls showed a clear right-hemi-field facilitation (the target P300 response peaked earlier, and was larger in amplitude, for the stimuli that were presented on the right side), in the Cystinosis group this included right-sided as well as a left middle location (Figure 2).

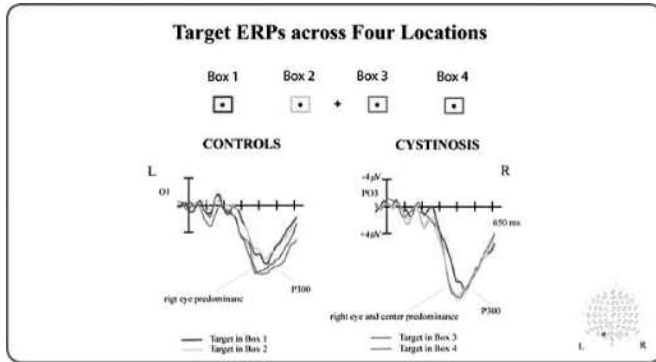


Figure 2

- Finally, response to novel stimuli, as indexed by the fronto-central Negative Component (Nc) was diminished in the Cystinosis group and, importantly, showed no spatial selectivity (Figure 3).

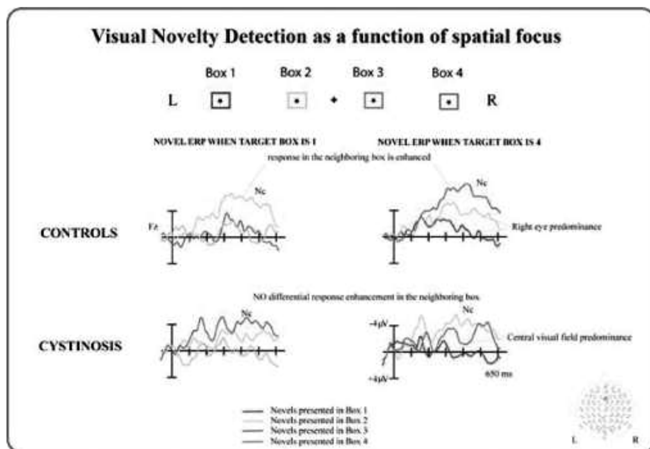
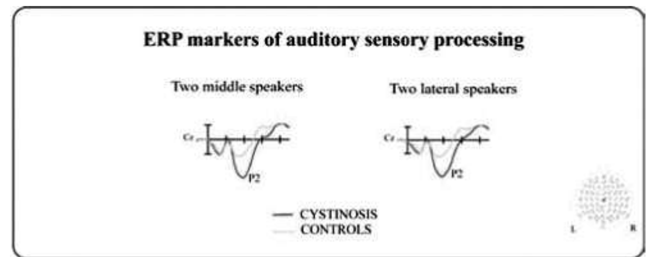


Figure 3

Auditory ERPs

- As in the visual modality, in the auditory modality the Cystinosis group showed strongly enhanced sensory P2 peak (Figure 4, top panel).
- As can be seen in the bottom panel of Figure 4, Spatial Attention effects differed substantially between the groups. PN is a Processing Negativity that indexes attention effects. It is assessed by comparing attended (but not responded to) and unattended stimuli. It has two components, the early component arising in the auditory cortex and the late component arising in the frontal cortex. Our data demonstrate that the early component is elicited in the Cystinosis group. However, the late PN component is either delayed, diminished in amplitude, or absent. These differences were more pronounced with attention to peripheral speakers.



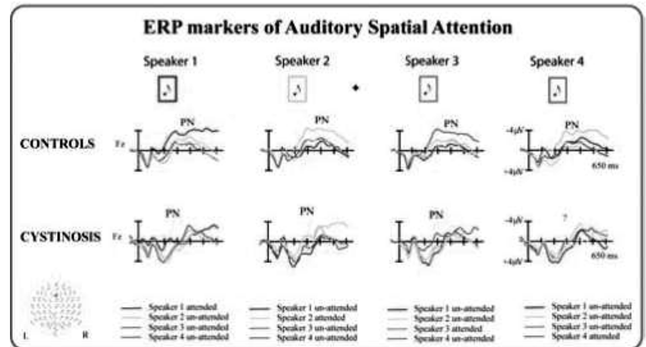


Figure 4

- Due to the low *target detection* rate in the auditory modality, brain's responses to targets in the Cystinosis group were noisy and difficult to interpret. This should improve once more participants have been tested. However, an overall pattern of results seems to indicate that in this group, target detection is more efficient in the middle locations than in peripheral locations. No such differences were found in the control group.
- The auditory novelty responses can manifest either as visual Nc (in younger children) or as a novelty P3 response. Novelty P3 reflects attentional orienting to potentially relevant sounds occurring outside the attentional focus while the Nc may reflect conscious processing of stimuli that triggered orienting. Novelty P3 was more consistent in our data. Further, it wperipheral speakers, indicating that they were more distracting they occurred at locations more spatially distant from the attentional focus. In contrasvisual modality in which Cgroup showed abnormal noveltydetection, in the auditory modality group differences were rather subtle (Figure 5). When the Nc was elicited, in the controls it showed location-dependent amplitude gradient. This pattern was less consistent in cystinoas data.

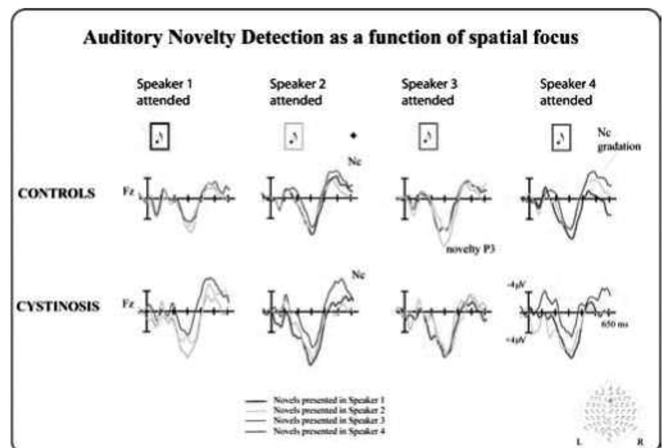


Figure 5

5. **Significance of findings**

Behavioral data indicate that, overall, individuals with Cystinosis have difficulties with perceptual localization. This is more severe in the auditory modality. Electrophysiological finding of overall larger ERP amplitudes in the Cystinosis group may be caused by function non-specific factors such as increased water retention and/or thinner scalp bones due to renal osteodystrophy.

In addition, function-specific electrophysiological differences between the Cystinosis and control groups were found at all levels processing, with those at the attentional level predominating. The sensory differences were similar across the auditory and visual modalities and consisted of enlarged P2 peak, a frequent finding in neuro-developmental populations. Although the significance of this finding is not entirely clear, it has been associated with increased stimulus feature non-specific sensory arousal, potentially leading to disruption of stimulus feature specific encoding and excessive distractibility. Spatial attention effects differed between the auditory and visual modalities. In the Cystinosis group, spatial selection was intact in the visual modality and deviant in the auditory modality. In general, spatial attention and selection is more important in visual than auditory modality since we attend were we look at. In contrast, auditory localization abilities are often complemented by vision. Therefore, auditory localization might be a more challenging ability in general and, as evidenced by behavioral and ERP data, for the individuals with Cystinosis.

Voluntary stimulus parsing appears to be least affected in this disorder; however, subtle spatial attention differences are a possibility. Finally, dampened response to visual novelty is striking. Although the neural generators of the Nc response are not known, its frontal predominance strongly suggests frontal lobe involvement. The auditory novelty P3 showed a more typical pattern. This response is strongly driven by afferent thalamo-cortical projections and has frontal, parietal, and temporal lobe generators.

Summarizing, our findings indicate that individuals with cystinosis have subtle electrophysiological deficits, with more challenging, higher-level functioning being affected the most. This includes auditory localization and visual novelty detection.

6. **Plans for the next funding period**

- We will continue with data collection. We are hopeful that we will have recruited and tested 2/3 of projected participants by the end of the next funding period.
- We will continue analyzing visual and auditory behavioral and ERP data obtained from typical children as well as children and adults with Cystinosis. The focus will be to clarify how behavioral and brain responses change with age. Is there an age-related decline in cognitive functions due to cystinosis?
- We will attempt to identify individuals with late-onset cystinotic encephalopathy and examine their brain response pattern.
- In collaboration with Dr. Trauners' lab, we will begin compiling behavioral and structural MRI data sets of the participants tested with ERPs with the goal of examining relationships between behavior, brain function, and brain structure.

PROGRESS REPORT

RITA CEPONIENE, MD, PHD, PRINCIPAL INVESTIGATOR
DORIS TRAUNER, MD, CO-PRINCIPAL INVESTIGATOR
AMY SPILKIN, PHD, CO-INVESTIGATOR

"Neural Functioning in Auditory and Visual Systems in Cystinosis: Linking Brain to Behavior "

Period: 3/5/08– 9/12/08

The main goal of this project is to investigate the neural functioning of auditory and visual systems in children with cystinosis. By employing a non-invasive, children-friendly technique of event-related brain potentials (ERPs) we are examining auditory and visual perception, the strength and the breadth of auditory and visual spatial attention, and attentional orienting elicited by unexpected, novel events. All of the above-mentioned functions are important for the successful day-by-day functioning as well as academic peformance in children and adults.

Our second main goal is to determine how, if at all, the observed neuro-functional abnormalities change with age. This information will help to understand the neural mechanisms of adult-onset cystinotic encephalopathy as well as will speak to the issue of early neuro-developmental insult vs. progressive brain injury as the pathogenesis of the disorder.

Our third main goal is to bridge the brain function and structure with cognitive and academic performance. We will correlate the ERP indices of brain function obtained in this project with structural brain measures obtained through an NIH grant awareded to Dr. Trauner, as well as cognitive measures obtained in the project "Academic Functioning in Cystinosis" funded by the Cystinosis Research Foundation.

The completed study will include 40 individuals diagnosed with infantile nephropathic cystinosis and 40 healthy age, gender, and SES matched control participants, age 6 years and above.

During funding period 3/8/08 – 9/12/08, the following progress has been made.

1. **Subject recruitment and data collection**
   - During this funding period, we made a major push towards completing our goal for data collection. We have recorded 24 individuals with cystinosis (n=48 experimental sessions) and 17 age and gender matched control subjects (=34 experimental sessions). As a result, we now have data from 37 individuals with cystinosis, just 3 short of our end goal. While we have a total of 39 control subjects, only 29 of them are suitable matches for the patients. The rest of them were tested during piloting and normative data collection stage.

2. **Data analysis and results**
   - All the collected electrophysiological as well as behavioral task performance data have been cleaned from artifacts and analyzed. The main electro-physiological results remain as reported previously and therefore are not repeated here in detail.
   - Briefly, we find that auditory modality is affected more than visual modality, with higher-order functions of spatial attention and attentional orienting affected the most.

3. Plans for the next funding period

- The data collection goal for the last 6 months of the project is to recruit 3 more individuals with cystinosis and 11 control subjects.
- The data analysis goals are critical for the final stage of the project. They are as follows:
  1) Analyze the newly incoming event-related potential (ERP) data;
  2) Measure the magnitude, timing, and distribution of ERP components reflective of the cognitive processes of interest; perform statistical analyses on these measurements;
  3) Perform age-related ERP analyses with the aim of clarifying the nature of cognitive deficits in cystinosis (e.g., co-morbidity vs. cause-and-effect of cystine accumulation).
  4) Measure and compile volumetric and brain connectivity data from the cystinosis patients that have undergone MRI and DTI scanning in conjunction with Dr. Trauner's project funded through NIH. To this end, we have secured Dr. Sach's collaboration from Dr. Trauner's group, who has extensive expertise in brain imaging area.
  5) Compute correlations between the observed electrophysiological brain abnormalities and volumetric and brain connectivity measures with the aim of elucidating brain-behavior links, and therefore brain regions most affected by the condition.
  6) Compute correlations between the observed electrophysiological brain abnormalities and academic performance data collected through the Academic achievement project lead by Dr. Ballantyne.

Finally, at least three manuscripts will be written that will report findings of the present project. Two of the manuscripts will describe auditory and visual ERP findings and their relations with academic abilities, respectively. The third manuscript will describe brain-behavior links. These are rather rough estimates, however. As the data is being statistically analyzed, some other major areas of findings might be uncovered resulting in differently focused publications. For example, we may find age-related changes or the salient between-modality differences warranting separate manuscripts.

PROGRESS REPORT

CHRISTINE CHIAVERINI, MD, PHD, CO-PRINCIPAL NVESTIGATOR
ROBERT BALLOTTI, PHD, PRINCIPAL INVESTIGATOR

"Molecular Mechanisms of Hypopigmentation in Cystinosis"

Date: 6/4/2008

Regulation of Tyrosinase expression by *CTNS*

CTNS silencing induces a dramatic decrease in tyrosinase protein expression, but does not affect the expression of tyrosinase messengers. We hypothesized that *CTNS* might control tyrosinase stability/degradation. Two pathways have been involved in tyrosinase degradation.

One involves the proteasome and the other one involves cysteine proteases. Using the specific proteasome inhibitor MG132, we showed (Fig. 1) that proteasome inhibition did not prevent the decrease in tyrosinase expression evoked by *CTNS* specific siRNA. As positive control of MG132 functioning, we have show an increase in HIF1a expression in these conditions (not shown). On the other hand, the inhibition of cysteine protease, by E64 prevented the degradation of tyrosinase.

***CTNS* controls the pH of melanosome.**

Using Lysotracker, we have previously shown that *CTNS* silencing led to an acidification of intracellular vesicles that remained to be identified. To do so, we changed our technical approach. We used the N-(3-((2,4-dinitrophenyl) amino)propyl)-N-(3-aminopropyl) methylamine, dihydrochloride (DAMP), a weak base that accumulates in acidic compartments. DAMP was then localized by anti-DNP (dinitrophenol) antibodies.

Melanosomes were identified by anti-Tyrp1 antibodies. In basal conditions, melanosomes (orange/red) appeared located around the nucleus and co-localized more or less with DAMP (green). After treatment with MSH, melanosomes were found mainly at the cell periphery and little co-localization with DAMP could be observed, indicating that during cAMP induced differentiation melanosomes became less acidic. Interstingly, when cells were transfected with siCTNS, most of the melanosomes appear yellow, indicating that *CTNS* silencing caused an acidification of the melanosomes.

Hypothesis. Scheme 1.

Taken together these data led us to propose the following hypothesis: Melanosomes derive from early endosomes. Four types of melanosomes can be identified by their morphology and their melanin contain. It seems that type II melanosomes are more acidic than type III which contain tyrosinase and synthesize melanins. *CTNS*, which seems to be preferentially located within type III melanosomes, might participates in the alkalinization of these melanosomes, favoring thereby melanin synthesis. In absence of *CTNS*, the pH of type III melanosomes remains acidic leading to the miss targeting of tyrosinase to lysosome and its subsequent degradation. Alternatively, the decrease in melanosome pH might led to the reactivation of lysosomal cysteine proteases such as cathepsins that work preferentially at acidic pH and that were found in melanososome.

***CTNS* controls growth of melanoma.**

We also investigated the effect of *CTNS* silencing on melanocyte growth. Using XTT method we observed a 50% decrease in cell growth after transfection with siCTNS (FIG 3.A). FACS analysis of DNA content showed that cells depleted in *CTNS* were blocked in G1 phase (FIG 3.B). No apoptosis (subG1) could be observed. Further western blot analysis with anti Phospho-pRB and anti-p27 antibodies showed that *CTNS* silencing decreases pRB phosphorylation and increased p27 expression (FIG 3.C), in total agreement with a G1 arrest. How *CTNS* can influence cell growth remain to be elucidated.

Note that *CTNS* silencing was verified in all the experiments by Q-PCR analysis. Si-CTNS induced 80 to 90% decrease in *CTNS* messengers.

Clinical evaluation

We started the creation of the database of patients with infantile cystinosis in order to better define the clinical characteristics of the cutaneous involvement. For that, various parameters will be collected for each patient and his (her) parents, using a standardized questionnaire. A complete dermatological examination (including phototype) will be carried out among all patients and their parents to report other skin symptoms.

- an evaluation of the color of the skin (3 measures for Each parameter) with a colorimeter in unexposed skin regionS (inner part of the arms) and in exposed regions (forehead). This apparatus allows the measurement of the 3 cutaneous parameters (clearness, redness and the blue and yellow color of the skin) representative of skin color.

- a quantitative analyze of eumelanin and pheomelanin levels by HPLC and spectrophotometry in hair. For that, we will cut 2 wicks of hair (50mg) in all patients and their parents.

We have just started patient inclusion. Two patients have been included. We will include 30 patients. Further, We took advantage of a small surgery that a patient affected by cystinosis had to undergo to take a 4mm2 biopsy of skin and isolate melanocytes. These cells will be very precious to confirm in patient's melanocytes the data that we will obtain in cell lines.

Figure Legends.

