Cystinosis Research Foundation SCIENCE REPORT

PUBLISHED BY THE CYSTINOSIS RESEARCH FOUNDATION ★ FEBRUARY 2008

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

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

Less than five 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. CRF is the largest non-profit funder of cystinosis research in the world. Our funding efforts have allowed talented doctors and researchers to initiate novel research studies and advance their research efforts.

In 2007, the Cystinosis Research Foundation raised more than $2.5 million. Since its inception, the Foundation has raised more than $6.5 million and has committed and funded $5.1 million for cystinosis research. CRF is now funding 17 research studies and 5 research fellows. Our researchers are working in five countries around the world and collaborating to ensure the sharing of ideas and energy.

2008 Call for Funding Proposals

The Cystinosis Research Foundation will announce *A Call for Research and Fellowship Proposals* in the spring and autumn of 2008. 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. 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 2008.

Cystinosis Research Foundation Fellowship Program

The Cystinosis Research Foundation has launched a post-doctoral 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.natalieswish.org for details.

The First International Cystinosis Research Foundation Symposium for scientists and researchers will be held April 3 and 4, 2008 at the Arnold and Mabel Beckman Center of the National Academies of Sciences and Engineering. 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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2007 RESEARCH STUDIES COMMITTED AND FUNDED $1,936,030

MARCH 2007

Taosheng Huang, MD, PhD
University of California, Irvine
"Study on Mitochondrial Function in Cystinosis"
$50,000 – Pilot research

Robert Ballotti PhD
Christine Chiaverini, MD, PhD
INSERM U 385
Faculte' de Medecine, Nice France
"Molecular Mechanisms of Hypopigmentation
in Cystinosis"
$125,000 – 2-year study

JULY 2007

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

Bruce Barshop, MD, PhD
Jerry Schneider, MD
University of California, San Diego
"Practical Aspects of Intracellular Cystine
Measurements"
$109,886 – 1-year study

Ranjan Dohil, MD
University of California, San Diego
"One Year Treatment Study of Enteric-Coated
Cysteamine
in Patients with Cystinosis"
$99,688 – 1-year study

Bruno Gasnier, PhD
Institut de Biologie Physico-Chimique, Paris, France
"Identification and Characterization of the Lysosomal
Transporter Involved in Cysteamine-Medicated
Cystine Efflux"
$243,000 – 2-year study

Taosheng Huang, MD, PhD, Mentor
Sha Tang, PhD, Postdoctoral Fellow
University of California, Irvine
"Molecular and Pathogenesis Study of Cystinosis"
$106,180 – 2-year fellowship

SEPTEMBER 2007

Corinne Antignac, MD, PhD
Hospital Necker, Paris, France
"Purchase of Centrifuge and Rotor"
$46,550 – Award Total

Meredith Fidler, PhD
University of California, San Diego
"Associate Project Scientist for Various Cystinosis Studies"
$79,729.10 – 1-year research support

DECEMBER 2007

Eric Moses, PhD
John Blangero, PhD
Southwest Foundation for Biomedical Research,
San Antonio, Texas
"Scanning the Human Transcriptome in Cystinotic
Cell Lines for Changes that are Associated with
Genetic Variation in the CTNS Gene"
$183,470 – 2-year study

Bruce Barshop, MD, PhD
Jerry Schneider, MD
University of California, San Diego, California
"UCSD Tandem Mass Spectrometry Cystine
Determination Resource"
$118,845 – 1-year study

David Pearce, PhD
Seasson Phillips, Postdoctoral Fellow
University of Rochester Medical Center, Rochester,
New York
"Yeast Model for Cystinosis"
$173,474 – 3-year study

Holger Willenbring, MD
John McLaughlin, PhD
University of California, San Francisco, California
"Parthenogenetic Embryonic Stem Cells as
a Source of Immunocompatible Renal Progenitor
Cells for Therapy of Nephropathic Cystinosis"
$248,000 – 2-year study

Allison Eddy, MD
Daryl Okamura
Seattle Children's Hospital Research Institute,
Seattle, Washington
"Cysteamine Effects on Extracellular Matrix
Accumulation in Chronic Kidney Disease"
$267,208 – 3-year study

For information about our 2008 Call for Funding Proposals or the Cystinosis Research Foundation Fellowship Program, visit www.natalieswish.org.

BRUCE A. BARSHOP, MD, PhD
JERRY A. SCHNEIDER, MD
University of California, San Diego
"UCSD Tandem Mass Spectrometry Cystine Determination Resource"
Total Gift Award: $118,845

ABSTRACT/SUMMARY

The UCSD Cystine Determination Laboratory has transitioned from the cystine binding assay to tandem mass spectrometry. The decreased assay turnaround time has allowed research activity to be carried out in addition to the same level of clinical service. In the upcoming year, research is scheduled to look for conditions which allow a reliable assay with minimal sample preparation at the point of care. The instrument is also available to support other research projects sponsored by the Cystinosis Research Foundation. Several other research applications are also possible. Until the lease is fully executed, the cost of operation is particularly high, and supplemental income from research or other sources will be required in addition to the income from the clinical assays.

BRUCE A. BARSHOP, MD, PhD
JERRY A. SCHNEIDER, MD
University of California, San Diego
"Practical Aspects of Intracellular Cystine Measurements"
Total Grant Award: $109,886 – 1-year study

ABSTRACT/SUMMARY

Measurement of leukocyte cystine is important for both diagnosis and monitoring therapy in cystinosis, as it has been established that regular compliance is correlated with lower leukocyte cystine levels (Kieta et al., 2004) and with improved renal outcome (Markello et al., 1993), and that frequent regular dosing of cysteamine is necessary to maintain low leukocyte cystine levels (Gahl et al., 200 1, Gahl et al., 2002, Levtchenko et al., 2006). For much of the past two decades, the preferred method for clinical leukocyte cystine assays worldwide was a competitive radionuclide binding assay with a bacterial cystine binding protein as developed in the Schneider laboratory (Oshima et al., 1976). That became the method of choice, largely because the sensitivity is greater than that of ninhydrin-based amino acid detection of cystine and even than that of fluorometric detection (de Graaf-Hess et al., 1999). However, there has been a new opportunity with the development of the very sensitive and rapid technique of tandem mass spectrometry. We have adopted the method originally applied by Dalton and Turner in 2004 (Dalton and Turner, 2005), using electrospray ionization and stable isotope dilution with an internal standard of tetra-deuterated cystine. We have validated the tandem mass spectrometry method and shown it to be equivalent to the cystine binding protein radionuclide assay, with a sensitivity extending to 0.005 gmol/L. The most important advantage of this method is the markedly faster throughput, so that now many experiments can be performed which would have been impractical with the more laborious cystine binding protein assay. This project proposes to capitalize on the capabilities of the tandem mass spectrometric assay to conduct investigations that will be of practical use in the clinical follow-up and investigation of patients with cystinosis.

There are differences in both the pre-analytical and analytical techniques for leukocyte cystine measurement as it is carried out in different laboratories world-wide. Early work indicated that it was critical not to delay the isolation of blood leukocytes and preparation of a white cell pellet before acidifying the sample for storage. If those steps were delayed and the blood stored in either EDTA or heparin solutions for 24 hours or more, the test results were found to be unreliable (Schneider, unpublished). Once the white cell pellet is acid precipitated, the material may be stored at -20'C without loss of stability over reasonable time frames (Schneider, unpublished, Kamoun et al., 1999). Those observations led to the practice of preparing leukocyte isolates at the point of care and shipping the frozen leukocyte lysate-acid precipitate, and while the results with that approach have proven to be reliable, it entails extra work at the point of care, which may discourage testing. Some groups have found that blood samples may be shipped in ACD (acid-citrate-dextrose) solutions (Kamoun et al., 1999), but that EDTA was not suitable for stability. Another trial showed that on storage in ACD for 24 hours at room temperature, there was an almost doubling of intracellular cystine in polymorphonuclear leukocytes (Levtchenko et al., 2004). In other laboratories (van't Hoff, doctoral thesis), a small study (N=6) of blood samples stored in lithium heparin showed some variability at 24 hours, with 2 samples having results within 10% of the values from immediate processing, 2 samples increasing between 25 and 50%, and 3 samples decreasing between 10 and 50%. Reportedly, the variability at 48 hours was more marked and made that an unusable approach (Dalton and Turner, personal communication). It is possible that the situation is worse if sodium heparin is used instead of lithium heparin, since intracellular cystine levels may change through stimulation of sodium-dependent amino acid transport systems (Jiang et al., 2000). A commercial laboratory in the U.S. requests whole blood in heparin (without specifying which cation, sodium or lithium), to arrive within 48 hours.

It is recognized that in cystinotic white blood cells, the excess cystine is stored in the granules (lysosomes) of the polymorphonuc tear leukocytes (PMNS) (Schulman et al., 1970). Therefore, methods that analyze PMNs specifically are preferable in principle, and indeed, such an assay more reliably distinguishes heterozygotes from normals (Smolin et al., 1987, Levtchenko et al., 2004). In order to follow that approach, however, there must be either even more work performed at the point of care, or assurance of stability during the time for transport of whole blood. We have always been concerned that the latter condition could not be assured given the large geographic dimensions of North America.

We have shown (in fibroblasts) that the recovered non-protein cystine decreases with time even at very cold temperatures. Cystinotic fibroblasts stored at -20, -70, and even -I 96'C show a decrease of intracellular cystine of approximately 50% in 24 hours of storage, with another approximately 50% lost over the next 6 days (Kroll et al., 1974). It is also notable that plasma cystine decreases with time in storage at freezing temperatures

(Perry and Hansen, 1969). This might represent protein binding, but the phenomenon has not been studied.

Accordingly, the question remains exactly what happens to intracellular cystine with leukocyte storage. It seems likely that there is more than one process, since variations may be increases or decreases. Are apparent increases due to accelerated protein breakdown? Are apparent decreases caused by protein binding and/or formation of disulfide bonds with protein cysteine moeities? Are apparent decreases due to lysosome breakdown or "activation" of the leukocytes causing degranulation?

There is considerable variation in cystine values measured in samples from individual referral laboratories, and in some cases there seem to be systematic trends, with some centers having consistently lower or higher values. Clearly, there may be factors in the handling and preparation of samples which could account for such variation, and one possibility is that PMNs may be activated, resulting in extrusion of granules (lysosomes) and hence loss of cystine.

Spreading and activation of human neutrophils is preceded by a wave of increased intracellular calcium (Kruskal et al., 1986). It is clear that the source of the calcium is intracellular, and though there have been systems in which removal of extracellular calcium abolishes the spontaneous intracellular calcium transients (Jaconi et al., 1993), use of chelating agents like EGTA are not sufficient to block the calcium accumulation (Kruskal et al., 1986). One of the most potent stimuli for PMN chemoxtaxis and activation is the tripeptide, N-formyl-methionyl-leucyl-proline (fN4LP), and potent phenyldihydroimidazolpyrazole inhibitors of fmLP effect have recently been described (Bruno et al., 2007). Ryanodine receptor (RyR) agonists stimulate neutrophils (Hauser et al., 2005), and the RyR antagonist dantrolene is highly effective at blocking stimulation of T lymphocytes (Conrad et al., 2004). Notably, heparin may be synergistic with other agents promoting PMN degranulation (Cairo et al., 1983). Human neutrophils in media containing hypertonic sucrose or saline attach normally, but spreading and intracellular calcium spike are reversibly inhibited (Kruskal et al., 1986). Media with 0.25 or 0.5 M sucrose inhibit PN4N activation (Kruskal et al., 1986). There are inconsistent results regarding the effect of urea on PMN activation, some indicating no effect at a concentration of 0.5 M (Kruskal et al., 1986), and others demonstrating inhibition of lysosome release, to a significant degree at a urea concentration of 0.275 M (Stoehr and Smolen, 1988). Whole blood samples collected in slightly hypertonic solutions have been shown to display reduced neutrophil oxidative burst and degranulation in response to fN4LP (Hashiguchi et al., 2007).

