Cystinosis Research Foundation SCIENCE REPORT

PUBLISHED BY THE CYSTINOSIS RESEARCH FOUNDATION ★ FEBRUARY 2007

Welcome to Our First Edition of the *Cystinosis Research Foundation Science Report*

Less than four 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. Our funding efforts have allowed talented doctors and researchers to initiate novel research studies and to advance their research efforts.

This is our first edition of the *Cystinosis Research Foundation Science Report,* which is designed to provide research updates on studies underway and list the Abstracts and aims of the newly awarded funded studies. Our researchers are working in four countries around the world and their efforts are making a difference in the lives of our children.

Today, because of your commitment, the Cystinosis Research Foundation is the largest non-profit funder of Cystinosis research in the world.

2007 Call for Funding Proposals

The Cystinosis Research Foundation will announce a Call for Research and Fellowship Proposals in the spring and autumn of 2007. The 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. The Foundation has approximately $500,000 available for funding proposals 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 2007.

Cystinosis Research Foundation Fellowship Program

In 2006 the Cystinosis Research Foundation formed the first Cystinosis Fellowship Program in the United States to encourage young 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.

CRF is currently planning the first International Cystinosis Research Foundation Symposium scheduled for spring of 2008.

For more information visit www.natalieswish.org.

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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CYSTINOSIS RESEARCH FOUNDATION
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2006 RESEARCH STUDIES FUNDED AND COMMITTED: $2,313,962

FEBRUARY 2006

Ranjan Dohil, MD
University of California, San Diego
"A Study to Evaluate Enteric-Coated Cysteamine Therapy in Patients with Cystinosis"
$253,685 – 1-year clinical study

MAY 2006

Corinne Antignac, MD, PhD
Hospital Necker, Paris, France
"Characterization of Cystinosin Intracellular Trafficking"
$85,000 – 1-year study

Angela Ballantyne, PhD and Amy Spilkin, PhD
University of California, San Diego
"Academic Functioning in Cystinosis: A Comprehensive Study of the Process of Achievement"
$213,527 – 2-year study

Bruce Barshop, MD, PhD and Jerry Schneider, MD
University of California, San Diego
"API-4000 Tandem Mass Spectrometer for Cystinosis Research"
$118,400 – 2-year lease and maintenance

Elena Levtchenko, MD, PhD
University Medical Centre St. Radboud Nijmegen, The Netherlands
"Pathogenesis of Interstitial Renal Damage Leading to Renal Failure in Cystinosis"
$72,423 – 1-year study

Daniel Salomon, MD and Stephanie Cherqui, PhD
The Scripps Research Institute, La Jolla, California
"Treatment of Cystinosis Nephropathy Using Genetically Modified Adult Stem Cells in Murine Cystinosis Model"
$709,170 – 3-year study

DECEMBER 2006

Rita Ceponiene, MD, PhD
University of California, San Diego
"Neural Functioning in Auditory and Visual Systems in Cystinosis: Linking Brain to Behavior"
$287,918 – 2-year study

Francesco Emma, MD, Anna Taranta, PhD and Elena Levtchenko, MD, PhD
Bambino Gesù Children's Hospital and Research Institute, Rome, Italy and Radboud University Medical Centre Nijmegen, The Netherlands
"Transcriptional and Post-Transcriptional Regulation of the *CTNS* Gene"
$138,824 – 2-year study

Eric Moses, PhD, Mentor
Katy Freed, PhD, Research Fellow
Southwest Foundation for Biomedical Research, San Antonio, Texas
"Complex Genetic Approaches to Monogenic Disease: Genomic and Transcriptomic Dissection of Normal Expression of *CTNS*, the Gene Involved in Nephropathic Cystinosis"
$148,535 – 2-year study

Vasiliki Kalatzis, PhD, Mentor
Claire Hippert, Pre-doctoral Research Fellow
Institut Génétique Moléculaire Montpellier, Montpellier, France
"Gene Transfer Studies for Cystinosis"
$168,290 – 2-year study

Holger Willenbring, MD, Mentor
Kentaro Okamura, PhD, Research Fellow
University of California, San Francisco
"Targeted Cell Fusion for the Correction of Tubulopathy Due to Cystinosis"
$84,530 – 2-year study

Jess G. Thoene, MD
The University of Michigan
"Lysosomal Cystine Enhanced Apoptosis in Cultured Human Mesenchymal Stem Cells"
$33,660 – Laboratory supplies

***Jess G. Thoene, MD**
(Reinstated after Hurricane Katrina)
The University of Michigan
"Lysosomal Cystine Enhanced Apoptosis in Cultured Human Mesenchymal Stem Cells"
$150,000 – 18-month study

** Dr. Thoene's study was originally approved for funding in 2005 but because his lab at Tulane University was destroyed by Hurricane Katrina the study was cancelled. In November, 2006 Dr. Thoene's study was reinstated at the University of Michigan.*

For information about our 2007 Call for Funding Proposals or the Cystinosis Research Foundation Fellowship Program, visit www.natalieswish.org.

CORINNE ANTIGNAC, MD, PhD

Hospital Necker, Paris, France
"Characterization of Cystinosin Intracellular Trafficking"
$85,000 – 1-year study

ABSTRACT

Cystinosis is an inherited lysosomal storage disorder characterized by a defective lysosomal efflux of cystine. The causative gene, *CTNS*, encodes a lysosomal membrane protein, cystinosin, that contains 7 transmembrane 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.

The global aim of the research project is to characterize intracellular trafficking of cystinosin, and its potential role in lysosomal fusion. For that purposes, we will study lysosome formation and fusion in cell lines already available in the laboratory, stably overexpressing HA or GFP tagged-cystinosins, either the wild-type cystinosin or cystinosin deleted of one or both lysosomal targeting signals, by confocal microscopy in fixed cells using markers of the different intracellular organelles and by time-lapse fluorescence in live cells using the FRAP (Fluorescence Recovery After Photobleaching) technique, to visualize the kinetics of trafficking. A potential expression of the cystinosin-GFP proteins at the plasma membrane will be detected by surface biotinylation experiments.

In addition, to better assess the trafficking of cystinosin within the cell, we will identify which adapter protein (AP) subunits interact with cystinosin and study the consequences of their inactivation on the subcellular localization of cystinosin, using RNA interference and AP deficient cells. In order to identify other specific proteins interacting with cystinosin possibly involved in lysosome fusion, we will develop two complementary approaches: Cystinosin immuno-precipitation in cells overexpressing cystinosin, followed by electrophoretic separation of coimmunoprecipitated proteins and mass spectrometry identification, and a Blue Native/SDS-PAGE-based proteomic approach, to solve the whole cystinosin complex in intact lysososomes. The identification of the "cystinosin proteome" will be a valuable resource to understand cystinosin function and pathophysiological mechanisms of Cystinosis at the cellular level. Altogether, this project will allow a better comprehension of the pathways involved in cell trafficking of cystinosin and more generally of multimembrane-spanning lysosomal proteins and, ultimately, might identify a new role for cystinosin.