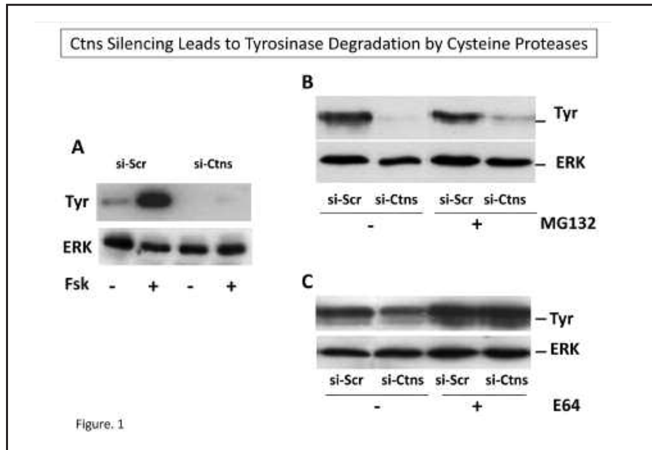


Figure 1. ***Effects of proteasome and cysteine protease inhibitors on tyrosinase expression.*** *(A) western blot analysis of B16 cells melanoma cells transfected with si-Ctns or Scrambled siRNA (Scr) and stimulated or not by FSK for 48 hours. Upper panel Tyrosinase (pep7 1/500), lower panel ERK (1/500) (loading control). (B and C) B16 cells melanoma were transfected by si-Ctns or Scrambled siRNA (Scr) and stimulated by FSK for 48 hours. Cells were incubated with 100 M MG132 (B) or 15 M E64 (C) during the last 24 hours. Then tyrosinase expression was analyzed by western blot as in A.*

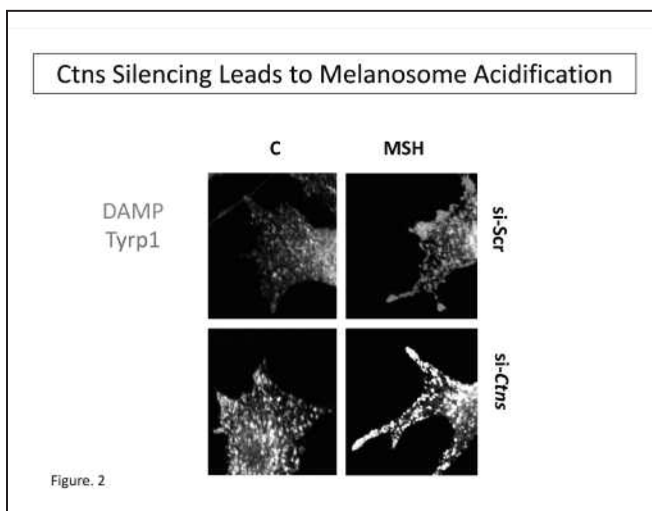
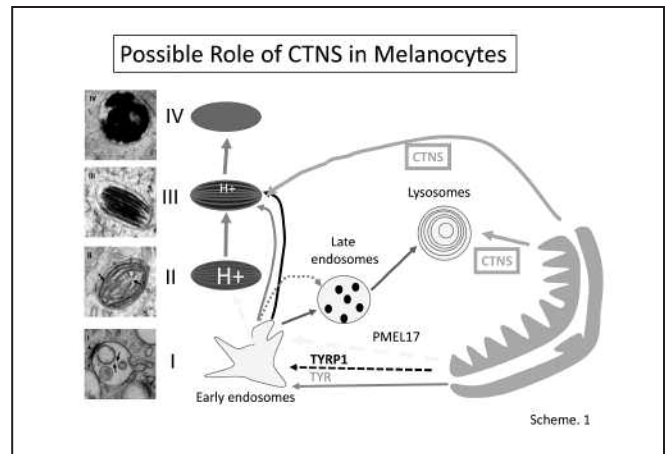


Figure 2. ***Effects of*** **Ctns** ***silencing on the pH of melanosomal.*** *B16 melanoma cells were transfected with si-*Ctns *or control (si-Scr), treated (MSH) or not by MSH, 0.1 M, for 48h and then incubated for 15 min with DAMP 100 M. Cells were fixed, permeabilized and labeled with anti-DNP (green) and anti-Tyrp1 (red) antibodies.*



Scheme 1. ***Possible Role of CTNS in Melanocytes.***

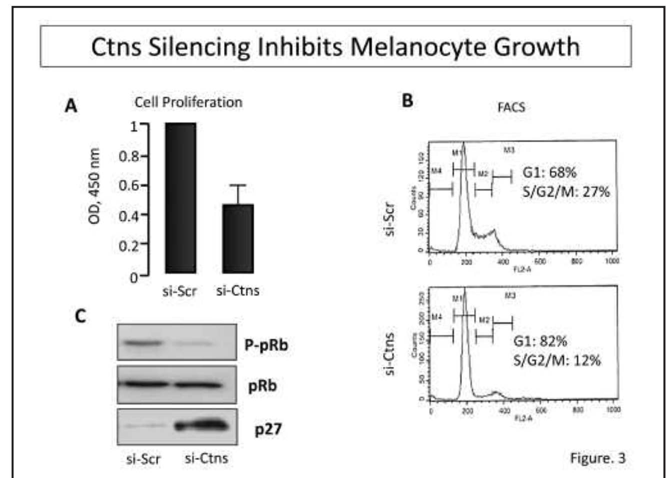


Figure 3. ***Effects of*** **Ctns** ***silencing on melanocyte growth.*** *(A). B16 melanoma cells were transfected with si-ctns or scrambled siRNA (si-Scr) and cell viability was assessed using the cell proliferation kit II (XTT; Roche Molecular Biochemicals) according to the manufacturer's protocol. (B).* Flow cytometry analysis. *B16 melanoma cells were transfected with si-ctns or scramble siRNA (si-Scr), detached in PBS/EDTA 1 mM and centrifugated for 5 minutes at 2000G at RT. Pellets were resupended in 200 L of citrate buffer pH 7.6 (sucrose 250mM, Tris-sodium citrate 40mM) and stained with propidium iodine at 4°C for 1 hour. Fluorescence was measured by using the FL2 channel. (C) B16 melanoma cells were transfected with si-ctns or scrambled siRNA (si-Scr). Solubilized proteins were analyzed by western blot using anti-pRB (total), anti phospho-pRB (807/811) and anti-p27 antibodies.*

PROGRESS REPORT

ALLISON EDDY, MD, PRINCIPAL INVESTIGATOR
DARYL OKAMURA, MD, CO-PRINCIPAL INVESTIGATOR

"Cysteamine Effects on Extracellular Matrix Accumulation in Chronic Kidney Disease"

Period: 7/1/08 – 12/31/08

The project is well underway. Breeding pairs of the two lines of genetically engineered mice that are required for the proposed studies (*Ctns-/-* and

vanin-/-) were obtained this summer and are now actively breeding in our vivarium. Nadia Bahrami, a 2008 graduate form the Bachelor of Science program in Molecular, Cellular and Developmental Biology at the University of Washington, was hired to work full-time on this project in September and she has made excellent progress with the experiments.

SPECIFIC AIM #1:

To perform preliminary studies to develop an optimal model and drug delivery strategy to investigate the effect of cysteamine in a mouse chronic kidney disease model.

a) Searching for a mouse model of cystinosis-associated nephropathy. Using PCR methods, the genotype of offspring the mice that were sent to us as *Ctns-/-* and *vanin-/-* were first verified (Figure 1). Both lines are now actively breeding in order to produce sufficient offspring to perform a study of the unilateral ureteral obstruction (UUO) model in male mice at 8 weeks of age, comparing fibrosis severity between the wild-type and knockout mice. At this time we currently have 17 *Ctns-/-* and 12 *vanin-/-* mice. The first litter of a *Ctns-/-* x *vanin-/-* mating was born on 12/16/08, as the initial step towards generating a colony of *Ctns-/- vanin-/-* double knock-out mice.

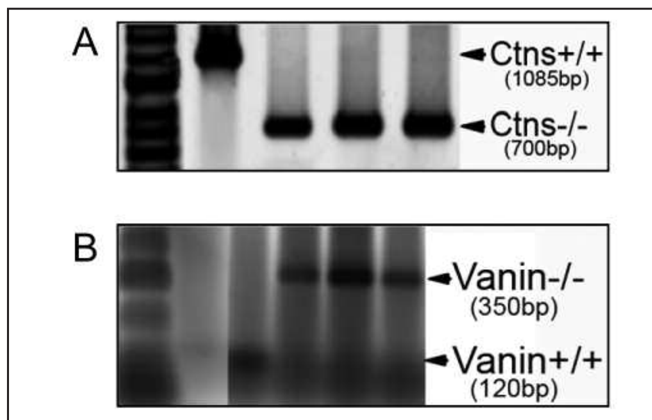


*Figure 1. PCR reaction product gels illustrate the wild-type (*Ctns+/+*) and mutant (*Ctns+-/-* cystinosin alleles (A) and the vanin wild-type and mutant alleles (B) for representative mice that are breeding in our facility.*

b) Optimizing the cysteamine treatment and protocol. The first pilot study (n= 4 mice/group) has recently been performed to determine the efficiency of cysteamine when administered orally in the drinking water (freshly made every 24h) in the 14-day UUO model in C57BL/6 wild-type mice. Our previous preliminary data was based on intraperitoneal administration once daily but given its short half-life, we believe that continuous oral exposure to cysteamine may be more effective. The tested doses were 200, 400 and 600 mg/kg/day. Initial results based on total kidney collagen levels indicated that fibrosis is reduced at the calculated dose by 15 percent and 11 percent, respectively (Figure 2). We are in the process of reconfirming these data in a larger group of mice. Kidney glutathione levels will also be measured using a glutathione (GSH) assay kit that measures both GSH and glutathione disulfide (BioVision, Mountain View, CA), as measure of cysteamine bioactivity, as cysteamine is known to promote glutathione synthesis from cysteine.

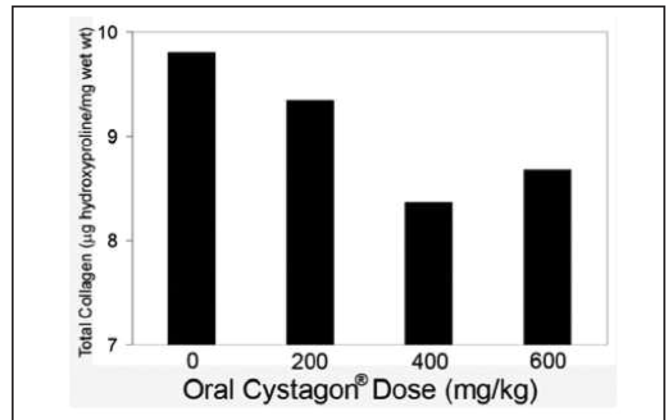


Figure 2. Total kidney collagen levels 14 days after unilateral ureteral obstruction. Results are from a pilot study (n – 4 mice/group) deigned to test the efficacy of oral Cystagon at the various estimated 24h doses shown. The drug was added to the drinking water.

SPECIFIC AIM #2:

To investigate the efficacy of cysteamine therapy for interstitial renal matrix protein reduction in chronic kidney disease and to determine its mechanism of anti-fibrotic action. As the male *Ctns-/-* and *vanin-/-* mice reach eight weeks of age (Aims #1a), the UUO studies will be performed as outlined in the original grant proposal. Once the optimal oral dose of cysteamine is established (Aim#1b), a UUO study will be performed as originally described. Outcome measures will be fibrosis severity, collagen synthesis rates, transglutaminase activity and lysosomal activity. In preparation for these studies, we have begun some in vitro experiments to investigate the ability of cysteamine to inhibit matrix protein synthesis by mouse tubular epithelial cells that are stimulated with the pro-fibrotic growth factor transforming growth factor beta 1 (TGF- 1). To date we have confirmed that MCT cells grown in DMEM plus 0.5%FCS develop a profibrotic phenotype with up-regulated procollagen I and fibronectin mRNA expression by 6.0 and 7.3 fold, respectively when exposed to TGF- 1 (Figure 3). A Cystagon dose trial study (50 to 250µM) is in progress to determine if cysteamine can inhibit extracellular matrix and thiol oxidation pathways in this model system.

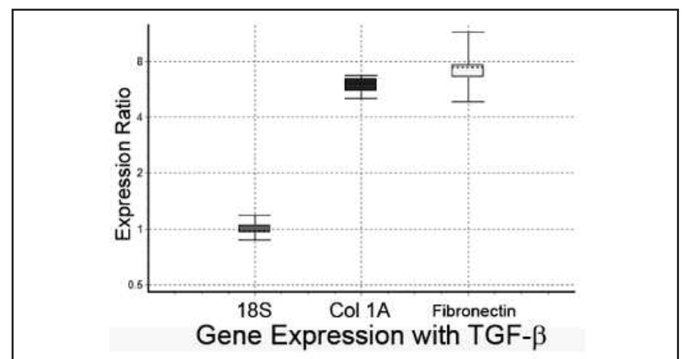


Figure 3. Development of an in vitro assay to test the ability of cysteamine to inhibit pro-fibrotic and pro-oxidant pathways. Shown in the graph, matrix protein mRNA levels in mouse proximal tubular cells exposed to transforming growth factor beta (5 ng/ml) for 48h are significantly increased. This assay will be used to investigate the ability of cysteamine to inhibit this and other renal fibrosis promoting molecular pathways. Col1A = procollagen 1(I) gene.

SPECIFIC AIM #3:

To investigate the effect of cysteamine on apoptosis of renal tubular epithelial cells, oxidant stress, and other novel target pathways of chronic kidney disease. These studies will begin together with the studies in the second aim once the *Ctns-/-* and *vanin-/-* mice are available and the optimal oral dose of cysteamine is established. As originally proposed, the effects on apoptosis, glutathione activity and novel cysteamine targets will be the primary focus of these studies.

In summary, the experimental models are now established to investigate the primary hypothesis that cysteamine reduces extracellular matrix accumulation in chronic kidney disease. Oral cysteamine appears to be protective is a small pilot study of chronic kidney disease. Additional studies are planned to be certain that a dose of 400mg/kg/day or 600 mg/kg/day is appropriate for large scale studies. We have not encountered any unanticipated problems and should be able to complete the proposed studies within the three year timeline.

PROGRESS REPORT

FRANCESCO EMMA, MD, PRINCIPAL INVESTIGATOR
ANNA TARANTA, PHD, CO-PRINCIPAL INVESTIGATOR
ELENA LEVTCHENKO, MD, PHD, CO-PRINCIPAL INVESTIGATOR

"Transcriptional and Post-Transcriptional Regulation of the CTNS gene"

Six-Month Progress Report: 4/1/08 – 10/1/08

The main goal of this project is to characterize a new cystinosin isoform (cystinosin-LKG) resulting from an alternative splicing of the exon 12 of the *CTNS* gene.

SPECIFIC AIM #1: Characterization of the cystinosin-LKG isoform

We have been successful in transfecting conditionally immortalized proximal tubular epithelial cells derived from patients with NC. However, efficient transfection could only be obtained in non differentiated cells grown at 33°C. For this reason, we are currently generating stably transfected cells in order to study the rescue effect of the 2 isoforms on the cell phenotype.

SPECIFIC AIM #2: Analysis of expression of the cystinosin isoforms

Expression studies of cystinosin isoforms have showed RT-PCR products spliced at the level of exons 3-5, which may indicate the existence of another cystinosin isoform. We are currently sequencing a 30 kD protein that was precipitated with different anti-cystinosin antibodies from human proximal tubular epithelial cells.

The localization of two known cystinosin isoforms has been analyzed under cystine, cysteine and methionine deprivation. We have observed under these conditions increased localization of the protein in the plasma membrane. These preliminary results need to be supported by quantitative analyses that are underway, and suggest that the localization of cystinosin-LKG is modulated by cystine content.

No differential expression of the 2 known isoform was observed, but tissue specific expression studies showed that the LKG protein is particularly expressed in the testis, where it accounts for 50% of the transcripts.

SPECIFIC AIM #3: Characterization of the cystinosin promoter

The *CTNS* promotor has been cloned into the luciferase pGL4 vector and tested under different conditions after transient transfection in HK2 cells. We have observed increased luciferase expression (+68±10%) upon cystine/methionine deprivation, indicating direct or indirect regulation of gene expression at the promoter level. Smaller regions of the promoter are currently under study, in the hypothesis that they contain a cystine sensitive sequence.

In addition, RNA stability experiments using actinomycin D have generated conflicting results suggesting that *CTNS* mRNA may be partially regulated by mRNA stabilization. In fact, our results indicate that the mRNA is markedly stabilized after 48 hour of incubation in cystine-free medium, but failed to show de-stabilization when these same cells were incubated back in a medium containing cystine.

PUBLICATIONS:

Taranta A, Petrini S, Palma A, Mannucci L, Wilmer MJ, De Luca V, Diomedi Camassei F, Corallini S, Bellomo F, van den Heuvel LP, Levtchenko EN, Emma F. Identification and sub-cellular localization of a new cystinosin isoform. Am J Physiol Renal Physiol. 2008 [epub ahead of print]

PROGRESS REPORT

BRUNO GASNIER, PHD, PRINCIPAL INVESTIGATOR

"Identification and Characterization of the Lysosomal Transporter Involved in Cysteamine-Mediated Cysteine Efflux"

Period: 1/10/09

Background and objectives

Biochemical studies performed in the 80s have implicated an unidentified lysosomal arginine/lysine transporter, named 'system c', in the cystine-depleting action of cysteamine. Identification of this lysosomal transporter would help improving current cysteamine treatments. In the first grant period, several members of the SLC7 transporter family were confirmed as potential system c candidates based on immunofluorescence data and, for functionally characterized members, on cysteamine-cysteine competition experiments. However, it is still unknown whether these transporters are active within the lysosomal membrane and whether they are involved in lysosomal egress of the cysteamine-cysteine conjugate formed in cysteamine-treated cystinotic lysosomes.