Whole blood has been treated directly with DMSO and the leukocytes have been shown to retain ability to respond to oxidative stress (Chuang and Hu, 2004). Leukocytes from whole blood preserved with 10% DMSO retain viability and integrity by flow cytometry and, at least for monocytes and lymphocytes, response to proliferative stimuli (Hayes et al., 2002, Schindler et al., 2004). Preservation of PMN functionality in DMSO, including phagocytic capability and oxidative burst, has been demonstrated in equine blood samples (Zerbe et al., 2003).

The biochemical abnormality underlying cystinosis was revealed from studies in cells which characterized the removal and reaccumulation of intralysosomal cystine. Dramatic depletion of lysosomal cystine stores was observed with the aminothiol, cysteamine (Thoene et al., 1976), essentially completely removing intracellular nonprotein cystine within one hour of adding 0. 1 to I mM cysteamine to cystinotic fibroblasts. The ability to load lysosomes with cystine by treating with cystine dimethyl ester (Steinherz et a]., 1982b) enabled the introduction of isotopic label, and to transiently elevate intralysosomal cystine in normal cells to levels comparable to cystinotic cells. The process of cystine egress was shown to be a transporter, since it demonstrated saturation kinetics and counter-transport (Gahl et al., 1983). Cystine loss was studied in isolated lysosomal particles from leukocytes and although the different rate of egress was not initially apparent in studies of isolated lysosomes (Steinherz et al., 1982b), a significant difference was observed in situ from the lysosomes of intact leukocytes (Steinherz et al., 1982a). Leukocytes loaded with 0.25 mM ^{35}S-cysteine dimethyl ester accumulate cystine at a rate of approximately 25 nmol/mg protein/hr (PNAS) (Steinherz et al., 1982a), and the normal cell lines had ty, = 42.7±3.1 min, whereas 5 of 10 cystinotic cell lines had $t^1/_2$, > 450 min. At the same time, cystine loss from cystinotic fibroblasts was shown to be negligible over a 90 minute time frame (Jonas et al., 1982a)., and essentially zero in lysosomes from cystinotic PMNS, with half-normal rates in samples from heterozygotes (Gahl et al., 1982), and minimal in a 120 minute time frame in cystinotic lymphoblasts loaded with cystine dimethyl ester (Jonas et al., 1982b). The source of accumulated cystine was known to arise from intracellular protein degradation, since treatment with an inhibitor of protein synthesis (100 µM cycloheximide) completely eliminated reaccumulation of cystine following depletion with cysteamine, and reaccumulation was also completely prevented with an inhibitor of intralysosomal proteolysis (50 ~µM chloroquine) (Thoene et al., 1977). Notably, it was observed in that study also that the rate of entry of carbon from ^{14}C-serine to cysteine was undetectable above background over 24 hours.

ROBERT BALLOTTI, PhD
CHRISTINE CHIAVERINI, MD, PhD

Faculte' de Medecine, Nice, France

"Molecular Mechanisms of Hypopigmentation in Cystinosis"

Total Grant Award: $125,000 – 2-year study

ABSTRACT/SUMMARY

Patients with infantile cystinosis have hypopigmentation with, for Caucasian subjects, blond hair, blue eyes and a clear skin. However it seems that some patients, in particular African American patients, but also few Caucasian patients have not hypopigmentation. Unfortunately no correlation between cutaneous phenotype, severity of renal disease and genotype was carried out. The causes of hypopigmentation have not been so far elucidated. In humans, pigmentation results from the synthesis and distribution of melanin in skin, hair bulbs, and eyes. Melanin synthesis or melanogenesis is an enzymatic process, catalyzed by tyrosinase, tyrosinase-related protein 1 (Tyrp1) and dopa chrome tautomerase (DCT), which convert tyrosine to melanin pigments. This process takes place in melanocytes within lysosomes-related vesicles named melanosomes.

Two types of melanin are produced by melanocytes. Eumelanins are black/brown melanins with high photoprotective properties and pheomelanins

are red/yellow sulfur containing pigments that provide no protection against the noxious effects of solar irradiation. Melanogenesis proceeds in three distinctive steps. The initial step is the production of cysteinyldopa, which requires dopa (the product of tyrosine hydroxylation by tyrosinase) and sulfur containing compounds such as cystine. The second step is the oxidation of cysteinyldopa to give pheomelanin, which continues as long as cysteinyldopa are present. The last step is the production of eumelanin, which begins only after most of the cysteinyldopa are depleted. Further, melanin synthesis is also regulated by the pH of melanosomes: alkalinization of melanosomes by an inhibitor of vacuolar ATPase increases melanin synthesis and stimulation of melanin synthesis by...MSH is accompanied by an increase in melanosome pH. These data indicate that melanin synthesis take place at neutral or alkaline pH. Since CTNS is a cystine/H+ co-transporter, alteration of CTNS function in cystinotic cells would also lead to an acidification of melanosomes and an inhibition of melanin synthesis. Taking into account that cystinosin transports cystine out of lysosome and that melanosomes are lysosome-related vesicles, it is tempting to propose that cystinosin is involved in the active melanosomal efflux of cystine and regulates thereby melanogenesis. In cystinosis, cystinosin dysfunction might lead to an intramelanosomal cystine accumulation that could favor pheomelanin synthesis compared to eumelanogenesis or to a decreased intramelanosomal pH, which is known to be an important parameter in melanin synthesis. Alternatively, accumulation of cystine in melanosome might toxic for melanocyte leading to a decreased cell growth or increased apoptosis sensitivity.

The aim of this project is to explain the molecular mechanisms of hypopigmentation in cystinosis. To reach this objective: A-we will determine the dermatologic phenotype of patients with infantile cystinosis to have an objective and quantitative evaluation of pigmentation disorders in these disease. This part of the project will start in January 2008.

RANJAN DOHIL, MD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"One Year Treatment Study of Enteric-Coated Cysteamine in Patients with Cystinosis"
Total Grant Award: $99,688 – 1-year study

SPECIFIC AIMS

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. 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.

The purpose of this study is to determine whether dose frequency can be reduced giving enteric-coated Cystagon™ capsules. 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 a single dose of Cystagon™. The patient will continue to take 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 treatment will be recorded by asking patients to keep a detailed diary.

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

Seattle Children's Hospital Research Institute, Seattle, Washington
"Cysteamine effects on extracellular matrix accumulation in chronic kidney disease"
Total Grant Award: $267,208 – 3-year study

ABSTRACT/SUMMARY

Cystinosis – a disease with a worldwide incidence of 1 in 200,000 – typically caused end-stage kidney disease by the end of the first decade of life until the drug cysteamine was tested and shown to dramatically improve kidney survival in cystinotic patients. However, it is still not understood how intralysosomal cysteine accumulation damages the kidney and if the kidney protective effects of cysteamine work directly by lowering cystine levels. Cystinosis-associated kidney disease, like all chronic kidney diseases, progresses due to the deposition of scar tissue (comprised of extracellular matrix proteins) in a process called fibrosis. Based on preliminary data and results from studies in non-kidney diseases, we hypothesize that cysteamine therapy directly blocks the fibrotic process. Using a mouse model of chronic kidney disease that develops in response to unilateral ureteral obstruction (UUO), the proposed studies will investigate this hypothesis in three specific aims.

(1) To perform preliminary studies in order to optimize a therapeutic strategy of cysteamine therapy in a mouse model of chronic kidney disease. In addition to varying the dose and route of drug administration, the UUO model will be tested in both normal mice and mice harboring the cystinosis gene mutation *(Ctns-/-)*. A new line of mice will also be genetically engineered in an effort to develop a cystinosis mouse model that spontaneously develops significant kidney disease, which the *Ctns-/-* mice do not.

(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. After UUO surgery, groups of mice will be sacrificed 7, 14, and 21 days later and a series of studies performed on their kidneys to determine the severity of the scarring and examine changes in candidate pathways of fibrosis.

(3) To investigate the effect of cysteamine on apoptosis, oxidant stress and other novel cysteamine target pathways of chronic kidney disease by performing additional studies on the tissue harvested. It is anticipated that by determining how cysteamine inhibits kidney fibrosis, a new understanding of how the kidneys are damaged in children with cystinosis will emerge. Our long term goal is to use the knowledge gained from these studies to design new therapies to prevent nephropathy associated with cystinosis.

BRUNO GASNIER, PhD, PRINCIPAL INVESTIGATOR
ELLEN CLOSS, PhD, CO-PRINCIPAL INVESTIGATOR

Institut de Biologie Physico-Chimique, Paris, France

"Cysteamine-Medicated Cystine Efflux"
Total Grant Award: $243,000 – 2-year study

ABSTRACT/SUMMARY

Cystinotic patients are characterized by defective cystinosin-mediated efflux of cystine from lysosomes. Their treatment by cysteamine, which depletes accumulated cystine, is based on the presence, in the lysosomal membrane, of non-affected amino acid transporters which remain unknown at molecular level. After entering patients' cells, cysteamine accumulates into lysosomes through cysteine/cysteamine transporters. In the lysosomal lumen, it reacts with cystine to form a cysteine-cysteamine conjugate and, because of its structural similarity to lysine, the mixed disulfide leaves lysosomes through another transporter selective for cationic amino acids. After reduction of the mixed disulfide to cysteamine and cysteine in the cytosol, cysteamine reenters lysosomes to remove more cystine. The lysosomal cationic amino acid transporter, known biochemically as lysosomal 'system c', thus represents a 'salvage pathway' which by-passes the need for a functional cystine transporter.

In preliminary experiments, we identified in a family of cationic amino acid transporters two members which localize to lysosomes, and thus represent candidate system c proteins. In this project, we will characterize their transport activity, in particular their ability to translocate the cysteamine-cysteine disulfide across membranes. For this purpose, we will develop whole cell transport assays based on the redirection of the proteins to the plasma membrane by mutagenesis of their intracellular sorting motifs. This approach, which facilitates the study of lysosomal transporters and has been previously applied to cystinosin and sialin, will be carried out using radiotracer flux or electrophysiological techniques. In parallel, we will alter the expression of the candidate system c transporters in cultured cells, using cDNA transfection or RNA interference techniques, and examine whether their expression level correlates with the velocity at which cysteamine depletes cystine from cystinotic cells.

This project should represent a first step to improve the pharmacological treatment of cystinosis. Indeed, the molecular identification of system c will provide a robust assay to screen mixed disulfides translocated more efficiently (lower KM, higher Vmax) than cysteamine-cysteine. It would thus enable us to identify sulfhydryl compounds acting on cystinotic cells at lower concentration than cysteamine, thus providing a rationale approach to reduce the side-effects and constraints of the current treatment (gastrointestinal distress, administration every 6 hours).