Specific Aims

The research program developed in this proposal is the continuation of the project funded by the Cystinosis Research Foundation in 2005. The global aim is to characterize intracellular trafficking of cystinosin. Lysosomal targeting of lysosomal membrane proteins as well as their intracellular trafficking has been widely studied for proteins with one transmembrane domain, such as Lamp-1 or Lamp-2. However, data concerning cystinosin, and more generally multimembrane-spanning proteins, are still lacking. Cystinosin represents a good model to study intracellular trafficking of complex lysosomal membrane proteins, since it contains two lysosomal targeting signals and since genetic studies in patients with various forms of Cystinosis allowed identifying multiple mutations altering its function or cell localization.

The specific aims of the projects are:

1. To characterize how cystinosin is sorted to the lysosome:
   - either by the endocytic or biosynthetic pathways (or both) ?
   - with which adaptors and within which protein complex ?
   - what is the respective role of each lysosome targeting signal we have identified ?
2. To identify proteins interacting with cystinosin. By characterizing the "cystinosin protein complex", we would like to address the question of an additional role for cystinosin apart from that of cystin efflux. We hypothesize that cystinosin (as detailed in the research project), possibly through this second sorting motif, might not only transport cystine across the lysosomal membrane, but might also play a role in lysosome fusion. The finding of a double function for cystinosin could explain why some symptoms of Cystinosis (i.e. the Fanconi syndrome) are not improved by lysosomal cystine depletion using cysteamine, and it could provide further insights into the understanding of the complex fusion machinery. This research program will be performed in the laboratory of Corinne Antignac, Inserm U574, Necker Hospital, Paris, France, in collaboration with Germain Trugnan (Inserm U538, Paris, France).

ANGELA BALLANTYNE, PhD, AND AMY SPILKIN, PhD

University of California, San Diego
"Academic Functioning in Cystinosis: A Comprehensive Study of the Process of Achievement"
$213,527 – 2-year study

ABSTRACT

Infantile nephropathic Cystinosis is a rare autosomal recessive disease of lysosomal storage, in which the amino acid cystine accumulates within the lysosomes of all cells in the body. Studies to date of cognitive functioning in Cystinosis have focused primarily on the areas of intelligence and visual processing skills. An area that has not been studied in depth, however, is that of academic functioning (i.e., school competence in areas such as reading, oral language, math, written language, and academic knowledge). The goal of this project is to be the first study to comprehensively examine academic functioning in children with Cystinosis using a comprehensive academic achievement battery. We will also assess academic competence 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).

Finally, we aim to examine processes underlying potential areas of deficit, by pinpointing where breakdowns in academic competence occur. The proposed study will provide parents and school personnel with a more thorough understanding of the academic profile of children with Cystinosis. Moreover, our findings may serve as the basis for the future application of intervention strategies that may lead to greater academic success.

RITA CEPONIENE, MD, PhD

University of California, San Diego

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

$287,918 – 2-year study

ABSTRACT

Infantile nephropathic Cystinosis is a rare autosomal recessive disease in which the amino acid cystine accumulates within the cell lysosomes. The most serious somatic condition is kidney failure. Nonetheless, brain, cognitive, and behavioral abnormalities are found even in individuals with compensated renal function. As our research has shown, among the most impaired cognitive skills are visuo-spatial abilities. In contrast, visual perception, reading, and spelling are relatively spared. However, to the best of our knowledge there have been no investigations of the neural mechanisms underlying cognitive and academic deficits in individuals with Cystinosis.

Therefore, 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 will be able to individually assess several brain functions. This unique opportunity is provided by the high temporal resolution of ERPs that register brain activity as it happens in real time. Specifically, we will assess auditory and visual perception, the strength and the breadth of auditory and visual spatial attention, and attentional orienting elicited by unexpected, novel events. By comparing brain functioning across the auditory and visual modalities, as well as across lower (sensory) and higher (attentional) cognitive levels, we will be able to determine the specific areas of weakness and/or sparing in this disorder.

Our second 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 the roles of early neuro-developmental insult vs. progressive brain injury in the pathogenesis of the disorder. *Our third goal is to bridge the structural brain abnormalities with cognitive and academic deficits in Cystinosis.* We will correlate ERP indices of brain function obtained in this project with cognitive measures obtained in the project "Academic Functioning in Cystinosis" funded by the Cystinosis Research Foundation, as well as structural brain measures obtained through an NIH grant awareded to Dr. Trauner.

Summarizing, this study will be the first investigation of neural mechanisms underlying cognitive deficits in children and adolescents with Cystinosis. It will determine specific areas of weakness and sparing across two main sensory modalities and two cognitive processing levels. These domains are among the major determinants of successful learning in everyday and academic settings. Importantly, the roles of neuro-structural and neuro-functional abnormalities in cognitive manifestations of the disorder will be determined. Therefore, our findings may serve as the basis for new medicinal and cognitive intervention strategies that may lead to greater personal and academic success of these children.

Specific Aims

1. Assess neural functioning in the visual domain. Obtain ERP and behavioral indices of visual sensory processing and visuo-spatial attention and define origins of the observed visuo-spatial deficits.
2. Assess neural functioning in the auditory domain. Obtain ERP and behavioral indices of auditory sensory processing and auditory spatial attention and determine the presence or absence of the deficit. Compare with the findings in visual modality.
3. Assess auditory and visual orienting to novel stimuli. Examine evidence for attentional in-flexibility and distractibility, frequent symptoms in neuro-developmental disorders.
4. Characterize age effects on auditory and visual sensory, spatial attention, and orienting functions in order to clarify whether progressive brain damage with age occurs.

RANJAN DOHIL, MD

University of California, San Diego

"A Study to Evaluate Enteric-Coated Cysteamine Therapy in Patients with Cystinosis"

$253,685 – 1-year clinical study

ABSTRACT

Regular cysteamine therapy is the mainstay of treatment for children and adults with Cystinosis. From measurements of leukocyte cystine levels and clinical experience we know that the drug works best if administered every six hours (1-3). However, results from a recent study showed that when cysteamine is administered directly into the small intestine the leukocyte cystine levels-lowering effect of cysteamine is prolonged (4).

The purpose of this study is to determine whether dose frequency can be reduced giving enteric-coated Cystagon™ capsules that dissolves rapidly in the small intestine. Cystagon™ is currently the only commercially available preparation of cysteamine. Patients taking Cystagon™ as part of their treatment for Cystinosis will be enrolled into the study and the effectiveness of their regular therapy will be assessed by measuring leukocyte cystine levels weekly for 4 weeks. After this period, the patient will be admitted to the Clinical Research Center at UCSD Medical Center for 1 week. Serial leukocyte cystine levels, plasma cysteamine and gastrin levels will be measured after three single doses of Cystagon™ given (1) at the usual dose in the usual form, (2) in the usual dose in the enteric-coated form, and (3) in twice the usual dose in the enteric-coated form. The patient will continue to take high-dose enteric-coated Cystagon™ twice daily for 4 weeks and leukocyte cystine levels will be measured weekly. The effectiveness of the treatments will be assessed by comparing leukocyte cystine levels during the four-week treatment periods. Side effects of the different treatments will be recorded by asking patients to keep a detailed diary.

Specific Aim

To determine the feasibility, efficacy and safety of specifically prepared enteric-coated Cystagon™ for the treatment of patients with Cystinosis.