We thus tried to develop an in situ cystine depletion assay in whole cells to address these two questions. Our aim was to load artificially the lysosomal pool with labeled cystine using an ester precursor and subsequently to deplete this pool with cysteamine. Such an assay would then allow testing whether overexpressed system c candidates accelerate the cystine-depleting action of cysteamine.

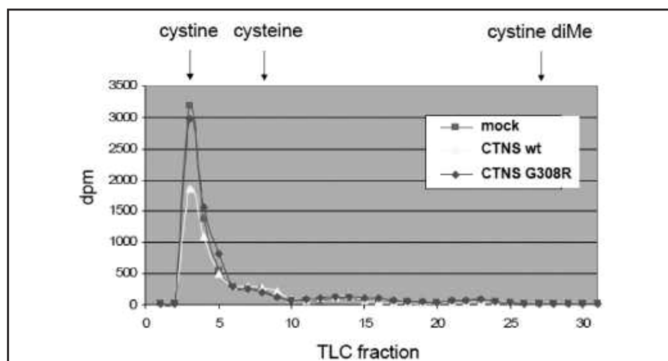
Development of an *in situ* cystine depletion assay in whole cells

A custom synthesis of [3H]L-cystine dimethyl ester (3 Ci/mmol) was purchased from Hartmann Analytic (http://www.hartmann-analytic.de/). Labeling with 3H, rather than 35S (half-life: 87.4 days), was chosen to avoid repeating costly custom syntheses. The [3H]cystine precursor was used to overload artificially lysosomes with [3H]cystine in readily transfectable cell

lines (HEK293, HeLa, COS-7). It was also used at lower concentration to label the lysosomal cystine pool of human *CTNS-/-* fibroblasts. Typically, cultured cells were incubated for several hours with the di-ester at 37°C followed by a brief (30 min - 1 h) chase period in the absence of radioactivity. Cysteamine treatment was performed during or after the chase period.

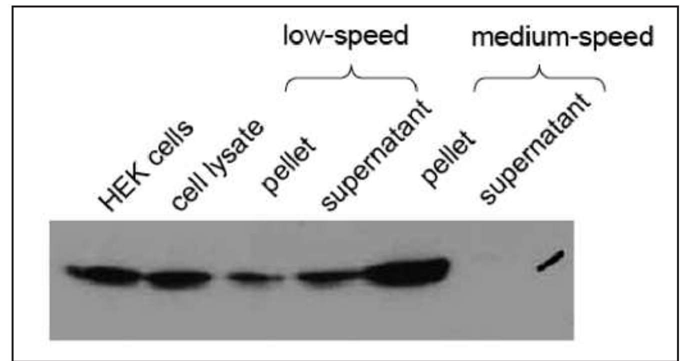
Analysis of the soluble cell content by thin-layer chromatography revealed that most of the accumulated radioactivity was recovered as free cystine, with no detectable remaining dimethyl ester (cells were post-treated with N-ethylmaleimide to prevent oxidation of cysteine to cystine during analysis). This data is consistent with diffusion of the [3H]cystine dimethyl ester into the lysosomal compartment and subsequent cleavage of the ester bond by lysosomal hydrolases. In agreement with this conclusion, we also found that neutralization of the lysosomal compartment by treating cells with chloroquine prior and during the loading step reduced the amount of accumulated radioactivity by 30 to 50% (a residual acidity may account for this partial effect).

However, subsequent cysteamine treatments (0.1 to 10 mM) yielded frustrating data because the radioactivity depletion effect was limited and partially masked by a high and variable level of spontaneous efflux. The origin of this spontaneous radioactivity efflux is unclear. The fact that overexpression of recombinant cystinosin decreases the steady state level of [3H]cystine in dimethyl ester-treated cells (Figure below) suggests that lysosomal export of [3H]cystine through endogenous cystinosin is limited in non-transfected cells.



Effect of overexpressed cystinosin on the radioactivity chromatographic profile of HEK293 cells treated with [3H]cystine dimethylester. Expression of wild-type cystinosin, but not of a non-active mutant, reduces the steady state level of [3H]cystine.

Despite intensive efforts, we were not able to find [3H]cystine dimethylester pulse-chase conditions which maximized the cysteamine-induced depletion effect in a reproducible manner. We alternatively tried to isolate the lysosomal [3H]cystine signal from 'nonselective' cytosolic radioactivity by perforating ester-loaded cells with digitonin. This compound is known to perforate selectively the plasma membrane. Surprisingly, this treatment abolished almost all radioactivity from the cells. Subsequent microscopy experiments after labeling cells with the fluorescent probe Lystracker revealed that this effect was due, in fact, to a concomittant disruption of lysosomes by digitonin, presumably because lysosomal membrane is also rich in cholesterol. To overcome these difficulties, experiments are now in progress to perform similar studies on isolated lysosomal fractions instead of whole cells in order to reduce the complexity of the cystine depletion assay. The figure below illustrates the enrichment of a lysosomal fraction from a large amount HEK293 cells.



Enrichment of cathepsin D during purification of a lysosomal fraction from 120 x 106 HEK293 cells.

Progress Report Mainz group (Ellen Closs and Jean-Paul Boissel)

Our focus in the second grant period lay on the generation and characterization of chimeras between the lysosomal, putative "system c" protein SLC7A14 and bonified members of the family of plasma membrane transporters for cationic amino acids (hCATs). Our previous attempts to direct SLC7A14 (a lysosomal localized orphan member of the SLC7 family of amino acid transporters) to the plasma membrane were without success (see previous report). We thus decided to make chimeras between SLC7A14 and hCAT-2A or hCAT-1 to address the following points:

- Investigate the transport activity of these chimeras
- Identify protein regions responsible for lysosomal localization
- Characterize transport properties of active chimeras

As system c has been discovered in lysosomes from skin fibroblasts, we also investigated the expression of SLC7A14 in these cells

Chimeras made and their transport activity

In our previous work, we used human cell lines to express SLC7A14. However, because the generation of stable cell lines is very time consuming, we decided to move to *Xenopus laevis* oocyte expression, previously used to characterize the hCAT proteins.

Chimeras between SLC7A14 and hCAT-2A

The so-called "functional domain" of SLC7A14 corresponds to a protein stretch of 81 amino acids that determines the transport activity of the hCAT proteins: chimeras between hCAT-1 and hCAT-2A (the CAT members with the most divergent transport properties) exhibit the transport properties of the respective donor of this functional domain (Habermeier et al., 2003). In contrast, exchange of this domain between hCAT-2A and SLC7A4 (a CAT-related plasma membrane protein without transport function) results in non-functional chimeras (Wolf et al., 2002). To prove if SLCA14 represents an amino acid transporter, we thus introduced the corresponding domain of SLC7A14 into the hCAT-2 backbone. This chimera (hCAT-2/SLC7A14-BK, see scheme in Figure 1) exhibited transport activity for the cationic amino acid L-arginine (see below). To create chimeras with a larger portion of SLC7A14, we exchanged either the N- or the C-terminus of hCAT-2 in this chimera for the corresponding terminus of SLC7A14, respectively. However, these chimeras had no transport function. Western blot analysis using an antibody against the C-terminus of hCAT-2A revealed that hCAT-2/SLC7A14-BK, both, in the glycosylated and deglycosylated form, migrated at the same speed as hCAT-2A in the SDS/PAGE, suggesting proper processing of the chimera. In contrast, the

chimera with the SLC7A14 N-terminus (SLC7A14-K-hCAT-2) was degraded. This suggests that the overall architecture of this protein was disturbed leading to misfolding and degradation. Expression of the chimera with the SCLC7A14 C-terminus could not be tested, because of the lack of an appropriate antibody.

Chimera between SLC7A14 and hCAT-1

Although the transport properties of hCAT-1 and hCAT-2A can be exchanged by exchanging the functional domains, this domain is not sufficient to mediate amino acid transport as demonstrated by the non-functional SLC7A4/hCAT-2A-BK chimera, carrying the functional domain of hCAT-2A in the SLC7A4 backbone. We were thus wondering if a chimera with the hCAT-1 backbone and the functional domain of SLC7A14 had transport activity and exhibited the same transport properties as the chimera with the hCAT-2 backbone. However, the hCAT-1/SLC7A14-BK chimera had no transport activity. Western blot analysis using an antibody against the C-terminus of hCAT-1 showed that the chimeric protein migrated very slowly in the SDS/PAGE and that the migration was not changed upon treatment of lysates with N-glycosidase. Only a very small portion of the protein migrated at the same speed as partly glycosylated hCAT-1 and was deglycosylated upon treatment with N-glycosidase. This suggests that, when fully glycosylated, the chimera may form aggregates that do not dissociate even under the denaturing Western blot conditions.

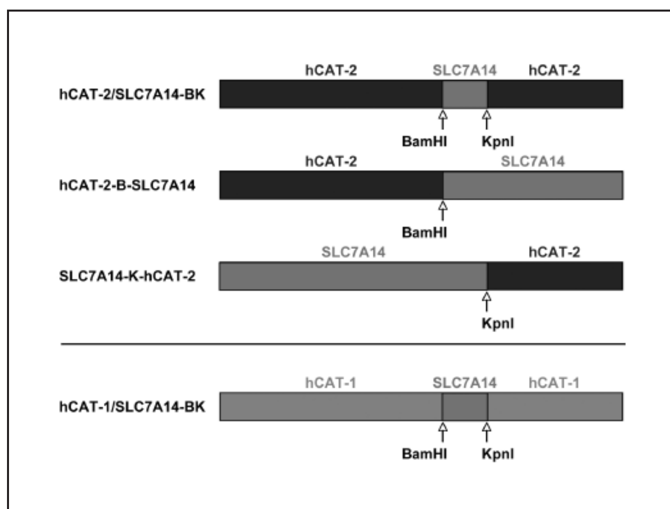


Figure 1: ***Scheme of the chimeras between either hCAT-2 or hCAT-1 and SLC7A14.*** *The scheme shows which protein fragments were exchanged between hCAT-2 or hCAT-1 and SLC7A14 and gives the names of the chimeras. First, all BamHI and KpnI sites were removed from SLC7A14 by silent mutations. Then, a BamHI and a KpnI sites were introduced into SLC7A14 at the respective position corresponding to the unique BamHI and KpnI sites of hCAT-2. This cDNA was used to exchange fragments with hCAT-2 or hCAT-1.*

Protein regions responsible for lysosomal localization

The hCAT-2/SLC7A14-BK chimera was the only chimera that mediated transport of cationic amino acids across the plasma membrane of oocytes. From this we conclude, that the protein regions confering lysosomal localization of SLC7A14 are located outside the functional domain. Introduction of smaller SLC7A14 fragments into this chimera will be necessary to narrow down the protein regions responsible for lysosomal localization and to find out, which protein regions interact with each other to give a functional transporter.

Characterization of the transport properties of the hCAT-2A/SLC7A14-BK chimera

The hCAT-2A/SLC7A14-BK chimera exhibited transport activity, which was similar, but not identical to the transport activity of CAT-2A (the low affinity splice variant of the CAT-2 gene). Noticeably, arginine transport by the chimera was pH-dependent, trans-stimulated and inhibited by epsilon-three-methyl-L-lysine, properties assigned to the lysosomal transport system c in human skin fibroblasts.

Expression of SLC7A14 in human skin fibroblasts

Expression of SLC7A14 was investigated in total RNA extracted from four different human skin fibroblast cell lines, two from cystinotic and two from healthy subjects. The RNA samples were provided by the lab of Bruno Gasnier. Quantitative RT/PCR showed strong expression of SLC7A14 mRNA in all these cells, further supporting the notion that SLC7A14 may indeed correspond to lysosomal system c. Noticeably, the expression was stronger in the cystinotic cells. However, more samples need to be examined to elucidate if SLC7A14 is really upregulated in cystinotic cells.

Research focus for the next 6 month:

New chimeras between hCAT-2A and SLC7A14

We will introduce small SLC7A14 fragments into the hCAT-2/SLC7A14-BK chimera to narrow down the protein regions responsible for lysosomal localization and to find out, which protein regions interact with each other to give a functional transporter.

Measurements of lysosomal transport

Although the chimeras give a first hint that SLC7A14 may function as lysosomal transporter for cationic amino acids, our studies show that it may be difficult to drag larger portions of the transporter to the plasma membrane for further characterization. We will therefore focus our work in the next six months on the establishment of a protocol for lysosome preparation (from cells with stable overexpression of SLC7A14) and subsequent transport assays. This will enable us to study the function of the protein in is native environment.

References

Habermeier, A., Wolf, S., Martine, U., Graf, P., and Closs, E.I. (2003). Two amino acid residues determine the low substrate affinity of human cationic amino acid transporter-2A. J Biol Chem 278, 19492-19499. Wolf, S., Janzen, A., Vekony, N., Martine, U., Strand, D., and Closs, E.I. (2002). Expression of solute carrier 7A4 (SLC7A4) in the plasma membrane is not sufficient to mediate amino acid transport activity. Biochem J 364, 767-775.

PROGRESS REPORT

TAOSHENG HUANG, MD, PHD, MENTOR
SHA TANG, PHD, POST-DOCTORAL FELLOW

"Molecular and Pathogenesis Study of Cystinosis"

Period: 7/7/07 – 12/7/07

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/disulfites, glutathione (GSH), thioredoxin and cysteine, are coupled. Cysteine is also one of the precursors for glutathione synthesis. 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.

STRATEGY:

To study the redox status in cystinotic cells, we measured GSH and GSSG levels. To investigate the apoptosis rate and cell cycle properties, we performed APO-BrdUTM TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) Assay (Invitrogen) and generated cell growth curve for cystinotic fibroblasts versus normal cells. Total intracellular ATP concentrations were also determined to analyze mitochondrial functions in cystinotic cells. In addition, expression arrays were utilized to identify differentially expressed genes in cystinotic cells.

PRELIMINARY DATA:

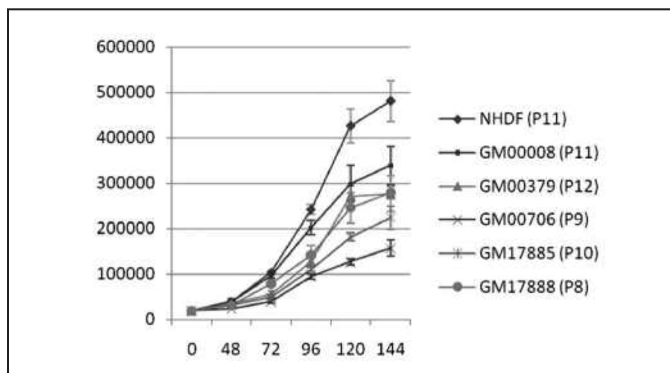
Cell cultures and intracellular cystine levels:

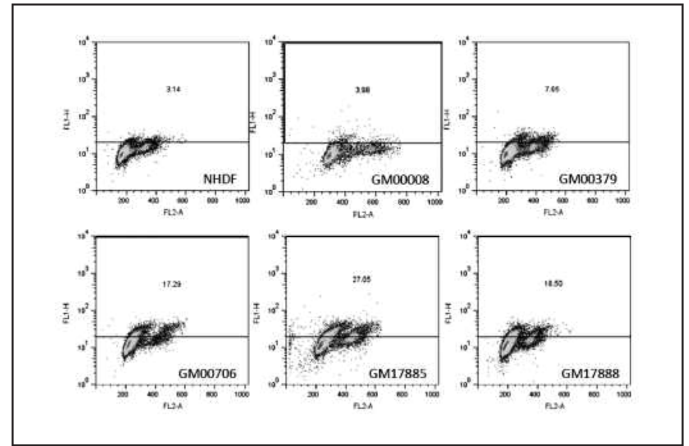
Cultures of normal and cystinotic human fibroblasts were obtained from the Coriell Cell Repositories and are maintained in the laboratory. The cystinotic lines represent different genotypes and total intracellular cystine levels of these fibroblasts were determined in Dr. Schneider's laboratory at UCSD.

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

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 (%)	UV only	CySH+UV
NHDF	3.14	53.79	36.56	7.51	8.74%	23.69%
GM00008	3.98	47.16	41.84	8.11	2.78%	4.25%
GM00379	7.05	55.87	33.21	6.28	9.80%	10.74%
GM00706	17.29	62.83	33.21	3.39	N/A	N/A
GM17885	27.05	48.21	44.52	5.22	34.41%	33.75%
GM17888	18.50	49.92	41.71	5.64	45.95%	16.06%

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.

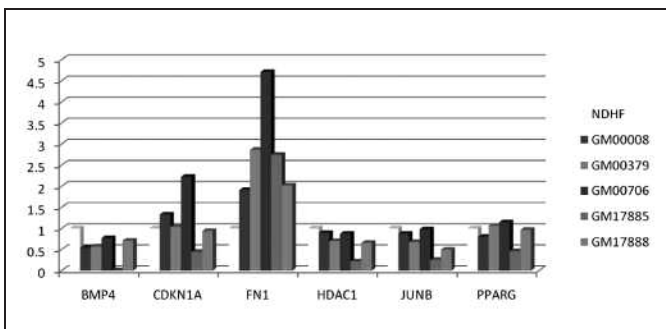
Gene expression analysis:

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.

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.

Summary and Future Directions:

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. However, the differences in GSH or ATP contents between cystinotic and normal cells were not definite; this further demonstrated the complexity of the disease and could partially explain the inconsistencies of similar data in previous reports. Meanwhile, 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.