TAOSHENG HUANG MD, PHD, MENTOR
DOUGLAS WALLACE, PHD, CO-MENTOR
SHA TANG, PHD, POSTDOCTORAL FELLOW

University of California, Irvine

"Molecular and Pathogenesis Study of Cystinosis"
Total Grant Award: $106,180 – 2-year study

ABSTRACT/SUMMARY

Cystinosis is a rare autosomal recessive disease. Mutation of cystinosin (CTNS) causes defects in the lysosomal cystine transporter and results in cystine accumulation in lysosome. The disease is manifested in two forms – nephropathic and non-nephropathic cystinosis. Nephropathic cystinosis presents with renal tubule failure, or renal Fanconi's syndrome, while non-nephropathic cystinosis is associated with cystine crystal deposition in the cornea. The development of cysteamine was a breakthrough in the treatment of patients with cystinosis. However, the pathogenesis of cystinosis is not fully understood. Accumulating evidence suggests that mitochondrial dysfunction is associated with renal tubular failure. The observation of glutathione depletion, increased apoptosis rate, and decreased ATP production in cystinotic cells indicates that mitochondrial dysfunction is an important step involved in the pathogenesis of cystinosis, as in many other diseases. Nevertheless, the link between intralysosomal accumulation of cystine and mitochondrial dysfunction is still unknown. In the proposed study, mitochondrial dysfunction will be studied by measuring redox with redox-sensitive Green Fluorescent Protein (roGFP); mitochondrial ROS productions, respiration rates, and enzyme activities will be analyzed; the effects of antioxidants and apoptotic inhibitors will also be investigated. These studies will facilitate our understanding of the pathogenesis of cystinosin mutations and may lead to a novel therapeutic target for cystinosis.

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

Southwest Foundation for Biomedical Research, San Antonio, Texas

"Scanning the human transcriptome in cystinotic cell lines for changes that are associated with genetic variation in the CTNS gene"
Total Grant Award: $183,470 – 2-year study

ABSTRACT/SUMMARY

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 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).

Due to the rare nature of Cystinosis, collecting samples from affected individuals and their families for research purposes is a challenge. In July 2007, the Cystinosis Research Network Family Conference took place in San Antonio, Texas. At that conference, scientists, clinicians and Cystinosis family members were in attendance. With University of Texas Health Science Center

San Antonio (UTHSCSA) Institutional Review Board (IRB) ethics approval, blood was collected from consenting individuals affected by Cystinosis and their first degree relatives. Within 3 hours of collection, the blood samples (147) were transported to the Southwest Foundation for Biomedical Research (SFBR), San Antonio, Texas where lymphocytes were isolated, transferred to vials and then snap frozen in Liquid Nitrogen. In the future, tissue culture techniques will be used to generate stable, long term viable cell lines from these lymphocytes. Each cell line will represent one Cystinosis family member, thus we will have the full spectrum of disease severity, from early onset severe Cystinosis to unaffected individuals. This will provide us with a unique biological repository for future genetic studies.

The generation and utilization of these cystinotic cell lines forms the basis of this proposal.

The Benefits :

We believe that the proposed project will produce important genetic information on the role of cystinosin and it is likely that novel genes, previously unknown to be involved in this pathway, will be discovered. These genes become potential targets for pharmacological intervention. In addition, an important outcome of this study will be the generation of the cystinotic lymphoblastoid cell lines. This unique biological repository will not only provide genetic material for molecular studies but will also establish an in vitro model for Cystinosis to test potential therapeutic agents.

DAVID PEARCE, PhD, MENTOR
SEASSON PHILLIPS, POSTDOCTORAL FELLOW

University of Rochester Medical Center, Rochester, New York

"Yeast Model for Cystinosis"

Total Grant Award: $173,474 – 3-year study

ABSTRACT/SUMMARY

Cystinosis is a lysosomal storage disorder resulting from mutations in the CTNS gene. Cystinosin, the protein product of the CTNS gene, encodes a cystine-H+ symporter which effluxes cystine out of the lysosome and the transport defect in cystinosin deficient cells causes accumulation of cystine in the lysosome. There are several forms of Cystinosis, the most severe being infantile nephropathic Cystinosis. This form is caused by the complete loss of function of cystinosin and is characterized by nephritic disorders, growth retardation, hypothyroidism, photophobias, neurological dysfunctions, and renal failure by age 10. The juvenile form of Cystinosis is slightly milder and is caused by partial loss of function mutations in cystinosin. Currently, the only treatment is cystamine which requires early diagnosis and is relatively difficult to administer.

We aim to use a yeast model of Cystinosis to gain a better understanding of CTNS function and the disease pathologies, ultimately resulting in the identification of novel therapeutic targets. The amino acid sequence of the yeast homolog Ers1p is 31% identical and 47% similar to cystinosin. Moreover, deletion of ERS1 causes yeast to become sensitive to hygromycin B which can be rescued by human CTNS expression, suggesting that these two proteins are functional homologs. Ers1p localizes to the vacuole, the analogous organelle to the lysosome. However, several questions about the mechanism of Ers1p and defects found in ers1-Δ remain unanswered.

HOLGER WILLENBRING, MD, PRINCIPAL INVESTIGATOR
K. JOHN MCLAUGHLIN, PhD, CO-PRINCIPAL INVESTIGATOR

University of California, San Francisco

"Parthenogenetic embryonic stem cells as a source of immunocompatible renal progenitor cells for therapy of nephropathic cystinosis"

Total Grant Award: $248,000 – 2-year study

ABSTRACT/SUMMARY

Early onset cysteamine therapy can preserve life-long kidney function in nephropathic cystinosis. However, several circumstances exist under which cysteamine therapy is not sufficiently applied. In addition, renal proximal tubular injury frequently occurs despite diligent cysteamine therapy. This leads to Fanconi syndrome which can have severe complications and impairs quality of life. Kidney transplantation is the only therapy currently effective at restoring failing kidney function but donor organs are sparse and organ transplantation necessitates life-long immunosuppression. Unlike other organs or kidney structures, the renal proximal tubular compartment is a promising target for cell therapy. Intriguingly, significant progress has recently been made in differentiating embryonic stem cells into progenitors of proximal tubular cells. What remains to be achieved is compatibility of the transplanted cells with the recipient's immune system. Several methods exist that establish pluripotency in recipient-derived cells but neither of these is currently efficient or safe enough for human application. By contrast, parthenogenetic embryonic stem cells can be efficiently derived from unfertilized human oocytes and establish a high likelihood of matching between relatives or even within the general population.

In this proposal, we line out the critical experimental steps that will establish the principal feasibility of using parthenogenetic embryonic stem cells for therapy of Fanconi syndrome due to nephropathic cystinosis.

Research Updates start on next page

PROGRESS REPORT

CORINNE ANTIGNAC, PRINCIPAL INVESTIGATOR

"Characterization of Cystinosin Intracellular Trafficking"

Persons working on the project :
Anne Bailleux (PhD – funded by the Cystinosis Research Foundation)
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

AIM 1 : SORTING OF CYSTINOSIN TO THE LYSOSOME

- MDCK and CaCo-2 cell lines stably expressing cystinosin-GFP or cystinosine-HA have been generated in the laboratory. In all cases, the tag is located at the C-terminus of the protein, since we have been unable to express constructs with HA inserted in the second inter-TM loop.
- To assess which pathway is used by cystinosine to reach the lysosomes and to identify which adaptor protein complexes are used, we want to inactivate each component of the adaptor protein complexes by RNA interference. Transfection of plasmids which contain sequences encoding short-hairpin RNA (shRNA) molecules specific of AP1 γ-adaptin and of AP2 μ2-adaptin has no measurable effect in MDCK and Caco-2 cells probably because of the poor efficiency of transfection. We thus decided to use a lentiviral vector system derived from HIV-1 to express shRNAs directed against AP1 and AP2 in tagged-cystinosin containing cells. In addition, enhanced GFP (EGFP) is incorporated in the system as a reporter gene, allowing easy check of transduction efficacy. In preliminary experiments, efficient transduction was observed, with more than 90% of cells transduced. We are now producing high-titre lentivirus batches in order to proceed to the next steps of the project.

AIM 2 : IDENTIFICATION OF PROTEINS INTERACTING WITH CYSTINOSIN

A lot of effort has been put on the setting up of the experimental procedures, which needed many adjustments, given the structure of cystinosin as a highly glycosylated, seven-transmembrain domain containing protein.

During the last six months, we have made the following progresses:

- We had initially planned to work on lysosomes purified by Percoll gradients, but we were unable to obtain a dry pellet of lysosomes, which was needed for BN-PAGE. We thus turned to the use of intracellular membrane pellets, which, as an additional advantage, contain not only lysosomes, but the various cell fractions involved in the intracellular traffic of membranes proteins. To obtain intact lysosomes in the pellet, we have tested several experimental conditions, especially by testing numerous detergents, various conditions of centrifugation and have shown that using DDM 0.05% allows the isolation of a membrane pellet that contains, on average, 46% of intact lysosomes (mean obtained from 27 experiments), with a three time enrichment in cystinosin-GFP compared to the total cell extract.
- Blue-native polyacrylamide gel electrophoresis (BN-PAGE) permits to separate intact and functional membrane protein complexes. This technique offers the advantage of separating native protein complexes present in membrane protein samples without dissociating them.

  The pellet of intracellular membranes obtained with DDM 0.05% was solubilized with NP40 1%, sonicated and submitted to BN-PAGE. Preliminary experiments revealed the presence of cystinosine-GFP on the BN-PAGE. Now, we have to analyze the size of the cystinosine-GFP containing complexes and to define the quantity of materiel needed to identify the partners of cystinosine-GFP.
- Co-immunoprecipitation is the alternative method we choose to identify the partners of cystinosine. HA- and GFP- tagged-cystinosin was immunoprecipited using antibodies directed against the tag (HA or GFP), but the experiments were hampered by the heavy background. We think we have overcome this difficulty by using cleaner protein preparation (see above) and by using magnetic beads directly coupled to monoclonal antibodies directed against GFP or HA, avoiding the secondary antibody.

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"

Six-Month Progress Report: Cystinosis Research Foundation
9/1/06 – 3/1/07

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 – 3/1/07

Over the first 6 months of the study we obtained IRB approval for the study, ordered testing supplies, and trained a research assistant on the measures to be administered. In addition, we have successfully recruited, inducted, and tested 12 individuals with cystinosis and 5 control participants. We have also made contact with Dr. Mark Pian, the pediatric pulmonologist at Children's Hospital, and to date he has referred 1 cystic fibrosis patient to us who we are currently attempting to schedule for testing (the patient is currently in the hospital). Hence, we have made substantial progress toward the goals of this study.

C. RESULTS

On all 17 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 in the first quarter of this research study, we have not yet analyzed any of 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 matched control participants, as we work toward the total projected participants (i.e., 30 with cystinosis, 30 with cystic fibrosis, and 30 controls).

PROGRESS REPORT

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

"Neural Functioning in Auditory and Visual Systems"

Period: 3/01/07– 8/15/07

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 awarded 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 3/01/07 – 8/15/07, following progress in the study has been made.

1. Setting up the experiments

- The two ERP experiments, auditory and visual, have been designed and programmed on a special stimulus presentation software (Presentation® software, Version 0.70, www.neurobs.com).
- The hardware needed for stimulus presentation was designed, parts purchased, and equipment was manufactured in-house. This included building a connected 4-loudspeaker system for sound presentation and a 4-box display for visual stimulus presentation.
- Additional hardware for data acquisition equipment was purchased and incorporated into the existing system. This included a new 64-electrode cable sets, electro-caps fit for children ages 6 to 18, subject monitoring device, data storage, and data transfer devices.
- Recruitment for a full-time research assistant was conducted, applicants interviewed, and an individual with an extensive prior experience in research with neuro-developmental populations, Ms. Mikaela Kinnear, was hired. She was trained on the ERP data acquisition technique and analysis. In addition, one lower-division student and two upper-division UCSD students were recruited to work on the project and trained on the ERP techniques.