FRANCESCO EMMA, MD, ANNA TARANTA, PhD ELENA LEVTCHENKO, MD, PhD

Bambino Gesu Children's Hospital and Research Institute, Rome, Italy and Radboud University Medical Centre Nijmegen, the Netherlands

"Transcriptional and Post-Transcriptional Regulation of the *CTNS* Gene"

$138,824 – 2-year study

ABSTRACT

In this project we propose a set of experiments aimed at characterizing a second isoform of the cystinosin protein, resulting from an alternative splicing of the exon 12 of the *CTNS* gene. This splicing modifies the amino acid sequence of the COOH tail, causing the protein to be expressed in other cell

compartments than lysosomes. Based on these preliminary results and on other results illustrated in the preliminary data section, the project is organized in 3 specific aims. **In specific aim #1** we will characterize the cell function of this newly identified cystinosin isoform, using rescue experiments, and analyze the relative contribution of the two known cystinosin isoform to the Cystinosis phenotype. Experiments proposed in **specific aim #2** are aimed at looking at the expression of cystinosin isoforms under various experimental conditions and in different human tissues. Together, these results will generate data that will be used in **specific aim #3** to define elements of the *CTNS* promoter that respond to physiological stimuli associated with increased *CTNS* transcription.

SPECIFIC AIM #1: Characterization of the cystinosin-LKG isoform *(hypothesis: cystinosin-LKG plays a role in cell cystine/cysteine metabolism and may be responsible, at least in part, for the cystinotic phenotype in the kidney).*

As stated before, one of the aims of this project is to begin studying the role of a newly identified cystinosin isoform, which is present in the renal cortex, although apparently less abundantly expressed then the "classic" lysosomal protein. Since all currently reported mutations in NC are predicted to alter the function of both isoforms, biological samples obtained from NC patients are not informative on the selective role of each isoform in the cell. We therefore propose to use immortalized proximal tubular epithelial cells derived from patients with NC that do not express the cystinosin protein (deletion of the *CTNS* gene). The phenotype of these cells will be alternatively rescued by one isoform. Cells will be tested under basal conditions and after exposure to apoptotic or oxidative stimuli. Measured parameters will include intracellular ATP levels, glutathione metabolites, lipid peroxidation, apoptosis rate and activities of mitochondrial respiratory chain complexes (I-V).

SPECIFIC AIM #2: Analysis of the relative expression of the cystinosin isoforms *(hypothesis: the analysis of the relative expression of the two known cystinosin isoforms will provide insights into the cell functions of these two proteins).*

In preliminary results illustrated in the following section, we have found that transcription of the *CTNS* gene is increased in conditions of intracellular cystine and/or glutathione depletion. In this specific aim, we propose to study the differential expression of the cystinosin-LKG and cystinosin-QLN isoforms in conditions of cystine depletion and after inhibiting or stimulating GSH synthesis. In addition, the relative expression of these two isoforms, which we have documented to date only in the renal parenchyma, will be studied in other tissues.

SPECIFIC AIM #3: Characterization of the cystinosin promotor *(hypothesis: transcription of the CTNS gene is stimulated by conditions that require increased delivery of cystine to the cell – the molecular characterization of stimuli that stimulate CTNS transcription will increase our knowledge on the function of this protein).*

In this specific aim we propose to start characterizing elements of the *CTNS* promoter that respond to specific stimuli. To perform these studies we will use a reporter gene system that will be stimulated based on our previous results that have shown increased *CTNS* gene transcription during cystine/GSH depletion and on results that will be obtained in experiments proposed in specific aim #2. The goals of this specific aim are limited to characterize sensitive regions of the promoter. If successful, these results will allow subsequently to analyze cis- and trans-acting factors that regulate *CTNS* gene expression.

ERIC MOSES, PhD, MENTOR
KATY FREED, PhD, RESEARCH FELLOW

Southwest Foundation for Biomedical Research, San Antonio, Texas

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

$148,535 – 2-year fellowship

ABSTRACT

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. Specifically, we hypothesized that it is likely that there are 'upstream' modifier loci that influence quantitative expression of *CTNS* and that genetic variation in *CTNS* influences the expression of other undefined 'downstream' genes playing key roles in the biological processes perturbed in this disease.

We argue that for those cystinotic individuals where there is incomplete loss of function of the *CTNS* gene product, cystinosin, 'upstream' trans-acting regulatory genes become possible targets for focused therapeutic intervention aimed ultimately at producing more cystinosin. Using a genomewide 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 project we propose 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. Genes downstream of *CTNS* will be directly influenced by the effects of genetic variation in *CTNS* and can therefore be identified by direct genetic association analyses. To this end, we have recently enumerated all sequence variation in the *CTNS* gene in our study families and we now propose to use this information to identify associated downstream genes. In this current study we propose to identify sequence variation within 2 kb of the proximal promoter of our top ten prioritized downstream genes as this is the most likely region to contain functional regulatory variants that may define a likely route to the identification of drugable targets.

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 and may be consistent with the previous observation that heat shock in cystinotic cells appears to reduce lysosomal cystine content "probably mediated by an altered substrate affinity for another lysosomal transport protein" (Lemons et al., 1986). Further characterization of Stub1 is now indicated for validation as a potential therapeutic target to take up more of the residual biological burden that is the consequence of a non-functional *CTNS* gene. When we have completed the characterization of these *CTNS*-interacting genes we will be in a strong position to undertake further studies to validate these functional relationships and associated (patho)biological sequelae using clinical material (e.g., human cystinotic cell lines) and the *CTNS* knockout mouse.

Specific Aims

Our proposed post-doctoral research fellowship proposal builds on several novel findings from the initial phase of our Cystinosis research program and involves several specific aims.

1. To comprehensively resequence the VPS13A gene, a putative upstream trans-acting regulator of *CTNS*, in182 individuals and type all identified sequence variants in 1,240 individuals. We will then perform Bayesian quantitative trait nucleotide analysis to identify the most likely functional variants and determine the downstream effects of such variation on other expression-based phenotypes (including *CTNS*).
2. We have identified a number of genes that are obligatorily downstream of *CTNS*. Several of these genes also show evidence of cis-regulation (i.e., self regulation). In order to clarify the downstream pathway we propose to re-sequence approximately 2 kb of the proximal promoter for ten such genes in 182 individuals. We will then type all identified sequence variants in 1,240 individuals. We will then perform association analyses to identify the most likely functional variants and determine the downstream effects of such variation on other potentially downstream expression-based phenotypes.
3. To comprehensively resequence the STUB1 gene, a putative functionally related gene to *CTNS*, in 182 individuals and type all identified sequence variants in 1,240 individuals. We will then perform Bayesian quantitative trait nucleotide analysis to identify the most likely functional variants and determine the downstream effects of such variation on other expression-based phenotypes (including *CTNS*).

4*. To verify by quantitative RT-PCR the down or up-regulation of the VPS13A, STUB1 and downstream genes identified in Aim 2 above, in patient and mouse cell lines.

5*. To perform experiments in human (including patient) and mouse (*Ctns -/-*) cell lines to determine the subcellular localization of the VPS13A and STUB1 proteins and if found to colocalize with cystinosin to test for interactions between them and cystinosin. We will perform the same experiments for proteins whose expression is modulated with cystinosin.