We are now analyzing the expression data in more details. We will obtain more control cell lines in the near future and will use quantitative RT-PCR to validate the differentially expressed genes. Apoptosis rate, GSH and ATP levels of additional control cell lines will also be investigated to further understand the differences in redox status and energy metabolism of cystinotic versus normal cells. Based on our preliminary data, mitochondrial malfunction could be a major factor in cystinosis. We will analyze mitochondrial respiration rate and complex I-IV activities to see if mitochondrial respiratory chain activities are compromised in cystinotic cells. ROS production will also be studied by measuring either aconitase activity or MitoSox fluorescence. We expect to see elevated ROS levels in cystinotic cells, since ROS can damage the mitochondria, reduce ATP production and initiate apoptosis. In addition, the effects of antiapoptotic reagents (broad-spectrum caspase inhibitors) on cystinotic fibroblasts will be investigated to see if they can reverse the increased apoptotic rates in cystinotic cells we observed.

PROGRESS REPORT

TAOSHENG HUANG, MD, PHD, MENTOR
SHA TANG, PHD, POST-DOCTORAL FELLOW

"Molecular and Pathogenesis Study of Cystinosis"

Period: 1/10/08– 12/10/08

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 synthesis. 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.

PRELIMINARY DATA FOR THE FIRST SIX MONTHS (Jul. 07 – Dec. 07):

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.

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 felt that the cell lines included in the previous experiments were not optimal for our purpose. 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 submitted to Genetic Testing and is in review right now. 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 *dOpa1* 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 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 *dOpa1* 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 Figure 1, treatment of cysteamine (1 mM) did not result in significant improvements in either the glossy or rough eye phenotypes.

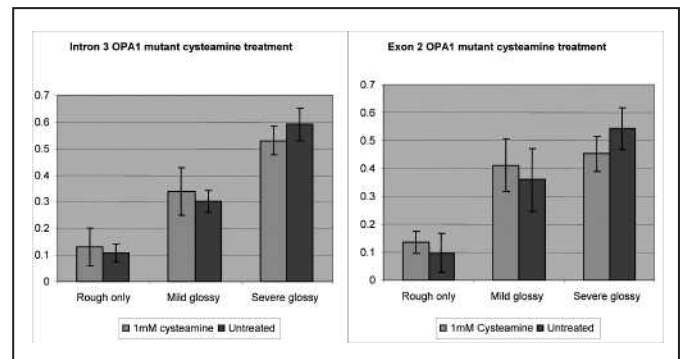


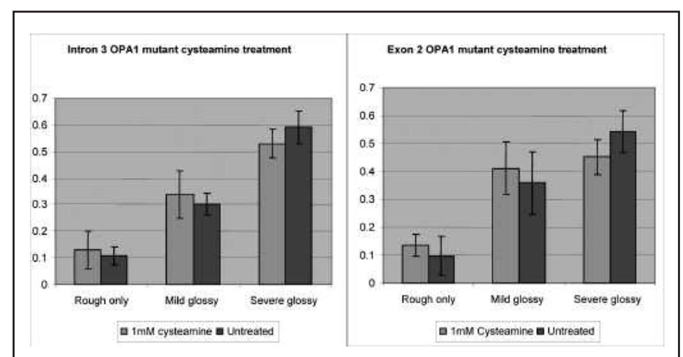
Figure 1. OPA1 mutants with cysteamine treatment. Neither mutant exhibits statistically significant variation from control in any of the phenotype categories.

PROGRESS REPORT

VASILIKI KALATZIS, PHD, MENTOR
CLAIRE HIPPERT, PRE-DOCTORAL RESEARCH FELLOW

"Gene Transfer Studies for Cystinosis"

Report 07/08/08 – 18 month progress



CRF 18-mo progress report

INITIAL SPECIFIC AIMS

I) Validate preliminary in vitro gene transfer studies on primary murine hepatocytes by in vivo gene transfer to the liver.

II) Generate viral vectors (helper-dependent canine adenovirus serotype 2 and adeno-associated virus serotype 8) expressing *CTNS* and perform eye-targeted gene transfer studies to correct the corneal anomalies of cystinosis.

III) Finish characterising the CNS anomalies in *Ctns-/-* mice, and begin CNS-targeted gene transfer to correct these anomalies.

ABBREVIATIONS

CAV-2	canine adenovirus serotype 2
HD CAV-2	helper-dependent canine adenovirus vector (devoid of all viral genes)
AAV8	adeno-associated virus serotype 8
E1	early 1 region of the adenoviral genome that encodes trans-activating factors
E3	early 3 region of the adenoviral genome that encodes immune-modulating factors
GFP	green fluorescent protein
Ctns-/-	homozygous deletion of the mouse *Ctns* gene
CAVGFP	canine adenovirus vector expressing the gene *GFP*
AdCTNS	human adenovirus vector expressing the gene *CTNS*
AdCTNSGFP	human adenovirus vector expressing the gene *CTNS* fused to the gene GFP
AdGFP	human adenovirus vector expressing the gene *GFP*
IRES	internal ribosomal entry site
CTNS-IRES-GFP	expression cassette containing *CTNS* and *GFP* separated by an IRES sequence
CAV-CTNS-IRES-GFP	canine adenovirus vector containing the CTNS-IRES-GFP expression cassette
AAV-CTNS-IRES-GFP	adeno-associated virus vector containing the CTNS-IRES-GFP expression cassette
AAV-GFP	adeno-associated virus vector containing the gene *GFP*

I) *IN VIVO* GENE TRANSFER STUDIES

Background:

As detailed in our 12-mo update, we showed for the first time that adenoviral-mediated *CTNS* gene transfer to the liver of *Ctns-/-* mice is feasible for reducing lysosomal cystine levels. Moreover, both our short-term and long-term studies suggested that the efficiency of cystine reduction was higher in younger mice. Over the last 6 months, we performed some additional experiments to complement this work prior to submitting the corresponding article for publication. These results are summarised below:

Results:

Detection of cystine crystals: We examined the role of cystine crystals, which form at elevated cystine concentrations, and asked whether the presence of crystals in older mice could interfere with cystine reduction. However, a transmission electron microscopy study showed that i) crystals were already present in young mice and that ii) at both ages crystals were only detected in a small number of hepatocytes in contrast to the predominant number of crystal-containing Kupffer cells. Thus it is unlikely that the age-dependent efficiency of cystine reduction was due exclusively to crystals.

Transgene expression: Although we detected GFP expression by epifluorescence studies in AdGFP-transduced mice 1-mo post-injection, we could not detect cystinosin-GFP expression from mice injected with AdCTNSGFP. Thus we also screened histological sections by immunohistochemistry (IHC) studies using an anti-GFP antibody. Although we found a strong GFP expression from AdGFP 1-mo-post-injection, we could not detect the cystinosin-GFP fusion protein. In contrast, we detected a strong cystinosin-GFP expression in mice 1-wk post-injection. More encouragingly, IHC studies with an anti-cystinosin antibody showed a persistent cystinosin expression in hepatocytes 1-mo post-transduction with AdCTNS. Taken together, these results suggest that GFP-tagged cystinosin is degraded more rapidly or is more immunogenic and should be avoided for viral-vector contructs.

Manuscript: The ensemble of this work represents the culmination of a project begun in 2003. Our manuscript detailing the first in vitro and *in vivo* viral vector-mediated gene transfer studies for cystinosis was published June 24th 2008 in "Molecular Therapy".

IIA) GENERATION OF CLINICALLY RELEVANT VECTORS (HD CAV-2 AND AAV-8) EXPRESSING *CTNS*

Background:

At the time that we submitted our initial proposal, we were optimising transfection conditions of canine cells to begin HD CAV-2 vector production (state-of-the-art CAV-2 vector devoid of all viral genes). This has been extremely laborious because i) canine cells are difficult to transfect in general and ii) transfection efficiency is further lowered by the use of a large (30 kb) plasmid. Project advancement was mainly hindered by the lack of technical help.

Results:

Since we hired Sandy Ibanes, a research assistant, in December 2007, HD CAV-2 vector production has advanced significantly. We optimised transfection efficiency with a control GFP-expressing HD CAV-2 plasmid to 5-10% and upscaled the transfection protocol to obtain 1.76 x 106 transfected cells. Following fluorescence-activated cell sorting (FACS) of the GFP-positive cells, cracking of the cells to liberate the vector, and re-incubation with a fresh cell monolayer, we obtained an increase in the number of GFP-positive cells with each successive amplification step. After the 6th amplification step, we produced and purified the control GFP-expressing HD CAV-2 vector. This has been the first time in two years that we have succeeded in producing a HD CAV-2 vector. Thus, following these encouraging results, we have now begun HD CAV-CTNS-IRES-GFP production. We transfected cells with the HD CAV-CTNS-IRES-GFP plasmid and obtained 2.8 x 106 GFP positive cells. We are currently at the 3rd amplification step and have obtained an increase in GFP-positive cells following FACS at each step. We should be able to finish production within the next month and we will then test if the vector is functional.

With regards to AAV-8 vector production, we received a first stock of AAV-CTNS-IRES-GFP and AAV-GFP (vehicle control) from the Vector Production Platform at the Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain) in July 2007. Unfortunately, due to a problem during the vector purification steps, we were provided with small amounts of each vector

at a low titre. Thus it was impossible to use these vectors for *in vivo* experiments. We requested a second batch that arrived in December 2007. Although, we received an appropriate volume of the vectors at a reasonable titre, the stocks arrived thawed due to a delay in the shipping process. Thus, although we began testing these vectors in vivo, see section IIb below, we are prudent as to the interpretation of the results as we do not know if the thawing affected vector viability. To avoid the risk of another shipping accident, Claire Hippert went to Barcelona in June 2008 and brought back a third (frozen) stock of AAV-CTNS-IRES-GFP, which we just began testing *in vivo.*

IIB) *IN VIVO* CORNEAL-TARGETED GENE TRANSFER STUDIES

Background:

Our gene transfer studies to the liver provided the proof-in-principal that viral vector-mediated gene transfer could reduce lysosomal cystine levels in vivo. The next step was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis. Although E1-E3-deleted adenovirus vectors were suitable for the proof-of-concept, for the corneal gene transfer studies we plan to use more stable, less immunogenic viral vectors (HD-CAV and AAV) to be as clinically relevant as possible.

Results:

We previously showed that we are able to transduce stromal keratocytes with an E1-E3-deleted CAVGFP vector ex vivo in human, microcebe and dog cornea, and in vivo in mouse cornea. These studies were performed by injection of CAVGFP directly into the corneal stroma. We have continued to optimise this strategy *in vivo*. In addition to intracorneal injection, we are also evaluating the possibility of reaching the cornea by intravitreal or intracamaral injections. We are trying to find the most clinically relevant way to reach the stroma whilst achieving the longest transgene expression and generating the least lesions. We have obtained a strong GFP expression in the cornea with CAVGFP via all three routes from 24 h. Intravitreal and intracamaral injection seems to have resulted in the transduction of the corneal endothelium in addition to the stroma, however we need to verify these results by epifluorescence studies on histological sections. We also tested our second AAV-GFP stock in vivo. We did not obtain GFP expression following intrastromal or intravitreal injection but we believe this was due to a technical problem. In contrast, intracamaral injection of AAV-GFP resulted in GFP expression in the cornea, which appeared at 72 h (the lag time is likely due to the time necessary for the single stranded AAV genome to convert to double-stranded). GFP expression was less intense from the AAV-GFP vector and disappeared relatively quickly. We believe this could be due to a problem with the viability of the AAV-GFP stock that had arrived thawed (see section IIa above). We have just begun to test our third AAV stock and do not yet have the results. We will begin tests with the HD CAV2 vector once production is finished. In the meantime, we are continuing the optimisation steps with CAVGFP and we are studying titre, duration of transgene expression (by histological studies and quantitative PCR), tissue specific expression and resulting inflammations/lesions. In this way, we will determine the optimal route of administration to obtain the longest duration of expression. Lastly, it should also be mentioned that we work together with eye surgeons who specialise in both the cornea and the retina, in order to be sure that our studies remain as clinically relevant as possible.

III) REFINE CHARACTERISATION OF THE CNS ANOMALIES IN *CTNS-/-* MICE

Background:

Our recent work suggested that the age-related progressive accumulation of cystine in the brain of *Ctns*-/- mice results in generalized deterioration of memory abilities in middle-aged animals, which are reminiscent of those in patients. These data suggested that the *Ctns*-/- animal model could be exploited to further investigate the evolution of the relatively poorly understood cystinosis-associated CNS anomalies. Having identified the brain regions affected, the next step is to identify the cell type.

Results:

We first set out to isolate individual cell types from the brain to assay their respective cystine levels. As our previous work evoked a hippocampal defect primarily, we are first concentrating on this structure. The procedure we are currently using involves labelling individual cell types with fluorescent-labelled cell markers and isolating these cells via FACS. To discriminate between cell types, we have selected antibodies directed to specific cell surface markers: NGF receptor for neurons; the glutamate transporter EAAT1 for astrocytes; myelin oligodendrocyte glycoprotein for oligodendrocytes, and CD11b for microglial cells. For our pilot experiments, we have concentrated on the isolation of microglial cells as we have prior experience with the anti-CD11b antibody.

We isolated the hippocampi from an 8 mo-old mouse and enzymatically dissociated the tissue by incubating with the digestive enzyme papain. We then mechanically dissociated the tissue by trituration with a 1-ml pipette and collected the cells after passage through a cell strainer. We labelled the cells with the fluorescent anti-CD11b antibody and sorted by FACS. We were able to see various populations in our cell suspension with a clearly separate, labelled population corresponding to the microglial cells. Following this encouraging and exciting data, we will now repeat the experiment with all four markers and determine whether we are able to clearly differentiate the other three populations.

We will then determine the number of cells we can isolate from one hippocampus to know whether we need to upscale in order to have enough cells for the cystine assay. Once the optimisation steps are finished, our plan is to isolate individual cell types from different-aged mice, sort the cells by FACS, and assay their respective cystine levels. We hope this will give us direct information about which cell type is most affected in the cystinotic brain.

PROGRESS REPORT

ERIC K. MOSES, PHD, MENTOR
KATY FREED, PHD, RESEARCH FELLOW

"Complex Genetic Approaches to Monogenic Disease: Genomic and Transcriptomic Dissection of Normal Expression of CTNS, the Gene Involved in Nephropathic Cystinosis"

Report 5/19/08

Overview

While rare human genetic diseases like cystinosis are caused by mutations in a single gene there is a growing realization that genes rarely work alone

but rather are posited within complex global regulatory networks in which they may potentially interact with many other genes. This realization underpinned the recently completed first phase of our cystinosis research program in which we set out to study the genetics of the *CTNS* gene in a large sample of unaffected families, employing normal human variation as a model for pathological human variation.

Using a genome-wide scanning strategy we identified the *VPS13A* gene on chromosome 9 to be a plausible positional and functional candidate for a trans-acting regulator of *CTNS* expression. In this current fellowship project our aim was to exhaustively enumerate all genetic variation in the *VPS13A* gene to confirm and ultimately identify those variants most likely to be functionally involved in the trans-regulation of CTNS expression.

Another gene of interest is *STUB1*. In many biological systems redundancy has been observed in gene function. This has been observed for the yeast homologue (ERS1) of human *CTNS*, with the recent identification of the functionally related *MEH1* gene. Using a combinatorial strategy involving database sequence interrogation and genetically correlated expression data from our transcriptome dataset we have identified *STUB1* to be a plausible candidate for the human homologue of yeast *MEH1*. This raises the possibility that Stub1 may work in parallel with *Ctns* having an overlapping or complementary function

Progress to date

One of the major aims of the proposal was to re-sequence the genes STUB1 and VPS13A to identify all known SNPs (single nucleotide polymorphisms) in our Mexican-American population. All of STUB1 has been re-sequenced, including 1.5 kb of the 5' promoter region. 32 novel SNPs (i.e. those not found in the public databases) and 4 known SNPs were identified in our population. To date, 1.5 kb of the 5' promoter region of VPS13A has been re-sequenced. The next phase of the project was to analyze all (known and novel) SNPs in STUB1 and VPS13A in 1,240 individuals derived from the Mexican-American population.

We used the BeadArray platform (Illumina inc. San Diego, California) to genotype our DNA samples. This technique utilize targeted regions of DNA that are immobilized on beads and the SNPs are visualized through fluorescent tags, which differentiate among alleles. Initial analysis is carried out using the BeadStudio software followed by association analysis.

1,240 DNA samples from our Mexican American population were genotyped. 250 ng of template DNA was plated into 15x 96-well plates. Initially, a pool of primers was generated, containing 278 VPS13A SNPs and 25 STUB1 SNPs. The BeadXpress method is a 2-day procedure, with pre-PCR carried out on Day1 and post-PCR with SNP detection on Day 2. On each day, 2 plates were processed. After the SNPs had been visualized on Day 2 using the BeadXpress Reader, the data was transferred to the Illumina BeadStudio software to generate the genotypes for each individual.

The genotyping results for STUB1 are presented in Table 1. For the 25 STUB1 SNPs that were analyzed, none showed statistically significant cis effects on STUB1 mRNA levels (Table 1) nor any trans effects on *CTNS* mRNA levels (data not shown).