2. Piloting

- Every new ERP experiment has to be piloted to adjust its various parameters in order to ensure that it is comfortable for the subjects, that subjects are employing task strategies intended by the researchers, and to ensure that the experiment is producing informative data of interest. To this end, half a dozen of UCSD college students and two children have been recruited and tested on auditory and visual experiments each (16 experiments total).

3. Subject recruitment

- Human subjects protection protocol was written and presented to the UCSD Internal Revenue Board for approval (approved).
- Recruitment flyers and posters have been designed, printed, and distributed in schools and daycares in the extended San Diego area. Existing subject databases have been screened and potential children contacted for participation in the control group.
- Ms. Mikaela Kinnear went to the annual Cystinosis Research Network meeting in San Antonio, Texas in July of 2007. There, she talked about our research to families. She was successful in recruiting 11 individuals with cystinosis.

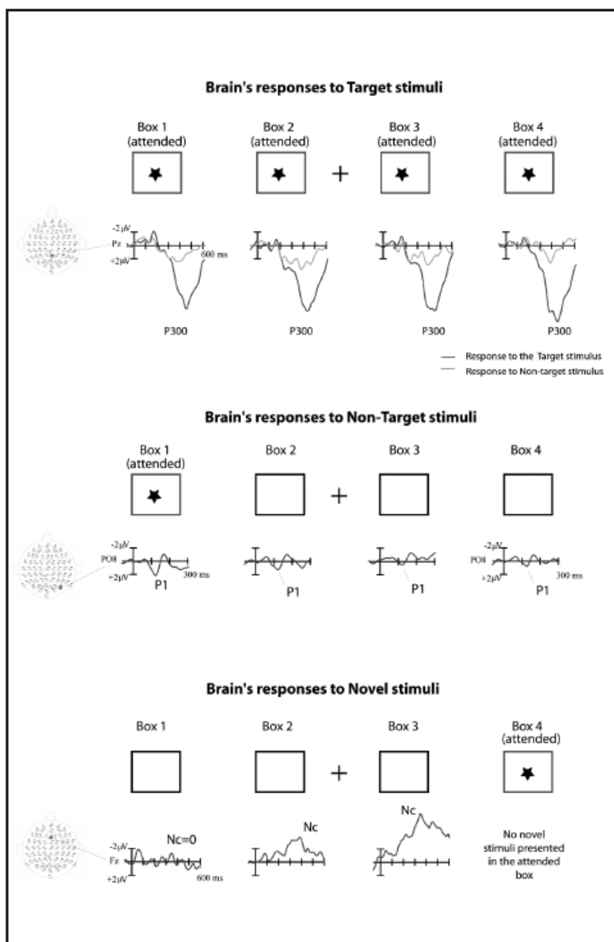
- Through Dr. Amy Spilkin, we are closely collaborating with Dr. Trauner's group in combining recruitment efforts and scheduling participants' visits to both laboratories during one trip.

4. **Data collection**

- We have collected data on auditory and visual ERP experiments from 10 young healthy adults (20 experiments). This is an almost full group (we will test 4 more individuals) that will provide the normative, or reference, data for our developmental and cystinosis populations.
- Data collection from children will proceed on an individual case-control matching basis. We will recruit a gender, age, and SES matched control for every child with cystinosis. Approximately 20 potential control participants have been pre-screened and lined up for participation. Two out of 10 adult subjects from the adult control group are matches to two adult cystinosis participants that have been already tested.
- So far, we have tested four individuals with cystinosis (8 experiments) and two control children (4 experiments). Three more individuals with cystinosis are scheduled to participate by the end of September.

5. **Data analysis**

- So far, we have analyzed adult control data from the Visual experiment. In this experiment, participants are presented with stimuli that occur randomly in four different locations on the computer monitor (represented by four boxes in the Figure below).



While the participants are asked to look at the fixation cross in the center of the screen at all times, their attention is covertly directed to one of the boxes during any given stimulus block (e.g., blue box in the middle and bottom panels). Three stimulus types are randomly presented. The first stimulus type was Targets. These were infrequent stimuli (10% of all stimuli) to which subjects had to respond by pressing a response button. The target stimulus was an upside down angle. Brain's responses to the target stimuli are presented in the top panel of the figure. The second stimulus type is Non-Targets, comprising majority of stimuli (80%). The non-target stimulus was an upside-down arch. Responses to these non-target stimuli are presented in the middle panel of the figure. The rest 10% of stimuli were novel, colorful shapes that occurred in all but the attended box.

- As it can be seen in the top panel of the Figure, Target stimuli elicited a robust positive-polarity response, called the P300 component. This response reflects brain's processes needed to identify the target stimulus and make a decision to respond to it. Please note that the size of this response is equal across the boxes since it was registered when participants attended to each of the boxes (at different times). Using the P300 response, we will be able to assess brains' capacity and processing speed related to the analysis of visual sensory information, stimulus identification, and decision-making, in children and adults with Cystinosis.
- The middle panel of the Figure demonstrates brain's responses to the frequent Non-Target stimuli. These data demonstrate that brain's response, here represented by the early P1 peak, is clearly enhanced in response to the stimuli in the attended (blue) box. Each box is attended at different times, but here we show only Box 1 as an example. The P1 gradually diminishes in size as the stimuli appear in the boxes more distant from the attended location. The size of this effect is a sensitive index of the strength of the visual spatial attention, and the slope of its diminution at more distant locations is a marker of attentional breath, or its extent in space around the visual focus. Using the P1 effect we will be able to assess the strength and the breadth of the visual spatial attention in children and adults with Cystinosis.
- The bottom panel of the Figure demonstrates brains' responses to the infrequent, Novel stimuli. These data demonstrate that novel stimuli elicit a very robust, negative-going response, usually termed the Nc, over the front of the head, but only when these novel stimuli occur in the vicinity of the attended location. This is another measure that will allow us to assess the breadth of the visual-spatial attention. However, differently from the P1 response, the Nc is an index of attentional orienting. Therefore, we will use this response to assess attentional distractibility in children and adults with Cystinosis.

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

- Given that both experiments are successfully set up and piloted, and that the groundbreaking recruitment efforts are fully on the way, during the next funding period we will have the opportunity to focus on more extensive data collection. By the end of year 1 of the study, we plan to have tested more than half of the projected 80 participants.
- We will continue analyzing visual and auditory ERP data obtained from typical children as well as children and adults with Cystinosis.

PROGRESS REPORT

PRINCIPAL INVESTIGATORS:

STEPHANIE CHERQUI, PhD AND DANIEL R. SALOMON, MD
THE SCRIPPS RESEARCH INSTITUTE

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

Period: July, 2007 – December, 2007

I – MICE

1. *Ctns-/-* mice

We have now a large colony of C57BL/6 *Ctns-/-* mice and we have achieved the necessary animal numbers to support the studies. We verified the integrity of *Ctns-/-* mice by measuring the cystine content in several organs. The *Ctns-/-* mice accumulate cystine in muscle, spleen, heart, liver and kidney (Table 1). The cystine measurements were performed by Jon Gangoiti at UCSD using a mass spectrophotometer (purchased with the support of the CRF). This experiment allowed Jon and I to optimize the parameters for cystine measurements in murine tissues.

	nmol/mg prot. ½ cystine	ratio accumulation KO/wt
wt muscle	0.002	**51**
wt spleen	0.018	**67**
wt heart	0.032	**25**
wt liver	0.001	**1642**
wt kidney	0.040	**43**
KO muscle	0.108	
KO spleen	1.207	
KO heart	0.794	
KO liver	2.444	
KO kidney	1.723	

Table 1: Cystine measurement in the muscle, spleen heart, liver and kidney of a widltype and Ctns Ko mice.

II – TRANSPLANTATION

We performed several bone marrow stem cell (BMSC) transplantations in *Ctns-/-* mice. BMSC were isolated from our 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 premise is that after transplantation, any cell derived from these BMSC will be fluorescent and detectable using flow cytometry or microscopy. 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 ureteral injections into the ureter of the right kidney (in which case the left kidney serves as the internal control).

1. Cells

Two kinds of stem cells were transplanted to test which one is the more efficient to generate renal cells. First, the BMSC represent the heterogeneous population of cells contained in the whole bone marrow. These were freshly harvested from the mice and injected into *Ctns-/-* mice. Two independent experiments have been performed with BMSC, n=3 animals/condition x 2 experiments for a total of 6 animals per condition as follows:

- *Ctns-/-* mice receiving GFP+ BMSC via tail vein injection
- *Ctns-/-* mice receiving GFP+ BMSC via ureteral injection
- *Ctns-/-* mice receiving Ctns-/- BMSC via tail vein injection
- *Ctns-/-* mice receiving Ctns-/- BMSC via ureteral injection

The second kind of stem cells used for transplantation were the MSC. Indeed, some authors have shown that these cells are more efficient to generate renal cells as discussed in the original proposal. We obtained MSC by culturing BMSC from GFP transgenic mice for 2 months, changing the media every 3 days to remove non-adherent cells. As clearly shown in Figure 1, after 12 passages the stem cells stopped expressing hematopoietic stem cell (HSC) markers (CD45, CD31, CD34) and started expressing MSC markers (CD90.2, CD44, CD105). Two independent experiments have also been performed using these cells. For one of these experiments, the MSC have been genetically modified to express a Luciferase gene. Luciferase allows us to follow the cells in live mice using the IVIS Imaging System 200 Series (Xenogen). Each experiment included:

- 3 *Ctns-/-* mice receiving GFP+ MSC (± Luciferase) via tail vein injection
- 3 *Ctns-/-* mice receiving GFP+ MSC (± Luciferase) via ureteral injection

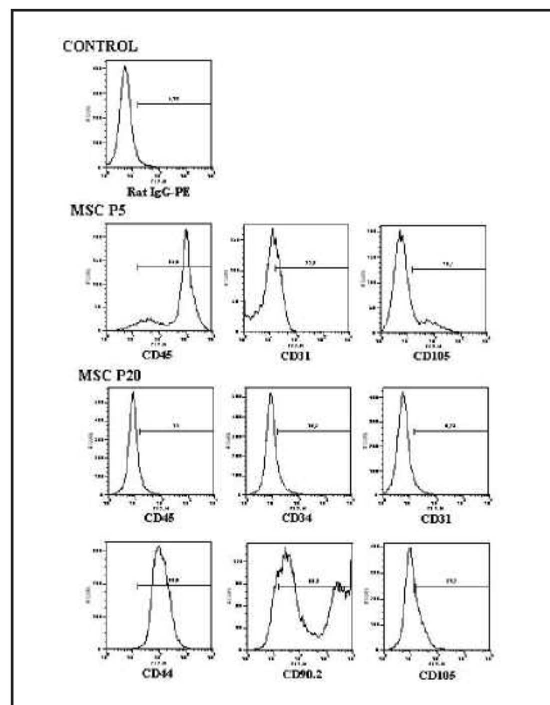


Figure 1: Flow cytometry analysis of the mesenchymal stem cells (MSC) at passage 5 (P5) and passage 20 (P20). At P5, the cells were expressing HSC markers as CD45 and CD31. At P20, the MSC express only MSC-specific markers (CD44, CD90.2, CD105) and not the HSC markers (CD45, CD34, CD31).

2. **Injection**

The cells have been injected into the mice using two different techniques. The first technique is injection via the tail veins of the mice after lethal (for BMSC) or sublethal (for MSC) irradiation. Thus, the transplanted cells replace all or part of the host hematopoietic or mesenchymal stem cells. The premise is that these stem cells will migrate to the kidney if there is ongoing renal cell injury and death. The second technique, ureteral injection, is aimed to target more precisely and more efficiently the kidney.