** These experiments will involve the proposed post-doctoral fellow (Dr. Katy Freed) traveling to Paris and working under the direction of our collaborator Dr. Corinne Antignac.*

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

Institut Genetique Moleculaire Montpellier, Montpellier, France

"Gene Transfer Studies for Cystinosis"

$168,290 – 2-year fellowship

ABSTRACT

The aim of our work is to explore the possibility of alternative treatments for Cystinosis using viralmediated gene transfer. This strategy has been successful in numerous cases of defective lysosomal hydrolysis but has never been used for correcting lysosomal transport. There will never be one viral vector that can treat all tissues equally well thus, rather than a multisystemic approach, we are initially concentrating on characterising and targeting the ocular and CNS anomalies in our animal model (*Ctns -/-* mice). Our recent data suggests that the ocular anomalies in *Ctns -/-* mice closely resemble those of patients. Furthermore, for the first time, we have brought to light marked age-related spatial and working memory defects.

We propose to compare the efficiency (in terms of titre, tropism and transduction) of two different viral vectors, which can effectively transduce different tissues of the eye and CNS: a helper-dependent (HD) canine adenovirus (CAV-2) and an adeno-associated virus vector (AAV-8). Aside from the fact that these vectors are both capable of long-term gene expression in the absence of toxicity, they have been chosen based on their tropism: HD CAV-2 vectors transduce the cornea, iris and retina of the eye, and are neurotropic and capable of a high level of retrograde transport in the CNS; AAV-8 vectors transduce the cornea and neurons. We will perform targeted gene transfer studies using both these vectors and compare the efficiency of cystine clearance in parallel with that of cysteamine. Taken together, this work will use the sole-existing Cystinosis animal model to further the understanding of the pathogenesis of Cystinosis, and to develop an alternative long-lasting treatment, which treats the cause of the disease rather than the symptoms.

Specific aims

The causative gene of Cystinosis, *CTNS*, encodes a novel protein, named cystinosin. Corinne Antignac's laboratory, of which I was previously a member, generated a knock-out mouse (*Ctns -/-*) model for the disease. Our research project is to use viral-mediated gene transfer to reduce lysosomal cystine levels in vivo. The efficiency of cystine clearance will be compared to that obtained with cysteamine, the drug currently administered to patients. Initially in this proposal, we will primarily target the ocular and CNS anomalies associated with this disease, which can be incapacitating or potentially life-threatening. Our project is divided into 3 main subjects:

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.

ELENA LEVTCHENKO, MD, PhD

University Medical Centre St. Radboud Nijmegen, The Netherlands

"Pathogenesis of Interstitial Renal Damage Leading to Renal Failure in Cystinosis"

$72,423 – 1-year study

ABSTRACT

End stage renal disease is the leading cause of morbidity in patients with Cystinosis. In this project we will focus on the mechanism of progressive renal damage in Cystinosis for identifying potential therapeutic interventions additional to cysteamine therapy to arrest the deterioration of the renal function in Cystinosis.

The project is based on the following observations:

1. The deterioration of the renal function in Cystinosis is associated with progressive development of tubulo-interstitial lesions.
2. Albuminuria, a major determinant of renal disease progression, is observed in patients with Cystinosis. Our previous study of urinary albumin excretion in Cystinosis demonstrated the glomerular origin of albuminuria.

3. Recent studies in cystinotic fibroblasts, granulocytes and proximal tubular cells demonstrated by us and others altered glutathione status, suggesting increased oxidative stress, which might up-regulate the secretion of pro-inflammatory cytokines and cause renal interstitial inflammation and fibrosis. Recently our group developed a valid in vitro model of Cystinosis – mature human proximal tubular cells carrying a well-defined genetic defect, which provides a powerful tool to study the pathogenetic mechanisms of Cystinosis. For the investigation of the pathogenesis of tubulo-interstitial damage in Cystinosis, via a concerted endeavour of the departments of Pediatrics, Pharmacology-Toxicology and Human Genetics of Radboud University Nijmegen

Medical Centre, we will address the following key-objectives:

1. Study of cytokines/chemokines and endothelin-1 production in mature proximal tubular cells from patients with Cystinosis compared to healthy controls at different stages of cystine accumulation and after albumin application The chemokines MCP-1, interleukin 8 and RANTES together with the cytokine TGF-_ and the vaso-active substance endothelin-1 are selected as being the possible mediators for the induction of interstitial fibrosis. These mediators will be measured in supernatants of the proximal cultured cells at different levels of cystine accumulation and with and without the addition of albumin.

   In order to induce interstitial fibrosis, the selected mediators should be transferred to the basolateral side of the proximal tubules. Using Transwell chambers, in which the apical compartment is separated from the basolateral compartment, it is possible to evaluate the polarity of the secretion. The mediators can be measured in both compartments after application of albumin at the apical side. With a cytokine antibody array we will be able to expand the number of possible mediators, initiating new directions of research.

2) Study of intracellular glutathione status, transport and generation of reactive oxygen species (ROS) in the cell model mentioned above

   In the cell model mentioned under 1, we will determine free and total glutathione and the activity of the enzymes of glutathione redox cycle. Secondly, we will perform a quantitative estimation of superoxide and ROS production using dihydroethidium and CM-H2DCFDA fluorescent probe at different stages of cystine accumulation and after albumin application. Furthermore, we will estimate ROS induced proximal tubular cell damage by measuring lipid peroxidation. The effect of antioxidants (N-acetylcysteine (NAC) and catalase) and cystine depleting agent cysteamine will be evaluated.

   The elucidation of cell biological mechanism leading to renal failure will set a paramount step towards improvement of therapy in patients with Cystinosis.

Specific Aims

The aim of this project is to reveal the pathogenesis of progressive renal damage in patients with Cystinosis. We will focus on the role of cytokines/chemokines, endothelin-1 and reactive oxygen species (ROS) generation as mediators of interstitial damage for identifying possible therapeutic interventions to prevent or to arrest tubular interstitial fibrosis. Our ultimate aim is to stop the progressive decline of renal function in Cystinosis.

HOLGER WILLENBRING, MD, MENTOR
KENTARO OKAMURA, PhD, RESEARCH FELLOW

University of California, San Francisco

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

$84,530 – 2-year fellowship

ABSTRACT

Recent findings have revealed that cell fusion between transplanted bone marrow cells and host proximal tubular cells produces functional cells that can regenerate the tubular compartment and cure renal Fanconi syndrome. In principle, by delivering a normal genome, cell fusion affords a much needed opportunity for the correction of genetically encoded tubulopathies including Cystinosis. Since cell fusion occurs independently of stable bone marrow engraftment facilitated by toxic myeloablation, it has potential as a safe and well-tolerated therapy. Moreover, it renders readily available and expandable cells such as bone marrow-derived macrophages useful for cell therapy.

Unfortunately, the realization of cell fusion as a therapy for nephropathic Cystinosis is hampered by its low frequency. However, it is conceivable that the severe tubular injury inflicted by Cystinosis could result in the proliferation of corrected cells, which would aid the regeneration process. To significantly induce fusion between donor macrophages and host renal proximal tubular cells I will utilize the targeted fusogenicity of measles virus. Measles virus fusion is initiated by an attachment protein that upon binding to a specific cellular receptor signals to a fusion protein that executes the fusion process. Importantly, both attachment and fusion protein sequences can be dissociated from the virus and are therefore safe for human application. I will transiently express these sequences in donor macrophages which I will transplant into healthy mice as well as models of renal Fanconi syndrome. Importantly, by utilizing mice transgenic for human CD46, I will exploit the narrow tropism of the measles virus attachment protein to induce cell fusion and limit its targets. These experiments will not only aim at increased fusion frequencies but they will also reveal the reprogramming efficiency and safety associated with this approach.