SNP name	P value for cis effect on STUB1
g_300c	0.011236
t2089c	0.012545
a_1892g	0.037012
11558085	0.063346
g1887a	0.076892
11642472	0.101045
10221050	0.161486
a_2224g	0.190639
c463t	0.202725
c309t	0.243782
g1292a	0.264715
c_2163t	0.325669
6597	0.331948
g2167c	0.342289
1060434	0.365002
28648987	0.399849
a_1410g	0.403282
g_2012a	0.42346
c_2555t	0.478511
11861355	0.527692
c_1369t	0.568837
g_1625a	0.608819
c399t	0.795027
11558083	0.807015
t1528c	0.865706

Table 1: Association analysis results for STUB1 SNPs in 1,240 Mexican-American individuals

Out of the 278 SNPs that were analyzed for *VPS13A*, 45 showed statistically significant cis effects on *VPS13A* mRNA levels (Table 2). That is, there are certain regions of DNA within the *VPS13A* gene that affect the levels of *VPS13A* mRNA.

SNP name	P value for cis effect on VPS13A
1048743	3.33E-18
1054368	2E-07
12684856	2.5E-07
4744830	1.08E-06
10781432	5.33E-06
9186	7.56E-06
2025846	9.38E-06
7034531	1.22E-05
10781423	3.05E-05
12686766	4.82E-05
2150903	6.04E-05
4744831	6.29E-05
7043232	7.63E-05
2150904	9.35E-05
9696759	9.83E-05
13286867	0.000114
7864334	0.000143
869241	0.000148
2153461	0.000158
10781429	0.000199
2275549	0.000221
10781433	0.000226
7044742	0.000289
11145408	0.000295
1890899	0.000342
12552898	0.000384
12347526	0.000422
4745628	0.000469
10869907	0.000644
11145357	0.000702
12551580	0.000739
12551400	0.000787
12156450	0.001059
11145388	0.00111
10869915	0.001127
2275547	0.001136
10869910	0.001562
7043951	0.001573
11145410	0.001783
3737289	0.002065
7855014	0.003077
12342273	0.003135
2275548	0.003676
11145353	0.00408
11145361	0.004419

Table 2: Association analysis results for VPS13A SNPs in 1,240 Mexican-American individuals

In addition, of the 5 best cis-acting *VPS13A* SNPs, 3 also showed statistically significant effects on *CTNS* mRNA levels (Table 3). This supports our previous data that *VPS13A* is a potential regulator of *CTNS* gene expression.

SNP name	P value for cis effect on VPS13A	P value for trans effect on CTNS
1048743	3.33E-18	0.27604
1054368	0.0000002	0.01323
12684856	0.00000025	0.19633
4744830	0.00000108	0.01443
10781432	0.00000533	0.00862

Table 3: Association analysis results for VPS13A SNPs shows evidence of cis and trans-regulation

The next phase of the project is to ascertain whether *VPS13A* exerts a biological influence on *CTNS.* Our first approach is to "knock down" *VPS13A,* that is to reduce the levels of *VPS13A* mRNA, then to determine what effect this has on the levels of *CTNS* mRNA. A detailed protocol and results will be presented in the next progress report.

PROGRESS REPORT

ERIC K. MOSES, PHD, MENTOR
KATY FREED, PHD, RESEARCH FELLOW

"Complex Genetic Approaches to Monogenic Disease: Genomic and Transcriptomic Dissection of Normal Expression of CTNS, the Gene Involved in Nephropathic Cystinosis"

Report 1/7/09

Overview

While rare human genetic diseases like cystinosis are caused by mutations in a single gene there is a growing realization that genes rarely work alone but rather are posited within complex global regulatory networks in which they may potentially interact with many other genes. This realization underpinned the first phase of our cystinosis research program in which we set out to study the genetics of the *CTNS* gene in a large sample of unaffected families, employing normal human variation as a model for pathological human variation. In order to do this, we generated a unique dataset of genome-wide lymphocyte transcriptional profiles using 1,240 samples from 42 Mexican American families

Progress to date

Using a genome-wide scanning strategy we identified a region on chromosome 9 which was suggestive of the presence of trans-acting genetic variation influencing *CTNS* expression levels. We have identified a candidate gene at this precise location (VPS13A at 76 cM) whose expression is highly genetically correlated to *CTNS* expression (G = -0.551, p = 9 ×10-6). Thus, *VPS13A* may be a potential inhibitor of *CTNS* expression. Our interest in this gene is further strengthened by the proposed function of the *VPS13A* protein which may control steps in the cycling of proteins through the trans-Golgi network to endosomes, lysosomes and the plasma membrane.

In order to identify genetic variants in the *VPS13A* gene, 1.5 kb of the 5' promoter region of *VPS13A* was re-sequenced in 189 DNA samples derived from the unaffected Mexican American families. Sixteen single nucleotide polymorphisms (SNPs) were identified, 6 of which were novel i.e. not in

the public SNP databases. The next step was to genotype the promoter SNPs and known (278) *VPS13A* SNPs in 1,240 individuals from the Mexican American population using the BeadArray platform (Illumina inc. San Diego, California).

Out of the 278 SNPs that were analyzed for *VPS13A,* 45 showed statistically significant cis effects on *VPS13A* mRNA levels. That is, there are certain regions of DNA within the *VPS13A* gene that affect the levels of *VPS13A* mRNA. Of particular interest, of the 5 best cis-acting *VPS13A* SNPs, 3 also showed statistically significant effects on *CTNS* mRNA levels. This supports our previous data that *VPS13A* is a potential regulator of *CTNS* gene expression.

The next phase of the project was to ascertain whether *VPS13A* exerts a biological influence on *CTNS*. Our first approach was to "knock down" VPS13A, that is to reduce the levels of *VPS13A* mRNA, then to determine what effect this has on the levels of *CTNS* mRNA.

Aim

The aim of the project was to test experimentally whether *VPS13A,* a gene that was statistically predicted to be upstream of the cystinosin gene *(CTNS),* plays a role in modifying the expression of *CTNS.* We proposed to 'knock-down' the expression of this putative upstream gene using molecular biology methodologies in human lymphoblast cell lines and measure the effect on the expression of the cystinosin gene.

A number of optimization experiments were carried out before testing of the candidate gene could commence. In the first instance, the growth characteristics of the lymphoblastoid cell lines were optimized. This was followed by establishing in our laboratory a relatively new method (QuantiGene Reagent System from Panomics) that was developed to determine the mRNA levels of genes without the need for a separate RNA extraction. Essentially, the cells are grown in a 96-well tissue culture plate, lysed then the lysate transferred to a 96-well Panomics plate where a gene specific probe is used to capture your gene of interest. The relative levels in each sample is determined by addition of a chemilumigeneic substrate that generates a luminescent signal that is proportional to the amount of target mRNA present in the sample.

We then needed to validate the silencing methodology in our laboratory and to establish whether the methodology was applicable to our experimental design. After a discussion with representatives from Dharmacon, the company that supplies reagents for RNA silencing protocols, they recommended a new 'knockdown' method (Accell siRNA) that had been used successfully in lymphoblastoid cell lines. We then initiated control 'knockdown' experiments using a well characterized housekeeping gene, *GAPDH.* Using 3 lymphoblastoid cell lines, we showed that the levels of *GAPDH* mRNA could be reduced by 92, 94 and 94% respectively in 3 cell lines.

Having established and validated the 'knockdown' and mRNA quantitation methodologies in our laboratory, we then tested the candidate gene using different 7 cell lines. Our hypothesis was that, given that *VPS13A* is genetically negatively correlated with *CTNS* mRNA levels, if we reduced the levels of *VPS13A,* the levels of *CTNS* mRNA would increase. Firstly we measured the levels of *VPS13A* mRNA levels in our chosen cell lines to ensure that the Panomics system was sensitive enough to detect our genes. Having established baseline mRNA levels of *VPS13A* and *CTNS* we commenced our 'knockdown' experiment. We did see a trend for the level of *CTNS* mRNA to increase when we reduced the amount of *VPS13A* gene expression. However, both the reduction in *VPS13A* mRNA levels (25%) and change in *CTNS* gene expression (9%) were modest and variability was high thus further experiments are required before final conclusions can be drawn.

PROGRESS REPORT

ERIC K. MOSES, PHD, PRINCIPAL INVESTIGATOR
JOHN BLANGERO, PHD, CO-PRINCIPAL INVESTIGATOR

"Scanning the Human Transcriptome in Cystinotic Cell Lines for Changes that are Associated with Genetic Variation in the CTNS gene"

Report 07/10/08

SUMMARY OF PROJECT

The Problem

Cystinosis is an inherited (autosomal recessive) disease with an incidence of around 1 in 100,000 live births. Mutations in the gene (*CTNS*) that codes for the lysosomal cystine transport protein, cystinosin, represent the known causes for this disease. A wide spectrum of causal mutations have been observed involving both complete elimination of the transport protein (in the most severe cases) and more subtle quantitative deficiencies of the protein (seen in less severe cases). While the *CTNS* gene was identified by a classical genetic approach, there has been only minimal scientific investigation into the broader effect that genetic variation in the *CTNS* gene has on other downstream phenotypes that may be more directly involved in pathology.

The Approach

There is a growing realization that genes rarely work alone but are positioned within complex global regulatory networks in which they may potentially interact with many other genes. In this current proposal we will comprehensively enumerate genetic variation in the *CTNS* gene in individuals with cystinosis and their first degree relatives and then test whether this genetic variation influences the quantitative expression of any other gene (via the measurement of genome-wide gene expression in lymphocyte-derived cell lines).

Specific Aims

The overall goal of this project is to determine whether the genetic variation of cystinosin (*CTNS*), the central gene involved in cystinosis, influences the quantitative expression of any other gene using cystinotic cell lines. There are 4 specific aims:

1. To transform lymphocytes derived from 147 cystinosis family members into cell lines
2. To comprehensively re-sequence the *CTNS* gene in our patient/family cohort using DNA isolated from cystinosis family members to identify all genetic variation
3. To perform whole genome transcriptional profiling using RNA isolated from the lymphoblastoid cell lines
4. To identify those genes whose mRNA expression is altered by *CTNS* disease mutations

PROGRESS TO DATE

Samples: In July 2007, the Cystinosis Research Network Family Conference took place in San Antonio, Texas. With University of Texas Health Science Center San Antonio Institutional Review Board ethics approval, whole blood (5-50 ml) was collected from consenting individuals affected by cystinosis and their first degree relatives. Blood was collected from 40 individuals with cystinosis and 107 samples from unaffected individuals. The blood was collected into anti-coagulant tubes (BD Vacutainer 10 ml EDTA plastic tubes), then centrifuged on a Ficoll gradient (Histopaque) to separate the white blood cells (lymphocytes) from other blood components. The cryoprotective agent, dimethyl sulfoxide (DMSO) (Sigma), was added to the purified lymphocytes then stored in Liquid Nitrogen.

CTNS DNA Re-sequencing: We have re-sequenced the entire genomic sequence of *CTNS* in addition to 1.5 kb of the 5' promoter region in DNA extracted from the cystinosis family members. Sequencing primers (59) were designed to cover the entire region at an average of 700 bp per fragment. PCR amplification of these segments was carried out using standard conditions. PCR amplicons were then used as templates in cycle sequencing reactions using the BigDye® Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Cycle sequencing reactions were performed on both the sense and anti-sense strands. Capillary electrophoretic separation of DNA sequence fragments was performed on an Applied Biosystems 3730 DNA Analyzer. Applied Biosystems supplied DNA Sequencing Analysis Software version 5.1.1 was used for first pass base calling quality assessment.

Identifying polymorphisms: Comprehensive sequence alignment for polymorphism identification was performed using Comparative Sequence Analysis (CSA) SeqScape Software version 2.5 (Applied Biosystems). Both strands were sequenced to promote resolution of polymorphisms in heterozygous individuals.

185 SNPs have been identified to date, including 5 insertions/deletions in the cystinosis families. The distribution of these SNPs within the *CTNS* gene is shown in Figure 1.

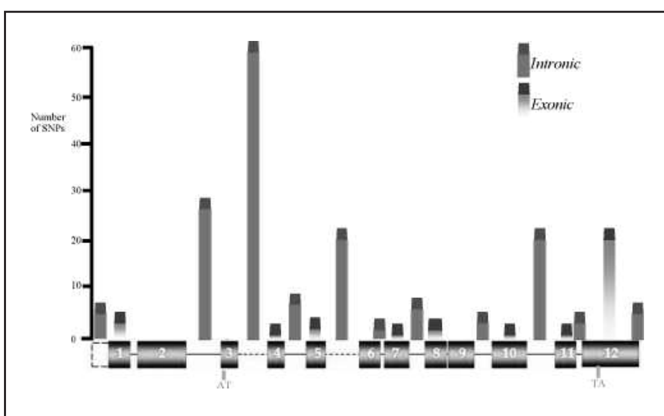


Figure 1. Distribution of CTNS genetic variants in cystinosis families.

Generation of cystinotic cell lines: Permanent cystinotic lymphoblastoid cell lines (147) will be generated using Epstein-Barr virus to transform the lymphocytes. Each cell line will represent one cystinosis family member. We have recently begun the transformation of the white blood cells and, to date, have generated 4 lymphoblastoid cell lines. These cell lines have been stored in Liquid Nitrogen until required.

Future work: From these permanent cell lines, RNA will be extracted using QIAGEN spin technology (QIAGEN Inc., Valencia, CA). The total RNA will be used for the whole genome transcriptional profiling.

PROGRESS REPORT

DAVID PEARCE, PHD, PRINCIPAL INVESTIGATOR
SEASSON PHILLIPS, POST-DOCTORAL FELLOW

"Yeast Model for Cystinosis"

Report 1/7/09

The yeast homolog of *CTNS* is ERS1 encodes the Ers1p protein. We aimed to study the cellular alterations that occur in the ERS1 deletion strain, ers1- . A phenotypic screen identified several cold-resistance phenotypes. Specifically, ers1- grew more robustly than the ERS1+ parental strain in the presence of calcium chloride, chloroquine, or increased glucose concentrations at 25oC (Fig. 1). Although these phenotypes need to be complemented by plasmid-borne ERS1 and *CTNS* expression, they are noteworthy because they indicate alterations in vacuolar pH and the vacuolar ATPase (V-ATPase). The V-ATPase is a highly conserved complex of proteins, which acidifies the lysosome/vacuole. It consists of a membrane-bound V0 subunit and a soluble V1 subunit, which assemble in the presence of glucose and disassemble under glucose deprivation. Upon assembly, ATP hydrolysis by the V1 subunit drives the proton pumping activity of the V0 subunit, consequently lowering the pH of the vacuolar lumen. In yeast, V-ATPase mutants are sensitive to calcium chloride when grown in media at pH 6, but not pH 4. In addition, chloroquine is a drug that raises the pH of the vacuole. It is interesting that these growth phenotypes occur in a cold-dependent manner, since cold-sensitivity in yeast often indicates alterations in complex formation.

Due to the cold-resistance phenotypes, there may be alterations in the vacuolar pH and V-ATPase complex formation in ers1- . Specifically, perhaps the vacuolar pH is decreased, which allows ers1- to grow in the presence of chloroquine. As expected, the vacuolar pH in ers1- cells decreases as the cells grow (Fig. 2). We speculated that perhaps the decreased pH may be due to increased V-ATPase subunit assembly, causing the cold resistance in the presence of calcium chloride. However, when V-ATPase subunit assembly was measured, we found a significant decrease in subunit assembly in ers1- (Fig. 3). We speculate that the V-ATPase is being down-regulated to correct for the decreased vacuolar pH, and the decreased pH allows for efficient calcium transport, which can be toxic if not sequestered in the lysosome.

Consequently, immediate plans include measuring V-ATPase-dependent proton pumping and ATP hydrolysis in ers1- to better characterize the role of the V-ATPase in those cells. Likewise, we plan to co-immunoprecipitate an endogenously expressed Ers1p-HA with the V-ATPase to determine if Ers1p physically interacts with the V-ATPase. In addition, because of the calcium chloride phenotype, we are deleting a protein that is part of the V-ATPase in ers1- to determine if ers1- suppresses the phenotypes seen in V-ATPase mutants (often referred to as the VMA phenotype), and thus interact genetically.

Along with identifying and characterizing alterations in the vacuole, we performed a synthetic genetic array (SGA) in collaboration with Charles Boone (University of Toronto), to identify genetic interactions with ers1- . Synthetic lethal or synthetic sick interactions are useful in identifying buffering or parallel pathways. In brief, synthetic sick or lethal interactions occur when deletion of one gene, such as ers1- appears to grow normally, but when deleted in combination with another gene deletion, there is reduced viability or lethality. SGA is a screen used to identify these interactions. In this screen, the ers1- strain was mated against every nonessential yeast deletion strain. The colony size of the resulting double deletion mutant is measured via densitometry, and compared to the size of the single deletion mutant colony size. Using SGA, 68 genes were identified as having putative interactions with ERS1. Candidate genes encode proteins involved in vacuolar and endomembrane function, nutrient limitation response, and mitochondrial function. Currently, these interactors are being verified by random spore analysis.