Moreover, this procedure would be the best to apply to cystinosis patients; it would be less invasive and less dangerous for the patient than a renal arterial injection. This technique has been shown to deliver virus vectors more efficiently to the kidney than intra-parenchymal or intra-arterial injections [Gusella et al., 2002a; Gusella et al., 2002b; Lai et al., 1997]. However, this method has never been used to inject cells. Therefore, as a proof of concept, we injected 293T (human embryonic renal cell line) expressing luciferase via ureteral injection in the right ureter of NOD/SCID mice. Four days later, we could observe Luciferase fluorescence in the area of the kidney in live mice (Figure 2A). To prove that the fluorescence came from the kidney, we sacrificed the mice and extracted the right kidney, which was clearly positive for Luciferase (Figure 2B).

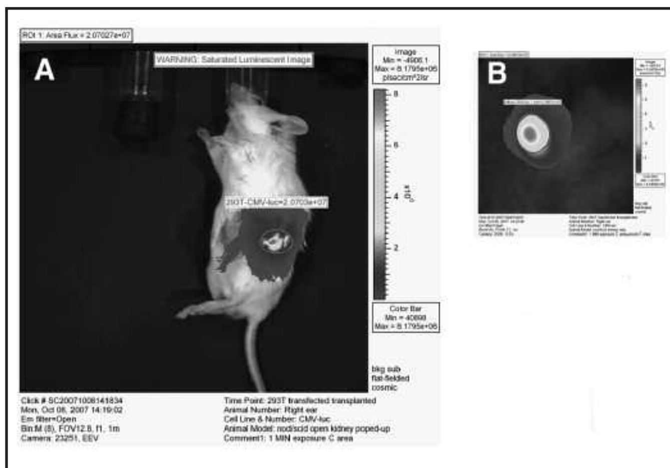


Figure 2: Proof of concept for cell delivery to the kidney via ureteral injection. ***(A)*** *NOD/SCID mouse after ureteral injection of 293T-Luciferase. The Fluoresecnce can be observed in the kidney area using the IVIS Imagin system.* ***(B)*** *Kidney after extraction, proving that the cells are in the kidney.*

3. **Results**

The mice transplanted were between 8 to 12 weeks old for each experiment. At 8 weeks post-transplantation, we measured urea and creatinine in the serum and urine as well as alkaline phosphatase in serum. As expected, no major difference was observed between the mice transplanted with GFP+ BMSC and Ctns-/- BMSC as no renal injury is observed at that age in *Ctns-/-* mice. However, it is important to note that the procedure was well tolerated by the animals and there were no problems encountered post transplantation if the mice successfully engrafted after irradiation. Therefore, we are leaving the mice to get older and we will measure their renal function at different time points with the assumption based on previous studies that renal injury in these animals will begin to accumulate after 6 months. We will eventually sacrifice the mice to verify and quantify the presence of GFP+ renal cells in the kidneys of *Ctns-/-* mice transplanted with either GFP+ BMSC or GFP+ MSC. We will also compare the cystine content and the renal injury of the kidneys of *Ctns-/-* mice receiving the GFP+ BMSC or GFP+ MSC to the results in the mice transplanted with Ctns-/- BMSC.

As shown in Figure 3, mice transplanted via a ureteral injection with MSC expressing luciferase demonstrated luciferase fluorescence in the kidney area 6 days after the injection (n=3). Luciferase fluorescence was observed in 2 of the 3 mice at 13 and 26 days post injection. The fluorescence intensity decreased between 6 and 13 days, which is not surprising because a substantial number of cells die after injection. However, between 13 and 26 days, the fluorescence intensity in the positive mice (# 1726 and 1727) remains stable (Figure 3). These results support our hypothesis the cells are integrated into the kidney. The mice are still alive and will be monitored every other week for at least two more months.

However, we strongly believe that we need a better model for renal injury so that there is a constant force driving potential stem cells to migrate to the kidney and repair an ongoing injury.

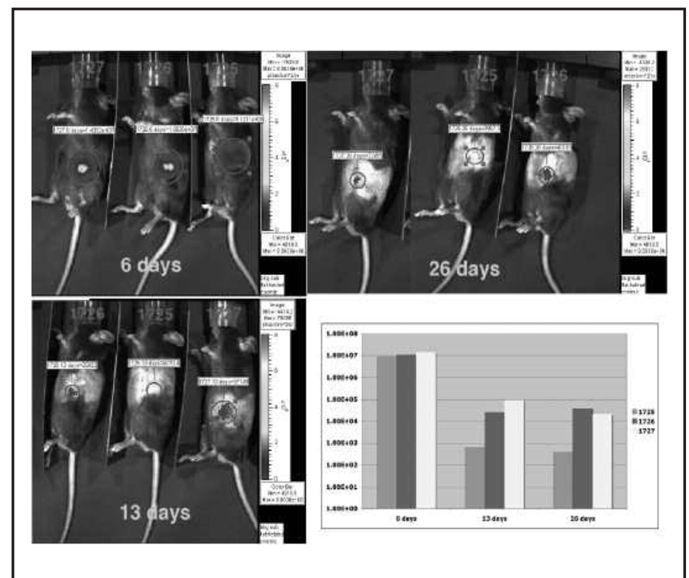


Figure 3: Following of mesenchymal stem cells expressing Luciferase after ureteral injetion in Ctns-/- mice. The cells can be observed in live animals using the IVIS imaging system at 6, 13 and 26 days post injection. The histogram shows the total flux intensity of Luciferase in each mouse.

III – OPTIMIZATION OF THE CYSTINOSIS MODEL FOR NEPHROPATHY

As described in the original project proposal, we hypothesize that the more progressive the kidney injury, the more robust the migration and engraftment of the bone marrow stem cells will be. Therefore, to test the efficiency of stem cell transplantation in cystinosis, it is important to enhance the nephropathy observed in the murine cystinosis model to obtain a proximal tubulopathy or frank renal failure as observed in human cystinosis patients.

1. **Generation of a drug-inducible proximal tubulopathy and renal failure via a novel transgenic mouse**

    We generated the genetic constructs for engineering a drug-inducible proximal tubulopathy and renal failure in mice. We subcloned a highly active form of the viral thymidine kinase gene (vTK) into a plasmid under the control of a proximal tubular cell specific promoter (Figure 4).

Figure 4: Genetic construct generated for drug-inducible proximal tubulopathy and renal failure. The constructs contain DsRed reporter gene with a Nuclear Localization Site (NLS) in 3', preceded by an Internal Ribosomal Entry Site (IRES) and a viral Thymidine kinase gene (vTK). The expression of these genes are under the control of either a kidney or proximal tubules specific promoter.

The rationale is that treatment with the drug, ganciclovir, will kill the cells where vTK is expressed. To follow the expression of the construct, a marker gene, DsRed, is subcloned downstream of vTK and the two genes are linked by an IRES (Internal Ribosomal Entry Site) sequence. Thus, the same promoter will drive the expression of both vTK and DsRed. We added a Nuclear Localization Site (NLS) at the end of the DsRed gene to direct the expression of DsRed primarily to the nucleus. Therefore, the intensity of the fluorescence of DsRed will be amplified and easily recognized in histology sections.

Based on a literature search, we chose two candidates for proximal tubular cell specific promoters: the promoter of the 25-hydroxyvitamin D3-1α-hydroxylase gene, CYP27B1, and the promoter of the glucose transporter, Sglt2. We generated the corresponding constructs and tested them *in vitro* by transfecting three different cell lines: NIH 3T3 (murine fibroblasts), MDCK (canine renal distal tubular cells) and LLC-PK1 (pig renal proximal tubular cells). Our goal was to verify if either one of the constructs, pIS-CYP or pIS-Sglt2, has a promoter specific enough to get DsRed expression only in the nuclei of the LLC-PK1 cells and to provoke cell death of LLC-PK1 in presence of the drug, ganciclovir. We will then use that construct to generate transgenic mice expressing this drug-inducible toxin specifically in the proximal tubule cells of the kidney. Thus, treatment of these mice with the drug, ganciclovir, should produce a proximal tubulopathy and eventually renal failure. If successful, these vTK transgenic mice will then be bred with the *Ctns-/-* mice to generate a controllable proximal tubulopathy in the murine cystinosis model. The current data suggests that the Sglt2 promoter is optimal for our purposes and we are finishing the final experiments.

We also decided to generate a construct that would lead to a general renal failure by using a kidney specific promoter that drives expression in different kinds of renal cells. We chose the promoter of CDH16 (ksp-cadherin), which is expressed in tubular epithelial cells in the kidney (distal and proximal), collecting ducts and thick ascending limbs of Henle's loop. Based on the literature, we tested two different sizes of the CDH16 promoter, one at 300 bp and one at 1300 bp. The larger one showed more specificity for renal cells. Our testing of this construct *in vitro* has been very promising and we are now doing final validations.

2. **Decreasing resistance to oxidative stress with SOD deficiency**

    Several lines of evidence suggest that cystine accumulation in the lysosomes of renal tubules results in increased oxidative stress, which results in cell dysfunction and tissue injury. The lack of tubulopathy in the *Ctns-/-* mice might be due to a better resistance of these animals to oxidative stress than human patients. Our hypothesis is that we can significantly enhance the progression of renal injury in our cystinosis model by decreasing the resistance of these animals to oxidative stress. Oxidative stress and mitochondrial dysfunction results in decreased of ATP levels and increased of superoxide dismutase (SOD) production, an antioxidant protein protecting the cells against oxidative stress. Therefore, to test our hypothesis we will inhibit SOD production in *Ctns-/-* mice by backcrossing them with SOD-deficient mice.

This work is done in collaboration with Dr Jeffrey Friedman (The Scripps Research Institute, La Jolla) who has the SOD1- and SOD2-deficient mice. SOD1 is a cytosolic form and SOD2 is a mitochondrial form of SOD. The backcrosses of the *Ctns-/-* mice with SOD1-/- mice and SOD2+/- mice (SOD2-/- are lethal) are currently going on. We want to obtain Ctns-/- SOD1-/- and Ctns-/- SOD2+/- mice, which will then be tested for their renal phenotype. These mice are difficult to obtain because the SOD knock-out are smaller and less efficient for the reproduction.

We obtained our 2 first double knock-out Ctns-/- SOD1-/- mice 2 months ago. We tested their renal function by measuring creatinine and urea levels in the urine and serum of the mice and alkaline phophatase in the serum. Two SOD1-/- and two *Ctns-/-* mice were used as controls. Urea and creatinine of the Ctns-/- SOD1-/- mice were normal and comparable to the ones obtained for SOD1-/- and *Ctns-/-* mice (Table 2). However, the alkaline phophatase levels were increased in Ctns-/- SOD1-/- mice compared to SOD1-/- and *Ctns-/-* mice (Table 2). These data are encouraging because the increased alkaline phophatase level in serum is one of the markers for proximal tubulopathy. Serum urea and creatinine levels are increased only when the kidney injury reaches the end-stages of renal failure. We are currently measuring the amino-acid levels in the urine of these mice. The premise is that if the Ctns-/- SOD1-/- mice really are presenting a proximal tubulopathy, we should observe an increase of amino acids in their urines. We will also measure the phosphate, calcium, sodium, potassium and chloride levels in the urine and serum of these mice, other indicators for proximal tubulopathy.

However, these results are only preliminary. They represent only two mice for the moment. We expect to have enough double knock-out Ctns-/- SOD1-/- mice for our studies in the next one or two months.