Finally, I will use a single-chain antibody that has recently been developed to effectively target a recombinant protein to rat renal proximal tubular cells in vivo to redirect measles virus protein-mediated fusion to these cells. These studies will reveal the efficiency and safety of targeting donor cell fusion specifically to renal proximal tubular cells. Provided it is efficient and safe, using readily available hematopoietic donor cells for targeted cell fusion would have potential as a therapy for genetically encoded, human tubulopathies such as Cystinosis.

Specific Aims

Aim 1: Induced fusion of donor BMM with renal proximal tubular cells in vivo.

a) Induced in vivo fusion of BMM to CD46 expressing renal proximal tubular cells using standard MV H and F proteins.
b) Assessment of the extent of donor BMM reprogramming to renal tubular function as exemplified by de novo expression of HNF4a.
c) Temporal limitation of expression of fusogenic MV 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 MV H protein by tethering it to a scFv for DPPIV.
b) Assessment of efficiency and safety of specific induction of fusion between BMM and renal proximal tubular cells in vivo.

DANIEL SALOMON, MD AND STEPHANIE CHERQUI, PhD

The Scripps Research Institute. La Jolla, California

"Treatment of Cystinosis Nephropathy Using Genetically Modified Adult Stem Cells in Murine Cystinosis Model"
$709,170 – 3-year study

ABSTRACT

Cystinosis is a metabolic hereditary disease characterized by intracellular accumulation of cystine. Affected individuals typically present proximal renal tubular dysfunction (the renal Fanconi syndrome) before one year old and, without specific treatment progress to end-stage renal failure by the end of the first decade. Other tissues are damaged by cystine accumulation. The drug cysteamine reduces the intracellular concentration of cystine. However, the need for regularly spaced doses and a number of undesirable side effects render its administration difficult.

Moreover, the proximal renal tubulopathy is not sensitive to treatment with cysteamine. Patients with renal failure require dialysis or renal transplantation, both of which have significant negative health effects and due to the severe shortage of donor organs, patients may wait three to six years for transplantation. Adult stem cells offer a promising alternative for the treatment of the nephropathy of Cystinosis. Indeed, it is now proven that bone marrow-derived stem cells can give rise to renal cells after migrating to the kidney, particularly in the setting of a progressive renal injury. Because bone marrow-derived stem cells also have the capacity to colonize essentially any organ, the possibility must be considered that this approach may also ameliorate the multi-systemic defects seen in children with Cystinosis.

The idea would be to isolate adult stem cells from the bone marrow of patients, genetically modify them ex vivo to introduce the defective gene (*CTNS*) and inject them back into the patients. As a proof of concept, we will use the *Ctns* -/- murine model for Cystinosis. These animals accumulate cystine and cystine crystals in all organs tested. *Ctns* -/- mice develop ocular changes similar to those observed in affected humans as well as bone and muscular defects and behavioral anomalies. However, the *Ctns* -/- mice do not develop proximal tubulopathy or renal failure despite the fact that they accumulate a significant quantity of cystine in the kidney and exhibit histological anomalies (i.e. giant mitochondria). Thus, we will determine cystine content and histology in the kidney as the measure of the efficacy of transplanting and repairing kidney injury with *CTNS*-expressing adult stem cells. We will test the hypothesis that certain growth factors known to drive differentiation of renal lineage cells will increase the functional engrafment and repair. The potential that *CTNS*-expressing stem cells will also engraft in other tissues and reduce cystine levels will be tested.

Finally, we will propose a strategy to optimize the murine model for the renal defects in order to better represent the nature of the human disorder and support this work as proof of concept for clinical application.

Specific Aims:

Cystinosis is a metabolic hereditary disease characterized clinically by generalized proximal renal tubular dysfunction (Fanconi syndrome) and biochemically by intracellular accumulation of cystine. Affected individuals typically present before two years old with symptoms of a severe fluid and electrolyte disturbance (vomiting, poor growth, rickets). Without specific treatment they will progress to end-stage renal failure by the end of the first decade. Other tissues (notably the cornea and thyroid gland) are also damaged by cystine accumulation. The drug cysteamine reduces the intracellular concentration of cystine, and, if used early in the disease and in high doses, can reduce the subsequent progression of renal glomerular damage and improve childhood growth.

However, the need for regularly spaced doses (every 6 hours) and a number of undesirable side effects such as digestive intolerance and persistent odor, render its chronic administration difficult. Moreover, the proximal renal tubulopathy is not sensitive to cysteamine. Patients with end-stage renal failure require dialysis or renal transplantation, both of which have significant negative health effects and due to the severe shortage of donor organs, patients may wait three to six years for transplantation.

Stem cell transplantation is a promising therapy now being considered for the treatment of renal disorders (Mollura et al., 2003). Indeed, it is now proven that bone marrow stem cells can migrate to a damaged kidney and there give rise to kidney cells. The objective of this proposal is to test the hypothesis that transplantation of autologous adult stem cells expressing a functional *CTNS* gene can be used to treat and prevent the renal defects of Cystinosis. It may also be possible to use the same approach to minimize cystine accumulation in other tissues. Thus, our strategy in the long term is to isolate stem cells from the bone marrow of the patients, genetically modify them ex vivo to introduce a functional version of the defective gene (*CTNS*). A key point is that the use of the patient's stem cells would avoid the risks of rejection, graft vs. host disease and the serious complications of immunosuppression.

One approach would be to inject these gene-modified cells directly into the renal artery of the patient. This method may allow the replacement of part of the renal cells of the patient by the genetically modified cells and correct the phenotype. If successful this intervention would delay or avoid the evolution of the tubular injury and renal failure.

However, as bone marrow stem cells have the capacity of colonizing essentially any organ, a second approach would be to engraft gene-modified stem cells into the patient's bone marrow compartment and correct the cystine accumulation in other organs. Indeed, successful engraftment of stem cells with the functional *CTNS* gene into the bone marrow compartment will serve as a source of stem cells to any injured tissue for the life of the patient. Thus, as renal injury is chronic and progressive, it makes more sense to us that this strategy be adopted, early in the course of the disease's progression. In the laboratory of Dr. Corinne Antignac (Hôpital Necker-Enfants Malades, France) I participated in the identification of the gene underlying Cystinosis, *CTNS*, in 1998 using a positional cloning approach (Town et al., 1998). *CTNS* encodes a novel, 367 amino acid protein called cystinosin that has 7 transmembrane domains. By construction of a cystinosin-GFP fusion protein, I demonstrated that cystinosin is a lysosomal membrane protein and that this sorting requires the presence of the GY-DQ-L motif on 3 the C-terminal cytoplasmic tail of the protein, and a novel sorting motif localized in the third cytoplasmic loop (Cherqui et al., 2001).

Next, we demonstrated that cystinosin represents a novel H+-driven transporter, which is responsible for cystine export from lysosomes (Kalatzis et al., 2001). I also cloned and characterised the murine homologue of *CTNS*, *Ctns* (Cherqui et al., 2000), and generated a *Ctns* knock-out mouse model (Cherqui et al., 2002). *Ctns* -/- mice accumulated cystine in all organs tested, and cystine crystals, pathognomonic of Cystinosis,

were observed. *Ctns* -/- mice developed ocular changes similar to those observed in affected patients, bone and muscular defects and behavioral anomalies. We tested the effect of oral administration of the drug cysteamine and demonstrated the efficiency of this treatment for cystine clearance in the *Ctns* -/- mice. Thus, this animal model is a unique tool for testing emerging therapies for Cystinosis. Though we acknowledge that *Ctns* -/- mice do not develop overt proximal tubulopathy or renal failure, they accumulate a very significant amount of cystine in the kidney as well as demonstrate histological anomalies. Therefore, measurements of kidney cystine content and histology will be used to determine the efficacy of our strategy using genemodified stem cells.