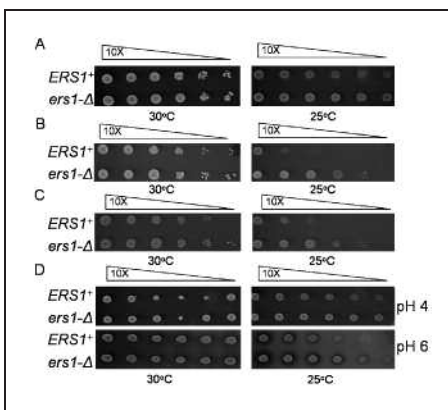


*Figure 1 – **ers1- exhibits cold-resistance phenotypes.** Ten-fold serial dilutions (indicated by wedge) of cells were plated on minimal (YNB) media containing yeast nitrogen base, 2% dextrose (unless otherwise indicated), and auxotrophic amino acids. Plates were incubated for 3-4 days at the indicated temperature. Media was at pH 6 unless otherwise indicated. (A) YNB (B) YNB with chloroquine (C) YNB with 10% glucose (D) YNB at pH 4 or 6 with 100mM calcium chloride.*

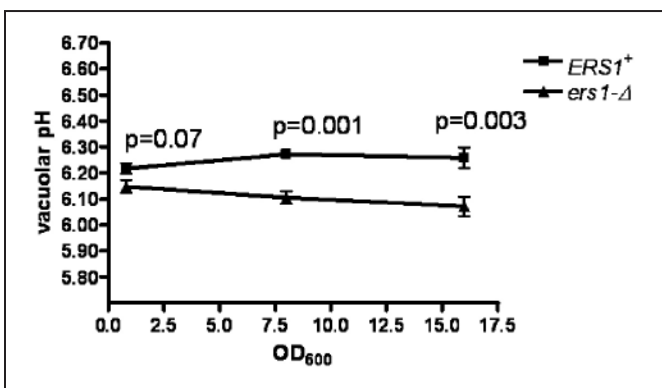


*Figure 2 – **Vacuolar pH is decreased in ers1-** Vacuolar pH was measured by growing cells in YPD (indicated by OD600), treating the cells with the ester BCECF, and measuring BCECF cleavage by a pH-dependent esterase fluorometrically. Squares represent the ERS1+ parental strain, while triangles represent ers1- . Differences were deemed significant when p<0.05 as determined by Student's t-test.*

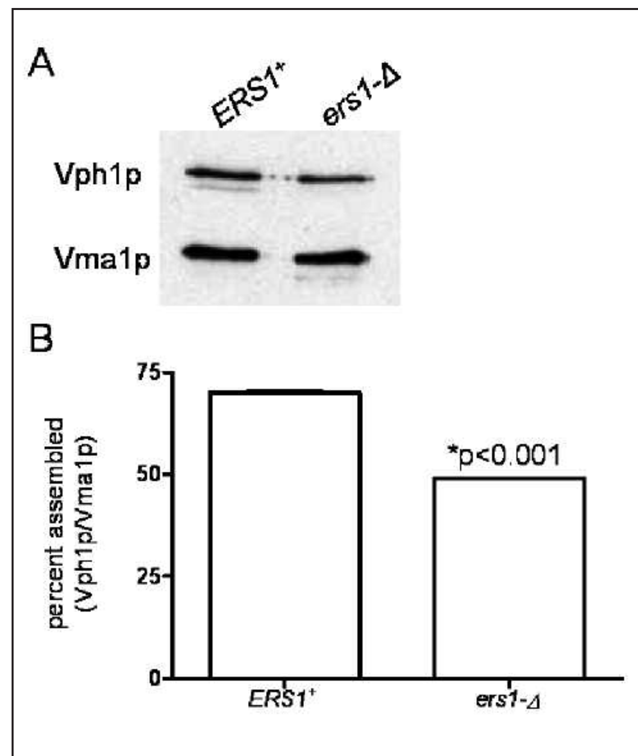


*Figure 3 – **V-ATPase assembly is decreased in ers1-** . V1 and V0 subunit assembly was measured by comparing the amount of V0 subunit that immunopurifies with the V1 subunit. (A) Immunoblot of the V1 and V0 subunits (represented here as Vma1p and Vph1p, respectively). (B) Percent assembly was calculated by the densitometry ratio of Vph1p to Vma1p. Significance was calculated by a Student's t-test.*

PROGRESS REPORT

DANIEL SALOMON, MD, PRINICPAL INVESTIGATOR

STEPHANIE CHERQUI, PHD, CO-PRINCIPAL INVESTIGATOR

"Treatment of Cystinosis Using Genetically Modified Adult Stem Cells in Murine Cystinosis Model"

Report 12/10/ 07 – 6/15/08

I- MICE

We are currently breeding several strains of mice for the cystinosis research project:

1- *Ctns-/-* mice

We have a large colony of C57BL/6 *Ctns-/-* mice to support all the different transplantation-based treatments that we are testing.

2- GFP transgenic mice

We generated a colony of mice transgenic for the Green Fluorescent Protein (GFP) expressed under the control of a ubiquitous β-actin promoter (C57BL/6-Tg(ACTB-EGFP)1Osb/J, Jackson Laboratory). The mice are used to isolate bone marrow stem cells, which express the wild-type *Ctns* gene, and that are GFP+ so that they can be followed after transplantation in lethally irradiated *Ctns-/-* mice.

3- Luciferase transgenic mice

We received in February 2008 the Luciferase transgenic mice provided by Dr. Michael Geusz (Bowling Green State University, Ohio). Firefly Luciferase reporter gene is expressed under the control of a CMV promoter [Collaco and Geusz, 2003]. The goal is to isolate bone marrow stem cells from these mice and follow them in live animals using the IVIS Imaging System 200 Series (Xenogen) after transplantation. This approach will be complementary to using stem cells obtained from the GFP transgenic mice and performed in parallel. The potential advantage of using the luciferase mice as the donors of stem cells is that we can image the engraftment of the stem cells and their locations in live mice in a serial fashion for any given experiment.

4- Galectin-3 deficient mice

In the laboratory of Dr. Corinne Antignac, they showed that cystinosin interacts with Galectin-3 in vitro (unpublished data). To prove this interaction in vivo, we generated the double knock out mice, *Ctns*-/- Gal3-/- at The Scripps Research Institute in collaboration with Dr. Antignac. We obtained the Gal3-/- mice from Dr. Dan Hsu [Hsu et al., 2000] in December 2007. The first pups were born in February 2008. These mice were heterozygous, *Ctns*+/- Gal3+/-. We then bred the double heterozygous mice together to obtain *Ctns*-/- Gal3-/- double knock out mice, the first of which were born on March 23rd 2008. Today, we have 6 male and 4 female *Ctns*-/- Gal3-/- animals, which are currently breeding. Once a larger quantity of *Ctns*-/- Gal3-/- mice will be achieved, we will analyze their renal function and their general phenotype, and tissues will be sent to Dr. Antignac for more specific analyses. We predict one of two outcomes. If the cystinosin/galectin interaction plays two separate and complementary roles in cellular cystine metabolism then we might see an increased renal disease in these animals. However, if cystinosin is required for galectin function in this pathway, then the double knock out animals should have the same amount of renal disease as the single cystinosin gene knock out. Nonetheless, once Dr. Antignac has all the tissues, there are additional possibilities for advancing our understanding of cystine metabolism and cystinosin function.

5- Vanin-3 deficient mice

Vanin genes encode pantetheinase that catalyzes the hydrolysis of D-pantetheine permitting the recycling of pantothenate (vitamin B5) and the generation of cysteamine in vivo [Martin et al., 2001]. There are three orthologous genes in humans (VANIN-1, -2 and -3) and two in mouse (vanin-1 and -3). In mice, vanin-1 and -3 have a different pattern of expression, vanin-1 is primarily found in epithelial cells and is abundant in liver, intestine and kidney, and vanin-3 is preferentially expressed in myeloid cells with additional ubiquitous, albeit lower, expression in many tissues [Min-Oo et al., 2007]. Thus, we now realize that human and mouse cells make some cysteamine naturally using the vanin gene pathway. Our hypothesis is that mice make significantly more than humans, particularly in the kidney, and that is why the disease phenotype of the mice is less severe. In collaboration with Dr. Allison Eddy, we will generate the triple knock out mice *Ctns*-/- Vnn1-/- Vnn3-/-. The experimental objective is to test our hypothesis that knocking out the vanin gene pathway in mice will increase the severity of the cystinosis phenotype especially the kidney defects in *Ctns*-/- mice. Dr. Eddy is generating the double knock out mice *Ctns*-/- Vnn1-/- and we are generating the double knock out mice *Ctns*-/- Vnn3-/-, which will subsequently be bred together. The Vnn3-/- mice are the A/J strain mice, which carry a unique nonsense mutation that leads to a truncated protein as well as a rearrangement in the Vnn3 promoter region [Min-Oo et al., 2007]. This mutation makes theses mice susceptible to malaria in contrast to the C57BL6 mice, and this susceptibility can be partially reversed by cysteamine. We obtained the A/J mice from Jackson laboratory in February 2008 and they are currently breeding with the *Ctns*-/- mice.

6- Sod1 and Sod2 deficient mice

As already described in the original proposal and in the previous progress reports, the proximal tubulopathy in cystinosis might be due to an oxidative stress that leads to a decrease of ATP levels in proximal tubules. Our hypothesis is that the inhibition of SOD, an antioxidant protein protecting the cells against oxidative stress production, in *Ctns*-/- mice, will lead to the development of a proximal tubulopathy in these mice. We thus backcrossed *Ctns*-/- mice with Sod1 and Sod2-deficient mice, and generated *Ctns*-/- Sod1-/- and *Ctns*-/- Sod2+/-.

We measured creatinine, urea and phosphate levels in the serum and in 24h urine collections, alkaline phosphatase in the serum and protein in urine of the *Ctns*-/- Sod1-/-, *Ctns*-/- Sod2+/- and the appropriate controls. This is currently done in our laboratory using the BioAssay Systems kits. We also measured aminoaciduria in selected mice using the UCSD Biochemical Genetics laboratory.

The *Ctns*-/- Sod1-/- mice exhibit an elevated alkaline phosphatase between 1 and 3 months that goes back to normal afterwards (Figure 1A). They do not present with any other defect in their blood and urine at least to the current time of follow-up, 6-months old. Thus, so far, there is no evidence that the genetic Sod1 defect is having any deleterious impact on renal function in the *Ctns*-/- background.

The *Ctns*-/- Sod2+/- had a normal urea, creatinine and alkaline phosphatase at 2 and 5 months of age. However, the mice exhibit an elevated proteinuria at 2 and 5 months and an elevated phosphaturia at 5 months old (Figure 1B). This might be signs of the beginning of a proximal tubulopathy, but more mice are necessary to confirm these potentially interesting data. We also plan to generate the triple knock out mice *Ctns*-/- Sod1-/- Sod2+/-. Our overall objective is to determine if oxidative metabolism is an important factor in the development and/or severity of cystinosis renal disease.

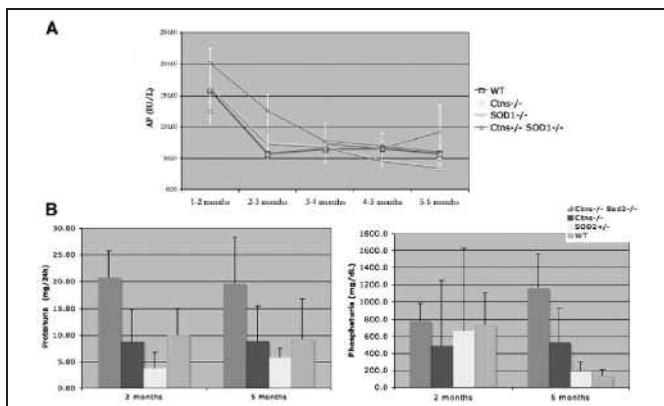


Figure 1: (A) Serum alkaline phosphatase in Ctns-/- *Sod1-/- mice compared to the controls. (B) Proteinuria and phosphaturia in* Ctns-/- *Sod2-/- compared to the controls.*

II- TREATMENT OF CYSTINOSIS NEPHROPATHY USING URETERAL INJECTION OF ADENO-ASSOCIATED VIRUS EXPRESSING *CTNS*

A grant has just been submitted for this part of the project to the Cystinosis Research Foundation with all the preliminary data and the research design and methods.

III - TREATMENT OF CYSTINOSIS NEPHROPATHY USING GENETICALLY MODIFIED ADULT STEM CELLS IN THE MURINE CYSTINOSIS MODEL

We performed bone marrow stem cell (BMSC) transplantations in *Ctns-/-* mice. BMSC were isolated from our colony of GFP transgenic mice or wildtype C57BL6 mice. For controls, we isolated BMSC from *Ctns-/-* mice. The premise is that we need to exclude the possibility that simply transplanting BMSC might have some unexpected impact on the *Ctns-/-* mice. We transplanted either BMSC or mesenchymal stem cells (MSC). We performed the cell injections by two methods: tail vein injection in sublethally irradiated mice or retrograde ureteral injections into the ureter of the left kidney.

Part of the results for this project has been presented to the International Cystinosis Research symposium in April 2008. A manuscript is currently being written for publication and will be sent to the Cystinosis Research Foundation before the end of August 2008.

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PROGRESS REPORT

DANIEL SALOMON, MD, PRINICPAL INVESTIGATOR
STEPHANIE CHERQUI, PHD, CO-PRINCIPAL INVESTIGATOR

"Treatment of Cystinosis Using Genetically Modified Adult Stem Cells in Murine Cystinosis Model"

Report 10/20/08

GRANT #1:

"Treatment of cystinosis using genetically modified adult stem cells" (funded July 2006 for 3 years)

The ultimate objective of this project is to cure cystinosis by performing non-myeloablative autologous bone marrow stem cell transplantation after introducing a functional *CTNS* gene into the patient's stem cells by gene delivery. To achieve this objective we are using the mouse model of cystinosis to demonstrate a proof of principle for a human clinical trial of this strategy.

RESULTS TO DATE:

Using the murine model for cystinosis, *Ctns-/-* mice, we performed autologous bone marrow cell (BMC) and mesenchymal stem cell (MSC) transplantation. BMC and MSC were from wildtype or GFP (Green Fluorescent Protein)-transgenic mice, both expressing a functional *Ctns* gene. At 4 months post-transplant, brain, eye, heart, kidney, liver, muscle and spleen have been analyzed.

Organ-specific cystine content was reduced in all organs tested of the BMC-treated mice going from 57% in the brain to 94% in the liver (Table 1). The natural progression of renal dysfunction was prevented and deposition of corneal cystine crystals was significantly improved in the treated mice. Confocal microscopy and quantitative PCR revealed a large quantity of transplanted BMC in all organs tested, from 5% in the muscle to 19% in the spleen of the total cells (Table 1). Most of these cells were not from the lymphoid lineage (specifically, not macrophages) even in the spleen, but part of the intrinsic structure of the organ such as tubular and glomerular mesangial cells in the kidney, reticulo-endothelial cells in the spleen, endothelial and neuronal cells in the brain and myocytes in the muscle. We also analyzed tissues from BMC-transplanted *Ctns-/-* mice at 2 months post-transplant. In comparison to the 2 month results, we observed a clear increased in quantity of engrafted cells and a decrease of cystine content by 4 months, showing these improvements continue through time.

In contrast, transplanted MSC did not integrate efficiently in any organ and did not decrease tissue cystine content.

	Brain	Eye	Heart	Kidney	Liver	Muscle	Spleen
Ctns expression, Percentage of total cells	5.9	13.4	11.0	12.9	9.6	5.4	19.3
Percentage decreased cystine content	57.3	70.4	81.9	70	94.4	65.6	86.8

Table 1: Ctns *expression and cystine decrease in the different tissues of Ctns-/- mice treated with BMC at 4 months post-transplant*

ONGOING EXPERIMENTS:

1 - Analysis of BMC-transplanted mice after 6 months and 1 year post-transplantation

Rational: The fact that we obtained an increased in engrafted cells and a decrease of cystine content in BMC-transplanted Ctns-/- mice at 4 months compared to 2 month post-transplant, suggests that BMC transplantation creates a reservoir of healthy cells that migrate and integrate to the targeted organ as a function of progressive cellular injury. Therefore, we hypothesize that the benefits of this treatment will increase with time and may eventually achieve normal levels of cystine content.

Twenty *Ctns*-/- mice have been transplanted with BMC from GFP-transgenic mice and will be analyzed at 6 months and 1 year post-transplantation.

2 - Transplantation of old *Ctns*-/- mice with BMC

Rational: In the previous studies, Ctns-/- mice have been transplanted at 2 months of age and were analyzed 2 or 4 months after. An important question for the treatment of cystinosis in human patients is if BMC-transplantation is as efficient in older mice than in young. Note that the life span of a laboratory mouse is approximately 2 years.

We transplanted 20 *Ctns*-/- mice between 6 and 10 months of age with BMC from GFP-transgenic mice and we will analyze them at 4 months post-transplant.

3 - Transplantation of *Ctns*-/- mice with hematopoietic stem cells (HSC)

Rational: We showed that BMC but not MSC efficiently integrated in multiple organs, replacing cells lacking the *Ctns* gene with cells expressing a functional Ctns gene, and resulting in significant decreases of cystine content in these tissues. BMC is actually a heterogenous mixture of cells that contains the true HSC. For a clinical trial, we need to know if whole bone marrow is necessary to achieve our objective or if similar results can be obtained with purified HSC only. This is important because human HSC are easily isolated from peripheral blood after growth factor-mediated mobilization (i.e. GM-CSF) and gene delivery to introduce a functional Ctns gene is also more efficient with HSC than with whole BMC.