Mice	Date of Birth	Sex	Genotype	Creatinine in serum (mg/dL)	Creatinine in urine (mg/dL)	Creatinine clearance (ml/min)	Urea in serum (mmol/L)	Urea in Urine (mmol/L)	Alkaline Phosphatase in serum (IU/L)
1731	10/8/07	M	Ctns-/- control	0.53	57.84	0.092	8.81	72.87	83.82
1732	10/8/07	M	Ctns-/- control	0.61	59.41	0.035	9.82	75.70	64.85
2401	10/16/07	M	Ctns-/- SOD1-/-	0.53	53.22	0.032	10.93	72.45	156.15
2402	10/16/07	M	Ctns-/- SOD1-/-	0.54	45.38	0.025	12.02	70.93	154.67
2476	8/1/07	M	SOD1-/- control	0.46	51.92	0.025	11.72	70.08	51.97
2490	8/1/07	M	SOD1-/- control	0.49	ND	0.000	10.24	ND	109.56

Table 2: Serum and plasma analyses for renal injury of the 2 double knock out Ctns-/- SOD1-/- mice compared to SOD1-/- and Ctns-/- mice.

III – A NEW DIRECTION FOR THE PROJECT: GENE THERAPY FOR CYSTINOSIS

We decided to test in parallel a new strategy to find a cure for cystinosis, focusing on the nephropathy as a proof of concept. In the original animal protocol we described a stem cell-based treatment for cystinosis. We now want to try a gene therapy-based treatment for this disease using the adeno-associated virus (AAV) as a gene delivery vector. These two methods are promising and will be tested in parallel to determine which one will be used in clinical application. AAV has the advantage of delivering the transgene (in our case CTNS) to a wide range of tissues with long-term expression and a lack of tissue pathogenicity. AAV has already been tested in preclinical studies in a large number of metabolic diseases and at least 20 clinical trials have been done or are presently underway in several hundred patients.

This project is being done in collaboration with Dr. Jude Samulski, an international expert on AAV vectors, and Director of the Gene Therapy Center (University of North Carolina at Chapel Hill). This facility ensures that investigators can have gene vectors available in the quality and quantities needed for preclinical or clinical studies. Research in his laboratory has centered on adeno-associated virus (AAV) in order to exploit the unique features of this virus to develop an efficient viral vector system for use in human gene therapy. Several clinical trials for a variety of genetic diseases such as Duchenne Muscular Dystrophy are currently being done using vectors produced by the Gene Therapy Center.

The most commonly used AAV vector in clinical trials is the AAV serotype 2 because it has a well established safety and efficacy and it does not generate significant immune reactions [Wu et al., 2006]. Moreover, AAV2 can successfully and efficiently transduce kidney cells [Koeberl et al., 2007; Takeda et al., 2004; Wu et al., 2006].

Our goal is to generate and test AAV serotype 2 expressing CTNS in our *Ctns-/-* mice. We want an AAV vector that can efficiently target the kidney but also other organs such as liver, muscle, pancreas and brain that are known to be involved in cystinosis. We will perform two types of injection of the vectors, a tail vein injection and a kidney injection via the ureter. These two procedures will mimic the potential options for a clinical procedure by comparing a systemic injection of the vector to target most of the organs vs. the injection in the ureter to specifically target the kidney. To determine the efficiency of AAV2-CTNS on the kidney and other organs, we will study the treated animals for the presence of CTNS gene in the different tissues and the impact of the treatment will be determined by histology, quantifying cellular anomalies and cystine crystals and by cystine measurements as described in the original proposal.

To develop such a project, we will have to submit another grant to The Cystinosis Research Foundation because each production of AAV2-CTNS virus by the Gene Therapy Center will be quite expensive as well as the analyses (qPCR, histology, cystine measurement). However, we will first develop the proof of concept and test the efficiency of the virus transduction in different targeted organs as well as determine the stability of the transgene in animals. To do so, we have already injected AAV2 vectors expressing the reporter gene Luciferase (AAV2-Luc) via the tail veins or ureters of *Ctns-/-* mice. By these approaches, we can observe the cells transduced in live animals using the IVIS imaging system and determine the stability of the transgene expression.

Thus, we recently injected 1x1012 AAV virus particles per mouse in 3 *Ctns-/-* mice via ureteral injection and 3 *Ctns-/-* mice via tail vein injection. Four days after the injection via the tail veins, luciferase could not be observed in the body of live mice except at the point of injection in the tail (Figure 5). However, this is very early for AAV expression of protein and the virus particles are distributed widely in the animal's tissues. Over time, AAV protein production should increase and it is still too early to conclude anything. In contrast, luciferase fluorescence is readily detected in the area of the kidneys in the animals injected via the ureters in 2 of the 3 mice (Figure 5). These mice will be kept alive for at least 3 months and monitored every over week for the presence of fluorescence. They will then be sacrificed to precisely determine the quantity and the phenotype of the cells infected.

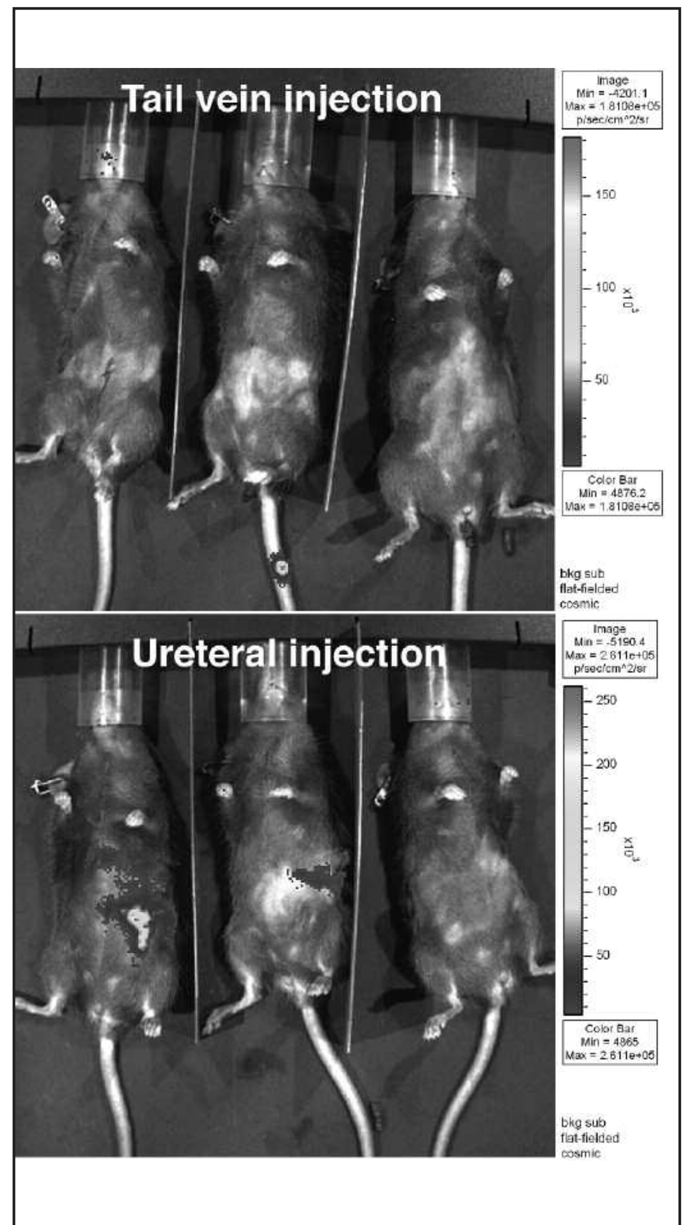


Figure 5: AAV2-Luciferase injection in Ctns-/- mice. The upper panel shows the mice that got the tail vein injection of the virus, the particles can be observed only at the site of the injection. The lower panel shows the mice that got the ureteral injection of the virus, the flurorescent particles can be observed in the kidney for 2 of the 3 mice.

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PROGRESS REPORT

RANJAN DOHIL, MD, PRINCIPAL INVESTIGATOR

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

Date: 1/14/2008

In the past six months two further cystinosis patients have been enrolled into the above mentioned study. Of these patients one has completed the pharmacokinetic study and the subsequent 1-month treatment trial successfully. The other patient is currently in the pre-pharmacokinetic study phase. One further patient has been recruited and is due to be enrolled within the first quarter of 2008.

Enrollment had to be halted in July 2007 due to a shortage in enteric-coated Cystagon™. Once further drug was received from Mylan and was shipped to The Coating Place, WI, for enteric-coating and enrollment was reopened.

In November and December three patients, who had previously completed the pharmacokinetics study and the subsequent 1-month treatment trial, were re-enrolled into the long-term enteric-coated therapy trial. The patients have not reported side effects at the doses prescribed. No other clinical results from the first study months are presently available.

One patient was excluded from the 12-month treatment trial after 10 months of treatment. Although the patient's white cell cystine levels were normal (0.1-0.3) on twice daily therapy, when we studied these levels over a 24 hour period it was felt that the patients cystine lievels were higher than we would expect. It was felt that this erratic profile was patient specific and due to abnormal stomach emptying.

One patient was enrolled into the open treatment trial. This treatment trial was added to the protocol after the patient had successfully been treated with enteric-coated Cystagon™ for 12 months. The patient continues to maintain low leukocyte cystine levels.

PROGRESS REPORT

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

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

First six month period: 3/1/07 – 9/1/07

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.

We have began the experimental work that was proposed, as briefly summarized below. We have also concluded with the support of the Cystinosis Research Foundation some complementary experiments that have been included in a manuscript describing the cystinosin-LKG isoform, which is currently under review. Support of the Cystinosis Research Foundation is acknowledged in the manuscript. Again, we are very grateful for the support and hope to produce interesting results in the very next future.

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

Proximal tubular cells (PTC) of two cystinosis patients and two healthy subjects have been cultured succesfully and have subsequently been conditionally immortalized with SV-40 T antigen and subcloned. The SV-40 T protein is not expressed at 10 days culturing at 37C. The conditionally immortalized cells express the proximal tubule markers aquaporin-1, p-glycoprotein and CD26 as shown by immunoblot techniques (fig 1) indicating their proximal tubular origin.

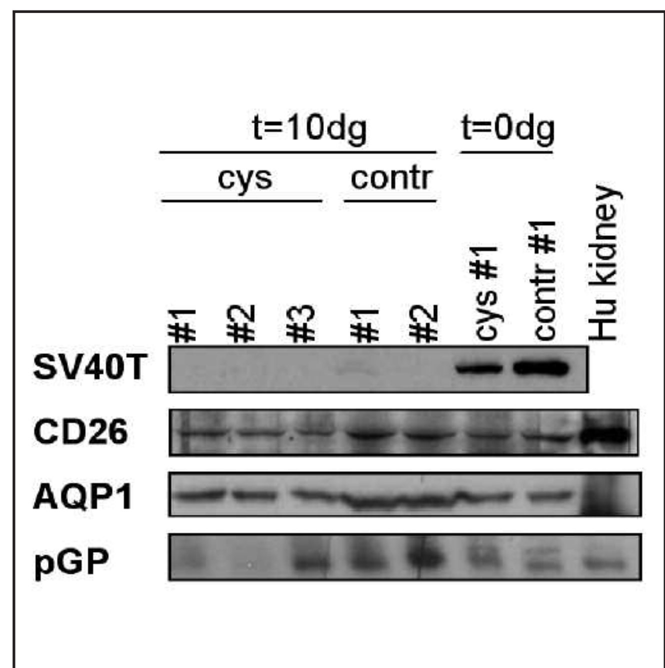


Figure 1. Immunoblots of cell homogenates of conditionally immortalized PTC of 3 cystinosis (cys) and 2 control (contr) clones cultured at 37°C for 10 days. Cells of cystinosis #1 and control #1 cell line are additionally harvested without maturation at 37°C. Results are compared to total human kidney homogenates.