Specific aim 1: Proof of concept and optimization. There is now evidence supporting transplantation of either hematopoietic stem cells (HSC) or mesenchymal stem cells (MSC) in experimental models of renal injury. Thus, we will isolate and compare both types of bone marrow-derived adult stem cells from mice transgenic for the Green Fluorescent Protein (GFP) and transplant them into lethally irradiated *Ctns* -/- mice. The kidneys will be analyzed by histology at different time points to determine the quantity and location of genetically modified bone marrow stem cells integrated and the differentiation of the cells as a function of time. The kidney will also be analyzed by measuring the cystine content. In parallel, we will test whether treating stem cells in vitro with growth factors known to drive renal cell differentiation will improve the parameters tested above.

Specific aim 2: *Ctns* gene delivery. The most efficient cells, HSC or MSC, determined in Specific aim 1, will be isolated from *Ctns* -/- mice and genetically modified ex vivo to express the *Ctns* gene using lentivirus vectors. The cells will then be transplanted into lethally irradiated *Ctns* -/- mice. The kidneys will be analyzed to test the efficiency of genetically modified bone marrow stem cells expressing *Ctns* to decrease the cystine content and to reverse the histologic anomalies in the kidney of the *Ctns* -/- mice.

Specific aim 3: Analysis of the other organs of the *Ctns* -/- mice for the colonization of transplanted, gene-modified bone marrow stem cells. The liver, the pancreas, the skeletal muscle, the heart and the brain from the mice will be analyzed for the integration of transplanted stem cells. The organs will be tested for cystine content and histology.

Specific aim 4: Optimization of the murine Cystinosis model for development of nephropathy (Fanconi syndrome and end stage renal failure). One hypothesis is that cystine-mediated cellular injury in renal tubular cells is accelerated by oxidative stress.

Thus, we will backcross *Ctns* -/- mice with two different strains of superoxide dismutase deficient mice. A second strategy would be to create transgenic mice with the viral 4 thymidine kinase (vTK) gene expressed specifically in renal tubular epithelial cells.

Gancyclovir treatment of the animals should create tubular injury and death in a dosedependent fashion as previously demonstrated by others in a liver failure model. The hypothesis is that the greater the kidney injury, the greater the integration and differentiation of the stem cells.

BRUCE BARSHOP, MD, PhD AND JERRY SCHNEIDER, MD

University of California, San Diego

$118,400 – 2-year lease and maintenance

"API-4000 Tandem Mass Spectrometer for Cystinosis Research"

ABSTRACT

This funding was for a two-year lease and maintenance of a API-4000 Tandem Mass Spectrometer needed for investigative research for Cystinosis at the Biochemical Genetics Laboratory at the University of California, San Diego. Dr. Jerry Schneider's Cystine Determination Laboratory has merged its operations with the Biochemical Genetics Laboratory directed by Dr, Bruce Barshop at UCSD. Samples are still prepared with the same stringent method to assure optimal reliability, but the last step of cystine quantitation now makes use of tandem mass spectrometry (MS/MS). As a result, the method no longer requires radioactivity, and the processing time is faster. The MS/MS method was adapted from that developed by Dr. Neil Dalton at Guy's Hospital in London, and it requires a particularly sensitive instrument. The generous support of the Cystinosis Research Foundation allowed the laboratory to lease an API-4000 for this purpose. The MS/MS looks at very specific fragmentation patterns that chemicals undergo, and by monitoring the molecular mass of a chemical and its fragment, its amount can be determined even in highly complex mixtures of other chemicals, e.g. the amount of cystine in the whole extracts of white blood cell. We extensively tested the new method in parallel with the previous radiochemical method, and found it to give virtually identical results with comparable sensitivity and considerably faster analysis time. We will be able to continue to serve the diagnostic and monitoring needs of our patients more quickly, but also because of the faster processing time, we will be able to study the stability of samples collected with simplified methods of white blood cell preparation. We also have developed an MS/MS method to assay cysteamine, and plan to look into details of the pharmacology and intracellular processing of cysteamine.

JESS G. THOENE, MD (Reinstated after Hurricane Katrina)

The University of Michigan

"Lysosomal Cystine Enhanced Apoptosis
in Cultured Human Mesenchymal Stem Cells"

$150,000 – 18-month study

$33,660 – Laboratory supplies

ABSTRACT

The Cystinosis Research Foundation awarded a two year grant to Dr. Thoene at Tulane in June, 2005 entitled " Lysosomal Cystine Enhanced Apoptosis in Cultured Human Mesenchymal Stem Cells". The award was for $200,000. Because of Hurricane Katrina, the work was interrupted shortly after it was begun. Initial progress showed apoptosis in these cells due to cystine loading, but the major part of the studies were suspended during the aftermath of the disaster. Now, Dr. Thoene has moved to the University of Michigan where he is a member in the Division of Pediatric Genetics and Director of the Biochemical Genetics Laboratory. The CRF grant has moved with him. There are, however additional costs to re-establish the lab at the University of Michigan, beyond what was present at Tulane.

Specific Aims:

To cover incremental lab costs associated with establishing a new laboratory at the University of Michigan

PROGRESS REPORT

A STUDY OF THE COGNITIVE DOMAIN OF EXECUTIVE FUNCTIONING IN INDIVIDUALS WITH CYSTINOSIS

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

18-Month Progress Report: 8/1/06 – 1/23/07

A. SPECIFIC AIMS

1) This study will provide the first set of comprehensive neuropsychological data on executive functioning in individuals with Cystinosis, from childhood through adulthood, using a cutting-edge assessment system (Delis-Kaplan Executive Function System). The importance of executive functioning cannot be underestimated because it is vital to the adaptive behavior and "real world success" of the individual, whether it be at home, school, or in the workplace.
2) This study will also provide meaningful questionnaire data from individuals, teachers, parents and/or caretakers on the daily functioning of individuals with Cystinosis. It is essential for parents and teachers to have the opportunity to describe the child's real world problems in executive functioning. This real world data will complement the neuropsychological testing data to provide meaningful clinical correlates to the test data, which in turn, will lead to a greater understanding of the implications of the executive functioning deficits.
3) This study has tremendous implications for everyday functioning and quality of life for individuals with Cystinosis and their families. It is only by understanding an individual's abilities to initiate, plan, organize, and solve problems that we can begin to implement intervention strategies that will optimize the functioning of the individual.

B. PROGRESS TO DATE: 8/1/06 – 1/23/07

Over the past 6 months of the study we successfully recruited, inducted, and tested 6 individuals with Cystinosis and 5 control participants. Currently, we have complete data on a total of 24 individuals with Cystinosis and 21 matched control participants. Hence, we have made substantial progress toward the goals of this study. We have 4 more Cystinosis participants scheduled (or in the process of scheduling) and have 2 others who have agreed to participate. Therefore we do not anticipate difficulty reaching our goal of 30 individuals with Cystinosis. We are also continuing to schedule matched control participants and should be able to reach our goal by the end of the grant period.