25 *Ctns*-/- mice have been transplanted with Sca1+ HSC isolated from GFP-transgenic mice. Six of them were sacrificed last week at 4 months post-transplantation for analysis along with non-treated *Ctns*-/- mice at the same age. Cystine content is currently being measured and tissues processed for quantitative PCR and confocal microscopy analysis. Results should be obtained in 2-3 weeks. We plan to carry the other mice in this study out to the 6 and 12 month time points.

4 - Adult stem cell gene delivery to introduce *Ctns* gene

Rational: We aim to use bone marrow stem cells from the patients (i.e. autologous) to avoid the problem of an immune response and graft vs. host disease and thus, obviate the need for immunosuppression. Therefore, we will genetically modify adult stem cells to express a functional *CTNS* gene by using a viral vector. Success of this strategy will require efficient gene delivery by a well-designed and safe vector delivery system.

We chose to use recombinant Adeno-Associated virus (rAAV) for this project because of its low immunogenicity, low toxicity and safety. We are collaborating with Dr. Arun Srivastava (University of Florida, College of Medicine). He recently was a member of the group reporting success in patients with blindness due to a retinal dystrophy caused by Leber's Congenital Amaurosis and treated with intraocular rAAV 1,2. He has also recently demonstrated the efficient and stable transduction of murine HSC using self-complementary (sc) AAV vectors 3.

We showed an efficient transduction of Sca1+ HSC by scAAV expressing GFP in vitro. We will now transduce Sca1+ HSC by scAAV expressing GFP and transplant them into *Ctns*-/- mice to test the stability and the efficiency of this vector for gene delivery in HSC. If we are satisfied with the results, we will use a scAAV expressing *CTNS* that we are currently generating, to transduce *Ctns*-/- HSC. Using quantitative PCR, cystine measurement and clinical analyses, we will test the efficiency of this treatment for cystinosis. We will also test its stability and safety by keeping the animals alive for 1 year with monthly immune response analyses and regular monitoring for any pathological abnormalities.

We anticipate being done with all these studies in approximately one and half years if no major problems occur. Therefore, we will need to apply for additional funding in early 2009 to complete these objectives. Then the next step will be a non-myeloablative autologous BMSC transplantation in Rhesus macaque monkeys (n=8) for a 1-year study for *CTNS* expression in different organs and for safety analyses. These studies represent the prerequisites steps before a clinical trial.

GRANT #2:

"Treatment of cystinosis using ureteral injection of scAAV expressing *Ctns*" (funded in July 2008 for 1 year)

The objective of this project is to test the hypothesis that retrograde ureteral injection of scAAV expressing a functional *CTNS* gene will treat or prevent the proximal tubulopathy and progression of renal defects in cystinosis when delivered very early in the disease and ameliorate the renal disease if administered to older patients. Retrograde ureteral adminstration would be a minimally invasive procedure and would complement systemic cysteamine therapy. This part of the project is performed in collaboration with Dr. Jude Samulski, Director of the Gene Therapy Center (University of North Carolina at Chapel Hill).

RESULTS TO DATE:

To determine the stability of transgene expression in animals, we started with rAAV2 expressing a luciferase reporter gene (rAAV2-Luc), the expression of which can be followed with the IVIS imaging system in live animals. Eight months post-injection, luciferase expression can still be observed within the kidney of the injected mice demonstrating that there is successful delivery of the vector to the kidney and stable expression of its luciferase gene.

We have also generated a new vector, scAAV2 expressing Ctns, and verified its functionality in vitro by transducing *Ctns*-/- fibroblasts. The *Ctns* gene was highly expressed in the fibroblasts and their cystine content was significantly decreased.

ONGOING EXPERIMENTS:

Dose determination

Rational: Before testing the efficiency of scAAV-*CTNS* to decrease cystine content, we need to determine the optimal viral particle dose to target the kidney using retrograde urteral injection. For this purpose, we are using another vector, scAAV2-GFP. Expression of Green Fluorescent Protein (GFP) has multiple advantages for a dose titration including ability to determine the location and phenotype of the transduced renal cells and measure their quantity.

The first experiment suggested that the 1x1011 viral particle dose is optimal. We are currently repeating this experiment.

Once the dose is determined, we will the prepare and inject scAAV-CTNS in a large number of *Ctns-/-* mice to answer two key questions:

- Can scAAV2-*CTNS* prevent the development and progression of renal dysfunction?
- Can scAAV2-*CTNS* reverse existing renal dysfunction?

We will also test vector safety by immune response analyses, histological and general health studies over a 1 year period.

If the results are positive, we will then apply for additional funding to inject 8 Rhesus macaque monkeys for a 1-year study of *CTNS* expression in the kidney and other organs, renal function, immune response and safety analyses.

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PROGRESS REPORT

MARY TAUB, PHD, PRINCIPAL INVESTIGATOR

"Mechanisms Underlying the Fanconi Syndrome in Cystinosis"

Report 01/20/09

This project is concerned with evaluating the mechanisms underlying the Fanconi Syndrome in Cystinosis. Small interfering RNA (siRNA) against cystinosin is being employed to knockdown the expression of cystinosin in primary cultures of rabbit kidney proximal tubule (RPT) cells. Subsequently, the effect of reduced cystinosin gene expression on transport function is to be examined.

Initially, the cystinosin expressed by primary RPT cells has been examined by means of Western analysis. Three antibodies against cystinosin were initially compared for their ability to recognize cystinosin in primary RPT cultures, including a rabbit anti-cystinosin antibody obtained from Lifespan Biosciences, a mouse monoclonal antibody against cystinosin obtained from Santa Cruz Biotechnology, and a rabbit anti-cystinosin antibody obtained from ProteinTech. The antibody against cystinosin from Lifespan Biosciences was raised against a KLH conjugated synthetic peptide against the sequence 268-285 in human cystinosin, and this sequence is retained in rabbit cystinosin (Ensembl website). Figure 1 indicates that 1) the Lifespan antibody recognizes a 55 kd protein present in primary RPT cells and 2) that this protein comigrates with the protein recognized by the antibody from Santa Cruz Biotech as well as the antibody from The ProteinTech Group.

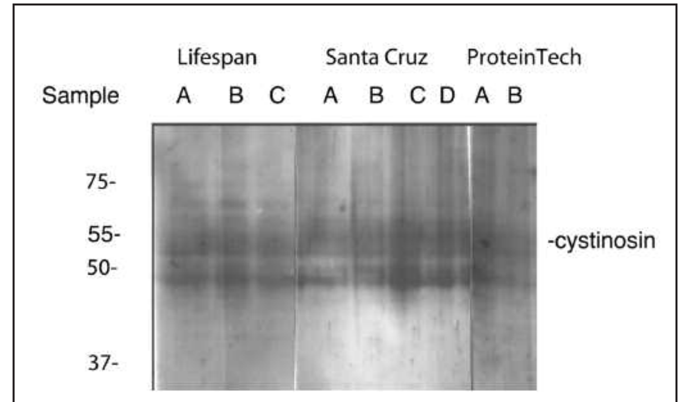

Figure 1. Primary RPT cell cultures in 35 mm culture dishes were employed for Western analysis. Cell lysates obtained from parallel cultures were separated by SDS-PAGE, and transferred to nitrocellulose. Strips of nitrocellulose obtained from the same blot were stained with antibody against cystinosin obtained from either Lifespan, Santa Cruz or ProteinTech. The bands were visualized by autoradiography, and molecular weights determined using Precision Markers (Biorad).

We have obtained 3 sets of siRNAs against rabbit cystinosin and their scrambled control siRNAs. The sequences of these siRNAs are illustrated in Figure 2. Previously, we examined the effect of one of these cystinosin siRNAs, 121 siRNA, as compared with its scrambled control. We obtained evidence that 121 siRNA reduced the level of cystinosin in primary RPT cells after the cultures were subjected to 2 transfections with this siRNA.

siRNAs	Sequence (5' to 3') Figure 5
121 Sense (S)	GACAAUACGUCUUGCUGCCCAGUUA
121 Antisense (A)	UAACUGGGCAGCAAGACGUAUUGUC
control_121 (S)	GACGCAUUUCUGUCGACCCGAAUUA
control_121 (A)	UAAUUCGGGUCGACAGAAAUGCGUC
430 (S)	UGGAGGAGAUGAGGUUACUCUUGCA
430 (A)	UGCAAGAGUAACCUCAUCUCCUCCA
control_430 (S)	UGGAGAGGAGUAUUGUCUCUGAGCA
control_430 (A)	UGCUCAGAGACAAUACUCCUCUCCA
509 (S)	CAGGACAGGUUGAAUUAGCUCAUUU
509 (A)	AAAUGAGCUAAUUCAACCUGUCCUG
control_509 (S)	CAGACGGUUAGUAAUCGCUAAGUUU
control_509 (A)	AAACUUAGCGAUUACUAACCGUCUG

Figure 2. The sequence of rabbit cystinosin mRNA was obtained from the Ensembl genome browser. Stealth siRNA sequences against rabbit cystinosin mRNA (as well as scrambled controls) were obtained using an Invitrogen web-based program.

Subsequently, we conducted a number of studies using 400 nM of these siRNAs. However, as shown in Fig. 3, the 121 siRNA did not have an affect at this concentration. Similar results were obtained with the other 2 siRNAs. When we observed the cultures under an inverted microscope, the cells had become elongated and groups of cells were very tightly clustered together.

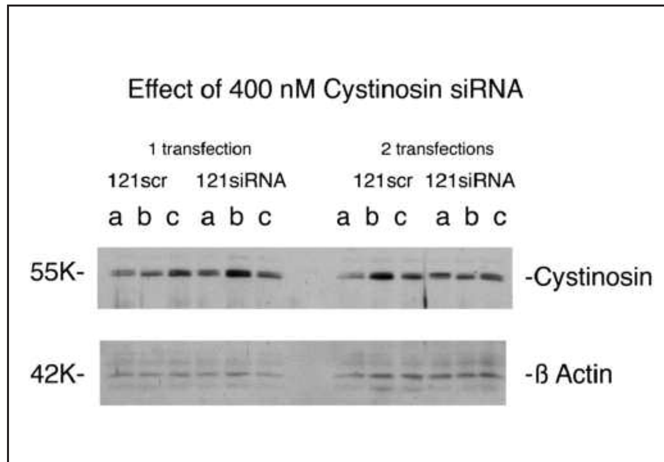

Figure 3. Individual primary RPT cell cultures were transfected in parallel with 400 nM of either 121 siRNA or its scrambled control, using lipofectamine reagent (20 l per 35 mm culture dish). A portion of the cultures were retransfected 24 hours later. Twenty four hours after either the first, or the second transfection, the cultures were harvested. Cell lysates (equalized with respect to protein) were utilized for Western analyses, using either rabbit anti-cystinosin antibody (Lifespan) or mouse monoclonal anti-beta actin antibody (Santa Cruz Biotech) on the same blot.

For this reason we subsequently began to use 100 nM and 200 nM siRNA, as well as a reduced the level of our transfection reagent (lipofectamine), adding 10 l per culture dish rather than 20 l. In addition to conducting studies with the original 121 siRNA, we have conducted studies with 509 siRNA. The results shown in Fig. 4A indicate that when the cultures are incubated with 200 nM of 509 siRNA, the level of cystinosin was reduced (as compared with the scrambled control). Similar results were obtained with the 121 as well as the 509 sequence (Fig. 4B). A number of studies have been conducted. We are presently adding another control siRNA (that has no homology to known mRNAs) to our studies to insure that the siRNA treatment has no adverse effects.

In the study described above (in Fig. 4) a cell lysate from a human cell line (HepG2) was run in the SDS gels in parallel with the primary RPT cell lysates. Fig. 4A shows that in the lane with the HepG2 cell lysate, a band was observed, which had a molecular weight that is similar to that obtained with the primary RPT cell lysates.

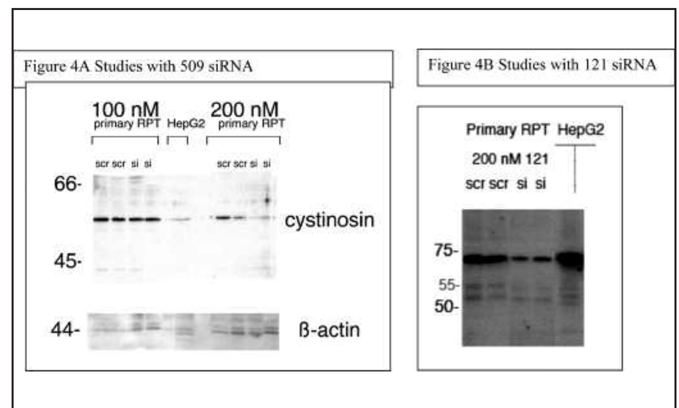

Figure 4A. Primary RPT cell cultures were transfected twice with 509 siRNA (or scrambled control 509) as described in Fig.3Experiments were conducted with either 100 nM or 200 nM siRNA. Twenty-four hrs after the second transfection, the cultures were lysed. Cell lysates were separated by SDS-PAGE. The protein was transferred to nitrocellulose, and the blot was subjected to a Western analysis of cystinosin and beta actin, using a rabbit anti-cystinosin antibody (Lifespan) and a mouse monoclonal anti beta actin antibody (Santa Cruz). Figure 4B. Results of a similar study with 200 nM 121 siRNA where the Santa Cruz Biotech antibody against cystinosin was used.

If cystinosin siRNA is to be useful in studies of progression of the disease, cystinosis, in culture, then knockdowns must occur over prolonged periods. To achieve these ends, the primary RPT cell cultures are being transfected over longer time periods, in parallel with functional studies (of cystine levels, apoptosis, and the localization of transport proteins). The transfection schema with siRNA is being modified so that knockdowns can occur over one week period. Fig.5 shows that the level of cystinosin is knocked down by cystinosin siRNA after 4 days of siRNA treatment. In the experiment, the second transfection is conducted 48 hrs after the first transfection, rather than 24 hrs. Now experiments are being conducted with a third transfection after 96 hrs (4 days), in parallel with functional markers.

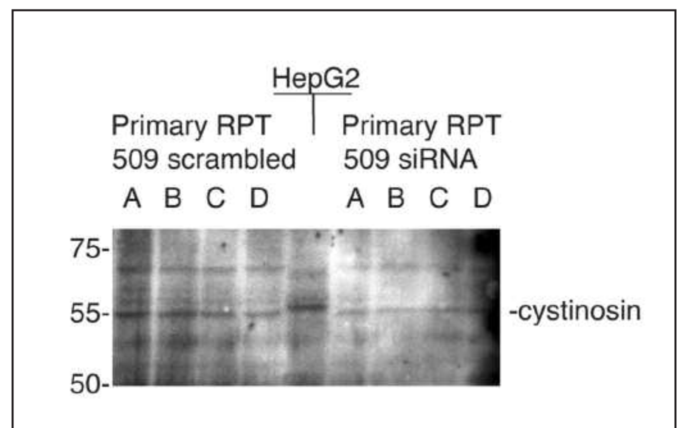

Figure 5. Primary RPT cells were transfected with 200 nM of either 509 cystinosin siRNA, or the scrambled control. A second transfection was conducted 48 hrs later. After 24 hrs parallel cultures were harvested, and subjected to a Western analysis using a mouse monoclonal anti-cystinosin antibody (Santa Cruz Biotech).

In order to obtain prolonged knockdowns of cystinosin, we have also conducted studies with primary RPT cells transduced with lentiviral particles containing a construct (pRNAT-U6). The lentiviral vector pRNAT-U6 utilized is illustrated in Figure 6. In our experiments, pRNAT-U6 contains a sequence encoding for shRNA complementary to either cystinosin 121 siRNA or the 121 scrambled control.

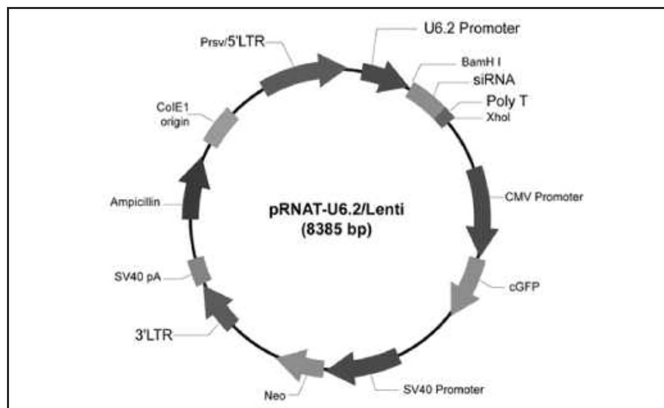

Fig 6. Lentiviral vector for in vitro delivery of siRNA to mammalian cells. To this end, a lentiviral vector (pRNAT-U6) containing siRNA targeting the cystinosin gene was prepared commercially (GenScript). pRNAT-U-6 containing either shRNA complementary to either 121 siRNA or 121 scrambled siRNA was incorporated into lentiviral particles, using the Block-It Lentiviral RNAi Expression System (Invitrogen).

Primary RPT cultures were transduced with lentiviral particles containing pRNAT-U6 with either cystinosin 121 shRNA or control scrambled 121 shRNA. After transduction, primary RPT cells containing the pRNAT vector were selected by incubation for 1 week in medium containing 0.4 mg/ml G418 (pRNAT has a neomycin resistance gene). The samples were harvested, and subjected to a Western analysis. The results (illustrated in Figure 7) are indicative of a knockdown of cystinosin by the shRNA. Subsequent studies are now in progress to examine cell growth, viability, as well as functional markers in the virally transformed primary RPT cell cultures.