The conditionally immortalized cells of both cystinosis patients and healthy controls are transiently transfected using Lipofectamin 2000 with vectors containing two isoforms of CTNS, called CTNS and CTNS-LKG, both with a RED fusion protein for localisation studies. Subsequently, transfected cells are incubated with antibodies raised again CTNS-LKG and visualized using conjugate go-a-rb-Alexa 488 (green). Cells are analyzed using immunofluorescence (fig 2). Both vectors are expressed in a intracellular vesicular pattern indicating succesful transfection. Only isoform CTNS-LKG perfectly co-localizes with CTNS-LKG antibodies.

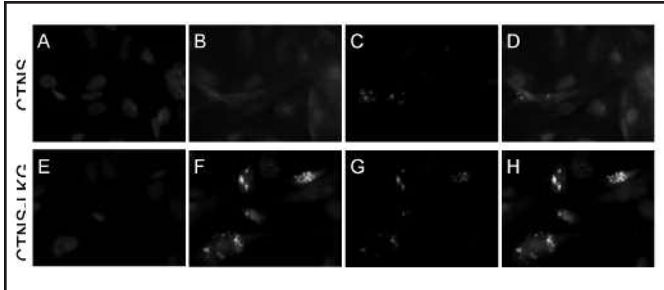


Figure 2. CTNS and CTNS-LKG expression in conditionally PTC after transfection. A-D shows transfected cells using CTNS-vector. E-H are cells transfected with CTNS-LKG. Nuclei are visualized using DAPI (A, E); CTNS-LKG is visualized using antibodies raised against CTNS-LKG. Vectors are expressed using a RED fusion protein (C, G). Only CTNS-LKG transfected cells show perfect co-localization (H).

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

The expression of the two cystinosin isoforms has been analyzed in different human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, speen, thymus, prostate, testis, ovary, small intestine, colon and peripheral leukocytes).

Cystinosin-LKG expression is tissue-specific, with the highest expression in the testis. This result may have important implications in explaining male hypogonadism in NC.

In addition, we have began analyzing the differential expression of the 2 isoforms under different conditions, including cystine deprivation and various forms of oxidative stress. So far, we have not detected significant changes in the relative expression of the two isoforms under the conditions that have been tested.

SPECIFIC AIM #3: Characterization of the cystinosin promoter

Constructs containing partial regions of the CTNS promoter have been prepared (from –769 bp, -348 bp, -316 bp, -254 bp, -81 bp to +1 bp) and cloned into the luciferase pGL4 vector. HK2 cells have been co-transfected with the pGL4 vector and the control phRL-TK vector that mediates the expression of the Renilla luciferase for normalization.

The activity of the promotor has been verified, reproducing the data that have been published by Phornphutkul et al. We are now in the process of testing the promoter under different stimuli and are also expanding the range of experiments that have been initially proposed including RNA stability experiments.

PROGRESS REPORT

BRUNO GASNIER (PARIS) AND ELLEN CLOSS (MAINZ)

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

Six-Month Progress Report: 2/6/08

Cysteamine depletes cystine from cystinotic lysosomes through formation of a mixed cysteamine-cysteine disulfide which exits lysosomes through the lysosomal cationic amino acid transporter, system c.

The molecular identity of system c is unknown. In preliminary experiments, the Mainz and Paris groups showed that several members of the SLC7 family of cationic amino acid transporters (CATs) localize to lysosomes and late endosomes, thus representing candidate system c transporters. In particular, two characterized CAT proteins, CAT2A or B, and an orphan SLC7 family member, SLC7A14, showed preferential lysosomal localization after transient expression in HeLa cells.

The aim of the project is to examine whether these SLC7 proteins are involved in lysosomal cysteamine-cysteine efflux, with the long-term goal to improve small-molecule treatment of cystinosis. Since these hypotheses on the identity of system c are not exclusive, they were examined in parallel.

The Paris and Mainz groups regularly met over the past 6 months to concert their efforts, share data and exchange tools (plasmids, etc).

PROGRESS REPORT PARIS GROUP (CHRISTINE ANNE, SAMIRA BOUBEKEUR, BRUNO GASNIER)

The Paris group focused on two objectives:

- characterize the interaction of cysteamine-cysteine with characterized CAT proteins
- search potential sorting motifs of SLC7A14 to induce plasma membrane expression as a preliminary step to functional studies

Recognition of the mixed disulfide cysteamine-cysteine by CAT transporters

A custom synthesis of 500 mg cysteamine-cysteine disulfide was purchased from the company Idealp-Pharma (http://www.idealp-pharma.com/).

The ability of cysteamine-cysteine to interact with human CAT1, CAT2A or CAT2B was examined by performing arginine transport assays in transiently transfected HEK cells using plasmids and protocols provided by the Mainz group. CAT1 was included as a control and because previous data from Mainz and Paris showed that it partially localizes to lysosomes and late endosomes in addition to the plasma membrane.

Surprizingly, hCAT1 turned out to be the most sensitive to cysteamine-cysteine, with ~80% inhibition at 10 mM concentration as compared to ~10% for CAT2B. The sensitivity of CAT2A is intermediate. These experiments thus suggest that CAT1 and/or CAT2A might be involved in cysteamine-mediated cystine depletion since they both share lysosomal localization and recognition of the mixed disulfide. The potential role of CAT1 is interesting since it is ubiquitously expressed.

Because of the high level of endogenous arginine uptake in mammalian cells, IC50 values of CATs for cysteamine-cysteine could not be determined in HEK cells. These experiments are thus now pursued in Xenopus oocytes, an expression system which provides a much higher signal-to-noise ratio, using plasmids provided by Mainz. Measurements will be repeated at acidic extracellular pH to mimic conditions of the lysosomal lumen.

Next step: The ability of CAT1, CAT2A and CAT2B to translocate cysteamine-cysteine across membranes using a custom-synthesized 3H-labeled molecule.

Development of an in situ cystine depletion assay in whole cells

The above transport experiments will tell whether CATs can export cysteamine-cysteine from lysosomes – and help focusing on particular isoforms – but not whether they actually do so. It is thus necessary to develop a lysosomal cystine depletion assay to provide a final conclusion on the identity of system c. This assay can be performed on isolated lysosomes or in whole cells, using an ester derivative to artificially load lysosomes with cystine.

To this purpose, we ordered a custom synthesis of [3H]L-cystine dimethyl ester labeled on the cystine residue to the company Hartmann Analytic (http://www.hartmann-analytic.de/).

However, because of purification problems the compound ordered in July was received only in late December.

Experiments are now ongoing to find optimal conditions for loading [3H] cystine into lysosomes of whole cells and perform cysteamine depletion experiments.

Screening of putative sorting motifs of the orphan transporter SLC7A14

As shown in previous studies from the Paris group (Kalatzis et al EMBO J 2001; Morin et al EMBO J 2004), the functional characterization of lysosomal transporters can be facilitated by redirecting them to the plasma membrane for transport measurement in whole cells. Paris and Mainz thus tried different approaches in order to redirect SLC7A14 to the cell surface.

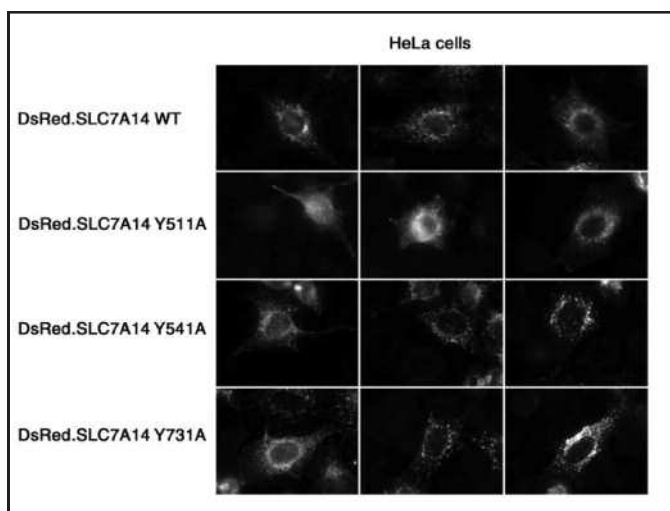
Attempts made in Paris were based on the mutation of potential lysosomal sorting motifs (see Bonifacino and Straub 2003 Ann. Rev. Biochem. 72:395-447). Several tyrosine-based and dileucine-based candidate motifs were identified in cytosolic loops or in the C-terminal end of SLC7A14.

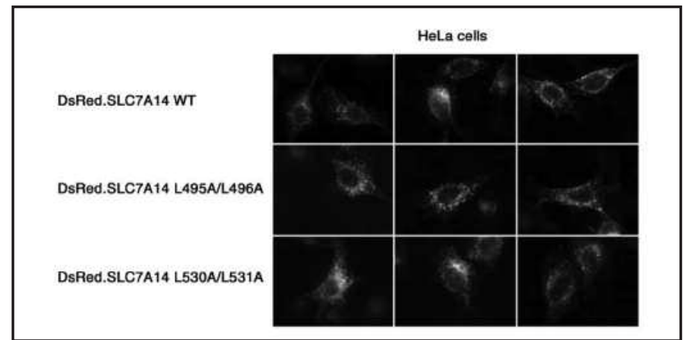
Their critical residues were mutated to alanine as follows:

L495A/I496A Y511A L530A/I531A

Y541A Y731A

The mutations were introduced into a DsRed-SLC7A14 construct provided by Mainz. However, as illustrated below, none of these mutants was redirected to the plasma membrane after transient expression in HeLa cells.





Two hypotheses may explain these results: either neither of the above motifs is involved (the lysosomal localization of SLC7A14 resulting from other signals) or several of them are involved. The latter hypothesis (motif redundancy) will be examined by combining several of the above mutations in a single construct. In parallel, we will explore a novel strategy bypassing knowledge of the sorting mechanism by inserting a dominant motif conferring surface expression into wild-type SLC7A14 (see Shikano et al 2005 Nat. Cell Biol. 7:985-992).

Other attempts by the Mainz group are described below.

PROGRESS REPORT MAINZ GROUP (ELLEN CLOSS AND JEAN-PAUL BOISSEL)

Our focus in the first 6 months of the grant period lay on SLC7A14, the orphan member of the SLC7 family that exhibited an exclusive lysosomal localization in our preliminary studies. The following points were addressed:

- Investigation of the expression pattern of SLC7A14 in human tissues and cell lines
- Subcellular localization of SLC7A14 in different human cell lines
- Attempts to induce plasmalemmal localization of SLC7A14
- Creation of stable cells lines expressing SLC7A14.EGFP fusion proteins

Expression pattern of SLC7A14 in human tissues and cell lines

To find out which tissues and cell lines express SLC7A14, its mRNA expression was assessed by qRT- real time PCR. High levels of message were detected in the CNS (brain, cerebellum, hippocampus, spinal cord), and in the bladder (Figure 1). Amongst various human cell lines tested, human umbilical vein endothelial cells (HUVEC), NB-OK-1 and TGW- 1 neuroblastoma cells exhibited considerable SLC7A14 expression (Figure 2A). Only little SLC7A14 mRNA was detected in NT2 neuron-committed teratocarcinoma cells under basal condition. However, when neuronal differentiation was induced in these cells by treatment with retinoic acid, a time-dependant upregulation of SLC7A14 was observed (Figure 2B). SLC7A14 thus seems to be expressed primarily in neuronal cells, endothelial cells and in the bladder.