C. RESULTS

On all 45 participants to date, we have collected D-KEFS, Wechsler IQs, and the age-appropriate executive functioning questionnaires (BRIEF or FrSBe). We have also scored, reliability checked, and data entered all of the test and questionnaire data collected so far.

Using the data collected for this project, we have had two scientific posters accepted for presentation at the 2007 American Neuropsychiatric Association (ANPA) 18th Annual Meeting (Feb 17 – 20, 2007).

PROGRESS REPORT

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

PRINCIPAL INVESTIGATORS:
DANIEL R. SALOMON, MD AND STEPHANIE CHERQUI, PhD

Period: 07/06 – 12/06

Abstract of the project: Cystinosis is a metabolic hereditary disease characterized by intracellular accumulation of cystine. Affected individuals typically present proximal renal tubular dysfunction (the renal Fanconi syndrome) before one year old and, without specific treatment progress to end-stage renal failure by the end of the first decade. Other tissues are damaged by cystine accumulation. The drug cysteamine reduces the intracellular concentration of cystine. However, the need for regularly spaced doses and a number of undesirable side effects render its administration difficult. Moreover, the proximal renal tubulopathy is not sensitive to treatment with cysteamine. Patients with renal failure require dialysis or renal transplantation, both of which have significant negative health effects and due to the severe shortage of donor organs, patients may wait three to six years for transplantation.

Adult stem cells offer a promising alternative for the treatment of the nephropathy of Cystinosis. Indeed, it is now proven that bone marrow-derived stem cells can give rise to renal cells after migrating to the kidney, particularly in the setting of a progressive renal injury. Because bone marrow-derived stem cells also have the capacity to colonize essentially any organ, the possibility must be considered that this approach may also ameliorate the multi-systemic defects seen in children with Cystinosis.

The idea would be to isolate adult stem cells from the bone marrow of patients, genetically modify them ex vivo to introduce the defective gene *(CTNS)* and inject them back into the patients. As a proof of concept, we will use the *Ctns* -/- murine model for Cystinosis. These animals accumulate cystine and cystine crystals in all organs tested. *Ctns* -/- mice develop ocular changes similar to those observed in affected humans as well as bone and muscular defects and behavioral anomalies. However, the *Ctns* -/- mice do not develop proximal tubulopathy or renal failure despite the fact that they accumulate a significant quantity of cystine in the kidney and exhibit histological anomalies (i.e. giant mitochondria).

Thus, we will determine cystine content and histology in the kidney as the measure of the efficacy of transplanting and repairing kidney injury with *CTNS*-expressing adult stem cells. We will test the hypothesis that certain growth factors known to drive differentiation of renal lineage cells will increase the functional engrafment and repair. The potential that *CTNS*-expressing stem cells will also engraft in other tissues and reduce cystine levels will be tested. Finally, we will propose a strategy to optimize the murine model for the renal defects in order to better represent the nature of the human disorder and support this work as proof of concept for clinical application.

This first period of time has been used mostly for getting the animals and materials necessary for the project. We started in parallel several specific aims of the project to optimize the time.

I – Mice

1. ***Ctns* -/- mice**

   The process to get C57BL/6 *Ctns* -/- mice, mouse model of Cystinosis, from France, started in May by the redaction of a Material Transfer Agreement (MTA) by Inserm and Scripps' attorneys. The MTA was signed by both parties on July 13th 2006 and we were authorized to ship the mice to San Diego. Four females and one male arrived the 19th of September. They had to stay in quarantine until November 8th and then were transferred to the Molecular and Experimental Medicine (MEM) vivarium where the experiments will take place. Our initial experience with the breeding of these mice has been difficult because the mothers are eating their pups right after birth (the reason why is unknown though it may be because the animals were relatively old by the time they were shipped). After several attempts and much consultation with local experts, we are setting up foster mothers for taking care of the new pups. If we can get past this first round of breeding, then we should have no further problems. Alternatively, we are also genotyping a recent litter from a *Ctns*+/- male and a C57Bl/6 wild-type mother. If we have *Ctns*+/- females from this litter, then we should be able to solve this problem too.

2. **GFP transgenic mice**

   The objective is to isolate bone marrow-derived adult stem cells from wild-type mice and transplant them into lethally irradiated *Ctns* -/- mice to obtain a replacement in the kidney (and other organs) of the cells lacking *Ctns* by cells expressing this gene. To verify the integration of the transplanted cells in the kidney and other organs by histology, we will isolate the bone marrow stem cells from mice transgenic for the Green Fluorescent Protein (GFP). Therefore, all the isolated cells will carry a functional *Ctns* gene as well as the GFP protein that can be tracked using a fluorescent microscope or flow cytometry after sacrificing the mice and processing the tissues.

   The mice C57BL/6-Tg(ACTB-EGFP)1Osb/J were purchased from the The Jackson Laboratory and were transferred to quarantine on October 24th. They should be released December 21th and transferred to MEM vivarium where they will be bred.

3. **Luciferase transgenic mice**

   In MEM vivarium, we have a new IVIS Imaging System 200 Series (Xenogen). This apparatus allows the tracking and monitoring of luciferase-expressing cells in live mice. To track the bone marrow-derived adult stem cells in vivo without sacrificing the mice, we would like to isolate stem cells from mice transgenic for the firefly luciferase and transplant them in lethally irradiated *Ctns* -/- mice. This would allow the tracking of the cells in the whole body and at different time points post-transplantation. Therefore, this approach would be complementary to using the GFP transgenic mice and performed in parallel.

   However, it is not sure that the apparatus will be sensitive enough to detect single cell engraftment and it has been reported that the luciferase is not well detected in kidney of live animals. Therefore, before purchasing the mice transgenic for luciferase, we will transfect a renal cell line with two plasmids expressing luciferase under either an actin or a human CMV promoter. It is possible that the actin promoter will be better for kidney expression based on some work we have done measuring actin expression in human kidney tissues by proteomics. We will inject these gene-modified cells directly into the kidney and measure the fluorescence in the live mice 48 hours after injection using the IVIS imaging system.

II – Optimization of the stem cells

In order to increase the quantity of bone marrow stem cells that migrate, engraft and differentiate in the kidney, we will try to first engage these cells in vitro in renal differentiation. Our hypothesis is that bone marrow stem cells can be cultured with specific growth factors known to initiate renal cell differentiation in vitro and when subsequently transplanted, will engraft and differentiate more efficiently in the kidney.

Based on the literature, we purchased several growth factors known to drive differentiation of renal lineage during kidney development. These factors are: retinoic acid, activin A, fibroblast growth factor-2 and 9, bone morphogenic protein-9 and Transforming growth factor-a. We will grow bone marrow stem cells in vitro using media supplemented with several different cocktails of these factors.

A second approach is based on our work on fetal liver endothelial progenitor cells (see copy of the article: Isolation and angiogenesis by endothelial progenitors in the fetal liver). Therefore, we collagenase digested murine fetal kidney to isolate a unique population of renal feeder cells just as we had done earlier with fetal liver. We kept the renal feeder cells in culture for 3 weeks and collected their conditioned media every 3 days, which we froze at 20oC after filtering to avoid the presence of any cells in the media. Our hypothesis is that the cells isolated using our method are fetal renal feeder cells that will produce the growth factors necessary for renal cell differentiation. We will grow bone marrow stem cells in vitro using the conditioned media collected from these feeder cells.