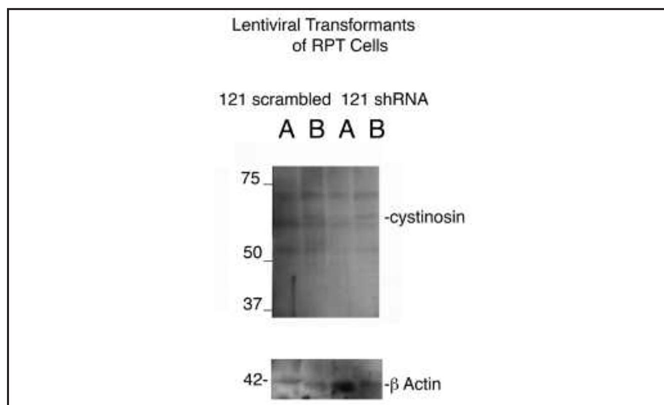

Figure 7. Primary RPT cells were transduced with lentivirus containing pRNAT-U6 with either 121 shRNA or 121 scrambled control shRNA. Subsequently, the cultures were incubated in medium containing 0.4 mg/ml G418 for 7 days. At the end of the incubation the cultures were harvested, and cell lysates were separated by SDS-PAGE. The blot was probed first with antibody against cystinosin (Life Span) and subsequently with anti-beta actin antibody (Santa Cruz Biotech.).

PROGRESS REPORT

HOLGER WILLENBRING, MD, MENTOR
SILVIA CARBAJAL, PHD, REARCH FELLOW

"Targeted Cell Fusion for the Correction of Tubulopathy Due to Cystinosis"

Period: 11/07/07-5/31/08

A. OBJECTIVE AND SPECIFIC AIMS

The objective of this work is use bone marrow-derived macrophages (BMM) to correct genetically encoded renal tubulopathies by delivering a healthy genome to renal proximal tubular cells. The specific aims of the project are:

Aim 1

To induce fusion of donor BMM with renal proximal tubular cells in vivo.

a) Induced in vivo fusion of BMM with CD46 expressing renal proximal tubular cells using the measles virus H and F proteins.

b) Assessment of the extent of donor BMM reprogramming to renal tubular function as exemplified by de novo expression of the transcription factor HNF4α.

c) Temporal limitation of expression of fusogenic measles virus proteins by means of an estrogen receptor specific for a synthetic ligand.

Aim 2: Targeting of donor BMM fusion specifically to renal proximal tubular cells.

a) Retargeting of the measles virus H protein by tethering it to a single chain antibody for DPPIV.

b) Assessment of efficiency and safety of specific induction of fusion between BMM and renal proximal tubular cells in vivo.

B. STUDIES AND RESULTS

Bone marrow-derived macrophages expressing MV H and F proteins fuse with CD46 positive renal cells in vivo.

In our previous report we showed that fusion between cells derived from the CD46+/- mouse model and cells expressing the H and F proteins of measles virus can be induced in vitro. We also demonstrated that bone marrow-derived nuclei are reprogrammed to express the renal tubular cell-specific transcription factor HNF4α, suggesting that bone marrow-derived cells can be used to genetically and functionally correct a wide range of tubulopathies including cystinosis.

To test if we can induce fusion between BMM and renal proximal cells *in vivo*, we transfected green fluorescent protein (GFP) expressing macrophages with a plasmid that expresses the MV H and F proteins, along with a plasmid that expresses Cre constitutively. After transfection, we injected the triple-transgenic BMM into the kidney of mice that express both human CD46 and a floxed lacZ reporter gene (CD46+/-, R26R+/-) in most cells. Seven days after injection, we found β-gal+ renal cells (Fig.1) reflecting activation of lacZ reporter gene expression by Cre and therefore cell fusion between BMM and recipient renal cells.

These results demonstrate that fusion of BMM with renal cells can be induced *in vivo* by transient expression of the measles virus H and F proteins.

C. ONGOING EXPERIMENTS

Optimization of fusion in vivo.

We are currently quantifying and characterizing the lacZ+ renal cells. We are also testing different conditions of the procedure with regard to the number of injected cells, volume of injection and analysis time after injection to optimize the experimental strategy.

Therapeutic potential of BMM expressing the measles virus H and F proteins in a mouse model of renal Fanconi syndrome.

Testing the therapeutic potential of fusion of BMM with renal proximal tubular cells requires a mouse model with kidney injury. To obtain this, we are currently breeding the *CD46+/- R26R+/-* mice described above with fumarylacetoacetate hydrolase *(FAH)* deficient mice. *FAH* is the last enzyme in the tyrosine degradation pathway and is normally expressed in both hepatocytes and proximal renal tubular cells. *FAH* deficiency causes mild renal cellular injury by an accumulation of the substrate fumarylacetoacetate *(FAA)*. However, the Fah-/- mouse develops chronic renal tubular disease if its hepatocytes are also deficient in homogentisic acid dioxygenase *(HGD)*, an enzyme upstream of *FAH* (1). As hepatocytes lacking *HGD* but not *FAH* have a strong proliferative advantage in the FAH deficient liver, they repopulate the livers of these mice. Hence, massive amounts of the *HGD* substrate homogentisic acid *(HGA)* are generated which are transported to the kidney and enter the tyrosine degradation pathway, thereby producing large amounts of toxic *FAA* in proximal tubules of the kidney.

As a result, *Fah-/-* mice repopulated with *HGD* deficient hepatocytes develop renal Fanconi syndrome. To use this model to test the therapeutic potential of induced fusion, we are going to transplant *HGD* deficient hepatocytes into *CD46+/-*, *R26R+/-*, *Fah-/-* mice to repopulate their livers and induce chronic renal tubular damage. Then, we will inject BMM expressing GFP, Cre and the measles virus H and F proteins into the kidneys of these mice to assess regeneration by fusion-derived renal proximal tubular cells.

We expect that fused *FAH* positive renal tubular cells will have a growth advantage over the *FAH* deficient cells, and therefore, will be able to repopulate and regenerate the damaged tubular epithelium of the recipient mice.

Targeting of donor bone marrow-derived macrophages specifically to renal proximal tubular cells.

In our initial specific aims we wanted to retarget BMM using measles virus H protein by tethering it to a single chain antibody specific for the renal proximal tubular cell antigen DPPIV. However, to continue to be able to use mice for these experiments and hence be able to detect cell fusion by X-gal staining (Fig. 1D) we decided to alter this strategy and use a bispecific antibody to target fusion of adoptively transferred BMM specifically to proximal tubular cells (2). This bispecific antibody binds to both a surface antigen specific f or proximal tubular cells (DPPIV) and macrophages (F4/80), hence forming a link between these two types of cells. In collaboration with Dr. Randall Lee at UCSF, we have tested the usefulness of this technology and we are going to inject BMM transiently expressing GFP, H and F and Cre and further armed with the bispecific antibody into the kidney, thereby directing their nonspecific fusion to proximal tubular cells. As *CD46+/-* mice, humans express CD46 on most cells and this strategy might be effective at targeting therapeutic cell fusion specifically to human renal proximal tubular cells.

D. REFERENCES

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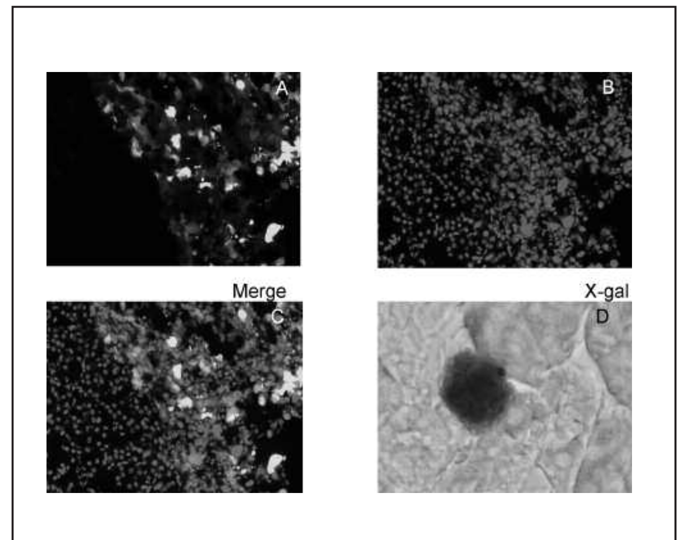

Fig. 1. Fused kidney cells after BMM transplantation. (A-C) Representative section from kidney of CD46+/-, R26R+/- mouse that was transplanted with GFP/H and F/Cre positive BMM stained for GFP (green) and the nuclear dye Hoechst (blue). (D) Representative image of β-gal+, that is fused renal cell.

PROGRESS REPORT

HOLGER WILLENBRING, MD, PRINCIPAL INVESTIGATOR
JOHN MCLAUGHLIN, PHD, CO-PRINCIPAL INVESTIGATOR

"Parthenogenetic Embryonic Stem Cells as a Source of Immunocompatible Renal Progenitor Cells for Therapy of Nephropathic Cystinosis"

Report 10/08

The goal of this research project is to achieve therapy of a mouse model of renal Fanconi syndrome (as it develops in nephropathic cystinosis) with kidney progenitors derived from immunocompatible pluripotent stem cells, specifically parthenogenetic embryonic stem cells (pESC) derived from mouse oocytes. For this purpose, we are (1) developing a cell culture protocol effective at directing pESC towards renal progenitor differentiation and we are (2) establishing a mouse model of renal Fanconi syndrome to test the therapeutic efficacy of the differentiated pESC. In the following, we describe the progress we have made towards achieving these aims.

SPECIFIC AIM 1: GENERATION OF KIDNEY PROGENITORS FROM PESC.

Aim 1.1: Derivation of pESC from oocytes carrying a reporter gene indicating mesodermal specification.

Our differentiation culture is based on a previously described protocol that uses mouse ESC with EGFP knocked into the brachyury (T) locus (*T-EGFP+/-*). This system enabled the identification of ESC that acquired mesodermal specification. In addition, these ESC carried a β-galactosidase marker gene for tracking after transplantation (*Rosa26*+/-, *T-EGFP*+/-). To generate pESC expressing the same marker genes, we have obtained *T-EGFP*+/- mice and are currently breeding these with *Rosa26*+/- mice to generate a cohort of female mice as oocyte donors for the derivation of pESC. As soon as a sufficient number of female *Rosa26*+/-, *T-EGFP*+/- mice is available, we will begin to derive pESC. To be able to establish cell culture conditions for renal differentiation of pESC during the time needed for expanding these mice, we have obtained the original *Rosa26*+/-, *T-EGFP*+/- ESC (Figure 1).

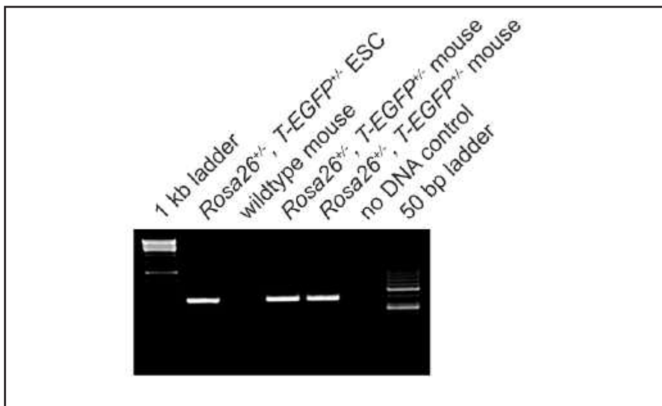

Figure 1: Genotyping (EGFP-PCR) of Rosa26+/-, T-EGFP+/- *mice and ESC.*

Aim 1.2: Differentiation of pESC into kidney progenitors in culture.

Rosa26+/-, *T-EGFP*+/- ESC were weaned of feeders and maintained under feeder-free and serum-free conditions on ultra low attachment plates. ESC were converted into embryoid bodies (EB) by removing LIF from the ESC medium. Subsequent culture in medium supplemented with Activin-A was used to induce mesodermal specification of EB cells. After several modifications of the original protocol, we were able to induce the characteristic pattern of initial up-regulation followed by down-regulation of brachyury expression in the ESC-derived cells (Figure 2). Brachyury is induced as cells commit to mesodermal specification but declines with further renal differentiation. As expected, progressive ESC differentiation was accompanied by loss of the pluripotency marker Oct-4. We are currently in the process of enriching the lineage-committed cells and optimizing their renal differentiation.

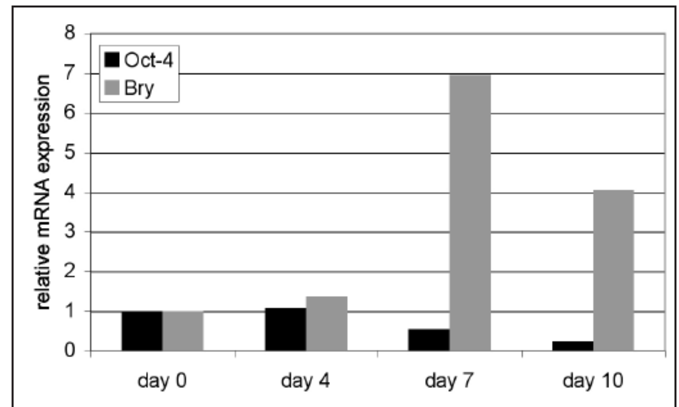

Figure 2: Quantitative reverse transcription PCR for brachyury and Oct-4 shows step-wise (day 0 to day 10) specification of cells in EB into mesoderm and further differentiated progenitors under culture conditions promoting renal differentiation.

SPECIFIC AIM 2: EVALUATION OF THE THERAPEUTIC POTENTIAL OF PESC-DERIVED KIDNEY PROGENITORS.

Aim 2.1: Transplantation of pESC-derived kidney progenitors into a mouse model of Fanconi syndrome to determine their function, proliferative capabilities, longevity and safety.

Since the mouse model of cystinosis does not recapitulate the kidney injury of the human disease, an alternative model is needed to test the therapeutic effectiveness of kidney cell therapy with derivatives of pESC. We proposed to induce renal Fanconi syndrome in *Fah*-/- mice by diverting tyrosine degradation from hepatocytes to proximal renal tubular cells. For this purpose, we aimed at repopulating the livers of *Fah*-/- mice with *Hgd*-/- hepatocytes (which are *Fah*+/+). In doing so, homogentisic acid, the substrate of *HGD*, will be diverted from hepatocytes to renal proximal tubular cells, the other cell type in the body capable of tyrosine degradation due to expression of the necessary enzymes including *FAH*. Renal proximal tubular cells remain *FAH*-deficient under these circumstances. Hence, these cells can be expected to encounter progressive damage due to the accumulation of homogentisic acid-derived metabolites.

When we transplanted hepatocyte suspensions isolated from *Hgd*-/- mice into *Fah*-/- recipients, we failed to observe liver repopulation, most likely due to immune rejection of the transplanted cells. Since *Hgd*-/- mice are on a pure C57Bl/6 strain background, the donor cell rejection was most likely caused by insufficient C57Bl/6 strain purity of our *Fah*-/- mice. (The *Fah*-/- mouse was originally generated in the 129S4 mouse strain. Hence, donor cells and recipient mice might differ in minor histocompatibility antigens.) To overcome this problem, rather than performing time-consuming back-breeding of the *Fah*-/- mice, we used immunodeficient *Fah*-/-, *Rag2*-/-, *c*-/- mice recently established in our mouse colony. After optimizing the postoperative antibiotic regimen, we achieved the desired progressive liver repopulation with hepatocytes from *Hgd*-/- mice in these animals (Figure 3). Based on our previous experience with this mouse model we expect liver repopulation to be complete at 6 weeks after transplantation. At that point we will begin screening these mice for signs of renal Fanconi syndrome including aminoaciduria (serine) and proteinuria. For this purpose, we have established in our laboratory an assay for the quantification of total protein in the urine (Figure 4).

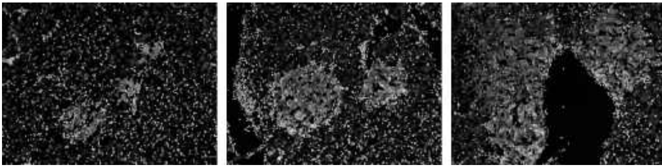

Figure 3: Progressive repopulation of livers of Fah-/-, Rag2-/-, γc-/- mice with donor Hgd-/- hepatocytes injected into the spleen. The donor hepatocytes have normal Fah alleles, hence express FAH (red immunostaining). Livers were analyzed at 2, 3 and 4 weeks after transplantation.

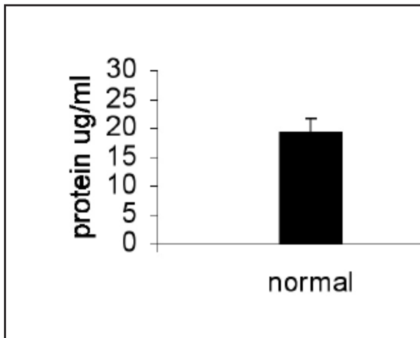

Figure 4: Normal range of proteinuria in the Fah-/-, Rag2-/-, γc-/- mouse strain used as recipients for the newly established model for renal Fanconi syndrome.

Aim 2.2: Transplantation of kidney progenitors derived from C57Bl/6 pESC into F1 hybrids of C57Bl/6 and Balb/c mice to determine the impact of hybrid resistance on kidney cell therapy.

We have not performed experiments pertaining to this sub-aim yet.