We will analyze the differentiation of bone marrow stem cells towards a renal phenotype after culture in growth factor cocktails or renal feeder cell-conditioned media by analyzing the expression of a panel of renal cell progenitor markers by q-PCR analysis. We thus purchased q-PCR primers for Wt1, Pax2, cadherin 6, Gdnf and Wnt4 genes. The culture conditions that will allow the highest expression of these genes will be used to culture bone marrow stem cells, isolated from luciferse or GFP transgenic mice, that will be subsequently transplanted in *Ctns* -/- mice.

III – Optimization of the Cystinosis model for nephropathy

As described in the project, we propose that the more progressive the kidney injury, the more robust the migration and engraftment of the bone marrow stem cells will be. This is the reason why it is important to enhance the nephropathy observed in the murine Cystinosis model to obtain a proximal tubulopathy as observed in Cystinosis patients.

We have started the genetic construction for engineering a drug-inducible proximal tubulopathy in mice. We subcloned a highly active form of the viral thymidine kinase gene (SR39) in a plasmid under the control of a proximal tubular cell specific promoter. Treatment with ganciclovir will kill the cells where SR39 is expressed. Based on a literature search, we chose two different proximal tubular cell specific promoters, the promoter of the 25-hydroxyvitamin D3-1a-hydroxylase gene, CYP27B1, and the promoter of the glucose transporter, Sglt2.

To follow the expression of the construct, a marker gene, DsRed, is subcloned upstream of SR39 and the two genes are linked by an IRES (Internal Ribosomal Entry Site) sequence. Thus, the expression of both SR39 and DsRed will be driven by the same promoter. We added a Nuclear Localization Site (NLS) at the end of the DsRed gene to obtain the expression of DsRed primarily in the nucleus. Therefore, the intensity of the fluoresence of DsRed will be amplified and easily recognized in histology sections.

The constructions CYP27B1 and Sglt2 are almost done. They will be first tested in vitro by transfecting three different cell lines: NIH3T3 (murine fibroblasts - control), MDCK (canine renal distal tubular cells) and LLC-PK1 (pig renal proximal tubular cells) that we purchased from ATCC. We will choose the construction that allows the expression of the DsRed reporter gene only in the proximal tubular cells, LLC-PK1, proving the specificity of the promoter. We will also verify that SR39 is expressed and functional by adding some ganciclovir to the medium of the transfected cells, expecting the death of the cells. If the results obtained in vitro are satisfying, transgenic mice expressing this construct will be generated and characterized. The final aim is to breed these mice with the *Ctns* -/- mice to generate a drug-inducible and controllable proximal tubulopathy in the murine Cystinosis model.

PROGRESS REPORT

MITCHONDRIAL DYSFUNCTION IN CYSTINOSIS MYOPATHY

PRINCIPAL INVESTIGATOR: DORIS A. TRAUNER, MD

Aims of the Study

The specific aims are to determine whether the myopathy associated with nephropathic Cystinosis is the result of mitochondrial dysfunction with resultant deficiency in respiratory transport chain function in adolescents and adults with Cystinosis, and to determine if a treatment regimen that provides additional co-factors for these enzymes will improve the strength and prevent deterioration in muscle strength.

Progress to Date

1. **Enrollment**

   Eleven patients (age range 16–43 years) have been enrolled in the study to date. One was unable to continue because of his prior participation in a study of cardiac function. He has subsequently died of cardiac complications of his disease. Of the remaining 10, all have completed the 6 month visit and nine of the ten have completed the 12-month visit. The one participant who did not complete the 12 month data point had been placed on additional medications by his physician that interfered with our ability to interpret the results of our data. All patients were randomized to treatment with a "mitochondrial cocktail" or placebo for the first 6 months, and then offered open-label treatment with the vitamin-cofactor combination if they wished.

2. **Results of Study to Date**

   We are still in the process of entering the data into the database and have not as yet broken the code for treatment. Preliminary qualitative data analyses reveal the following:

   a. Strength: of the 10 participants, 4 had clinical evidence of muscle weakness on neurological examination. The weakness was mild to moderate in 3 and severe in one. One had shoulder girdle weakness, one mild leg weakness, one mild hand weakness, and one severe upper greater than lower extremity weakness. Grip strength testing at baseline was weaker than expected for age and gender in 5 of the 10 participants. At the 6 month follow-up, 2 of the four with initial weakness had improvement in strength on muscle strength testing, but no change in grip strength. One person who had initially had normal strength on clinical exam was thought to have slight hand weakness at 6 months. At 12 months, the subject with the most severe weakness had improved to mild to moderate weakness. Four additional subjects had mild to moderate weakness, including 2 who had normal exams previously. One person with an initial finding of mild leg weakness no longer exhibited that at 12 months. No individual had worsening of lower extremity strength over the 12 months, and weakness appeared to be confined to the upper extremities when present at all.

   b. Muscle pathology: Seven of the 10 individuals had abnormal results on muscle biopsy. The most common findings were muscle atrophy, fiber type disproportion, ring fibers, and filamentous bodies. Two had increase red granular staining and an increase in lipid droplets, findings suggestive of mitochondrial disorders. Biopsy findings did not always correlate with clinical examination or patient report of strength. Interestingly, no cystine crystals were found in muscle tissue.

   c. Muscle biochemistry: Muscle carnitine levels were normal in all 10 patients. Plasma carnitine levels were normal as well. Mitochondrial respiratory chain activity for Complex I, II, III and IV was normal in all patients.

3. **Plans**

   We plan to break the treatment code and discuss the longitudinal results to determine whether there was any relationship between treatment and improvement. All data will be analyzed and manuscripts prepared within the next 6 months.

PROGRESS REPORT

LYSOSOMAL CYSTINE ENHANCED APOPTOSIS IN CULTURED HUMAN MESENCHYMAL STEM CELLS

PRINCIPAL INVESTIGATOR: JESS G. THOENE, MD

It is a great pleasure to write to you from my new (old) office at the University of MICHIGAN! Some of you may know that I was a member of the U of M faculty for 22 years before moving in 2000 to New Orleans to become Director of the Hayward Genetics Center at Tulane University. While there I became interested in how lysosomal cystine storage causes the devastating condition we know as Cystinosis, and have now published four papers demonstrating that lysosomal cystine augments a natural process, called "apoptosis" leading to inappropriate cell death, and potentially causing the entire Cystinosis clinical picture, including renal Fanconi Syndrome, renal failure, retinal problems and poor growth. Knowledge about disease causation in molecular terms is now beginning to take center stage in many fields of medicine as we seek better treatments. It is possible that understanding in fine detail which actual molecules mediate inappropriate cell death in Cystinosis can lead to new avenues of treatment, precluding the need for cysteamine, and its attendant problems. Funding from the Cystinosis Research Foundation was critical in bringing our work this far.

Now I am starting over in a new lab, which must be furnished from scratch, and with all new people, because the team I had assembled at Tulane was dispersed by Hurricane Katrina. Moving 1000 miles and unpacking many boxes was a daunting task, but it is now accomplished, and I look forward to resuming work caring for Cystinosis patients and resuming research on this important aspect of the disease.