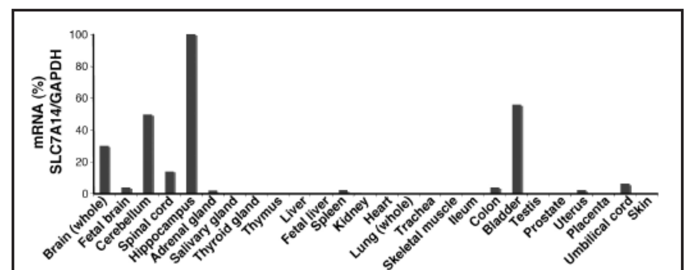


Figure 1 Expression of SLC7A14 in various human tissues was assessed by quantitative RT/PCR using a primer pair and a taqman hybridization probe specific for SLC7A14.

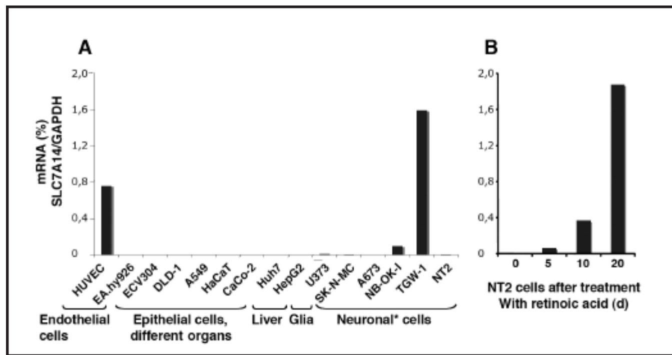


Figure 2 Expression of SLC7A14 was assessed as described in Fig.1: A) in various human cell lines, B) in human NT2 teratocarcinoma cells after treatment with retinoic acid for the time periods indicated. Retinoic acid induces neuronal differentietion in NT2 cells.

Localization of SLC7A14 in different human cell lines

Our preliminary studies indicated an exclusive lysosomal localization of SLC7A14 in human HeLa cells. To find out if this subcellular distribution is also seen in other cell types, a fusion construct between the SLC7A14 cDNA and enhanced green fluorescent protein (EGFP) was transfected in various human cell lines. In addition, the fusion protein was also expressed in oocytes from *Xenopus laevis.* In all cell lines tested (U373 glioblastoma, A673 neuroepithelioma, NT2 teratocarcinoma, Huh7 hepatoma and TGW neuroblastoma cells), SLC7A14 exhibited an exclusive intracellular staining. This was also true when the fluorescent protein DsRed (dimeric or monomeric) was fused to the C- or N-terminus of SLC7A14, indicating that the subcellular localization was independent of the position or type of fluorescent protein in the fusion protein. An example is shown for TGW neuroblastoma cells and NT2 teratocarcinoma cells (that exhibit endogenous expression of SLC7A14) in Figure 3. In all cases, the localization of the fusion proteins overlapped with the lysosomal markers Lamp1 (a type 1 transmembrane protein that is primarily localized in lysosomes and late endosomes) and the fluorescent dye "lysotracker" (that accumulates specifically in compartments with low pH) (Invitrogen). These results indicate that SLC7A14 is truly a lysosomal resident. However, antibodies against SLCA14 are necessary to examine the subcellular localization of the native protein in primary cells and tissues.

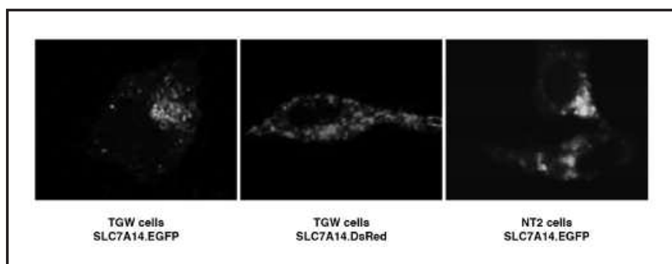


Figure 3 TGW neuroblastoma and NT2 teratocarcinoma cells were transiently transfected with expression constructs coding for fusion proteins between SLC7A14 and EGFP or DsRed as indicated.

Attempts to induce plasmalemmal localization of SLC7A14

The subcellular localization of SLC7A14 is clearly different from that of the related CAT proteins. Although the latter exhibit also intracellular staining compatible with a lysosomal localization, all CAT isoforms localize to the plasma membrane, where they mediate Na+-independent transport of cationic amino acids. Co-expression of SLC7A14 with CAT-1 revealed nearly 100% co-localization of the two proteins in intracellular compartments. However, only CAT-1 was expressed in the plasma membrane (Figure 4).

SLC7A14 exhibits extended C- and N-termini as compared to CAT-1 (Figure 5). To find out if either extension was responsible for the lack of plasma membrane localization, deletion mutants were constructed. However, DsRedmonoC1-SLC7A14 fusion proteins lacking the 20 C-terminal, 74 N-terminal or both, C- and N-terminal amino acids of SLC7A14 remained completely intracellular. Finally SLC7A14 was fused to rBAT, a glycoprotein necessary to pull SLC7A9 to the plasma membrane. This approach has recently been used to force a new SLC7 member to the plasma membrane and to subsequently show transport activity of the fusion protein (Chairoungdua et al. J Biol Chem 2001, 276: 49390-9). However, cells expressing SLC7A14/rBAT fusion proteins underwent apoptosis. We thus plan to create chimeras between SLC7A14 and CAT-1 in order to localize the protein region responsible for the exclusive intracellular localization.

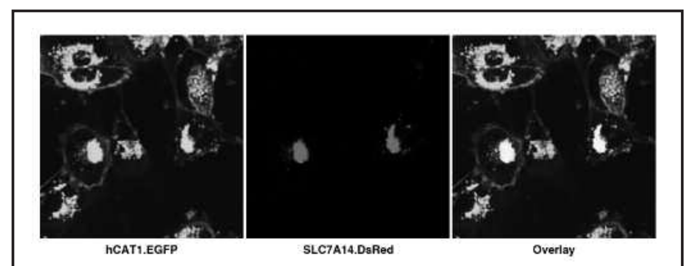


Figure 4 U373MG glioblastoma cells stably expressing a fusion protein between human CAT-1 and EGFP were transiently transfected with an expression construct for a fusion protein between SLC7A14 and DsRed. The overlay shows co-expression of the two proteins in intracellular compartments. However, only CAT-1 is also expressed in the plasma membrane.

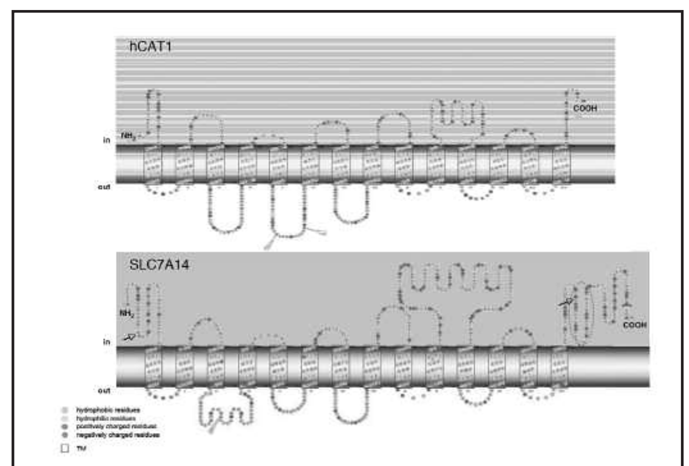


Figure 5 Comparison of the amino acid sequences of human CAT-1 and SLC7A14. Both proteins are predicted to span the plasma membrane 14 times. SLC7A14 exhibits extended N- and C-termini as compared with CAT-1. The arrows indicate the N- and C-terminal deletions.

Creation of stable cells lines expressing SLC7A14.EGFP fusion proteins

Our results so far indicate that SLC7A14 is a lysosomal resident and may be difficult to divert to the plasma membrane. It is thus important to try an alternative approach to measure transport by this protein directly in the

lysosomal compartment. We have thus generated a U373MG cell line with stable overexpression of the fusion protein EGFP-N1-SLC7A14 (Figure 6). The subcellular localization of the fusion protein in this cell line is identical with the localization of the transiently expressed protein. We are now planning to prepare lysosomes from this cell line as well as from untransfectd cells and cells stably expressing EGFP-CAT-1 fusion proteins to perform transport studies. The CAT-1 lysosomes are expected to exhibit increased transport of cationic amino acids and can thus be used as positive controls.

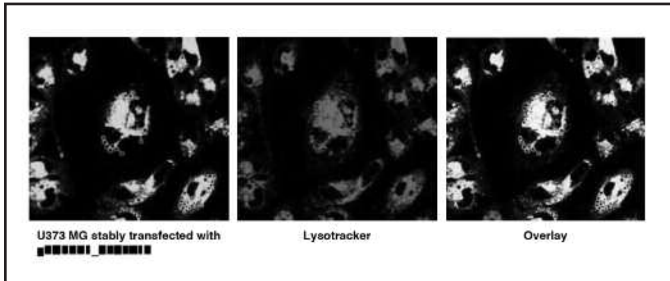


Figure 6 EGFP-N1-SLC7A14 stably expressed in U373MG glioblastoma co-localizes with lysotracker. Its localization is thus similar to the transiently expressed protein.

Ongoing projects

Antibodies against SLC7A14 and hCAT-1/ hCAT-2A

Previously, we have generated antibodies against the CAT proteins using fusion proteins between the CAT C-termini and the bacterial protein TrpE. However, the immune sera obtained against these fusion proteins have relatively low titer. In an attempt to increase the immune response, we are now planning to use fusion proteins between the CAT C-termini and glutathione-S-transferase (GST) in the first immunization and the TrpE fusion proteins for the boosts. If this strategy is more successful, GST and TrpE fusion proteins containing different regions of SLC7A14 will be generated for subsequent immunizations.

Subcellular localization of SLC7A14 in primary cells with endogenous expression

The localization of SLC7A14-EGFP or -DsRed fusion proteins will be examined in primary cells that express SLC7A14, e.g. HUVEC and primary neurons.

Chimeras between SLC7A14 and hCAT-1

We are currently subcloning the different SLC7A14 deletion and fusion constructs into an vector for *Xenopus laevis* oocyte expression. Especially for the rBAT fusion protein, we hope that the oocytes will support overexpression better then the mammalian cells. We will also generate chimeras between SLC7A14 and hCAT-1 to localize the protein domains important for transport and subcellular targeting. These experiments will also be carried out in oocytes and represent an alternative approach to find the transport substrates for SLC7A14.

Measurements of lysosomal transport

We are also planning to establish a protocol for lysosome preparation (from cells with stable overexpression of SLC7A14 and hCAT-1) and subsequent transport assays.

PROGRESS REPORT

VASILIKI KALATZIS, PhD, PRINCIPAL INVESTIGATOR
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"Gene Transfer Studies for Cystinosis"

CRF 12-mo detailed progress report. Date funding awarded: 1/31/07

INITIAL 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.

RESULTS OBTAINED OVER THE LAST 12 MONTHS

I) Validating *in vitro* gene transfer studies by in vivo gene transfer

BACKGROUND:

We previously showed that viral-mediated gene transfer is feasible for reducing cystine levels *in vitro* in *CTNS-/-* human fibroblasts and *Ctns-/-* murine hepatocytes. Furthermore our data from the murine hepatocyte studies suggested that the efficiency of cystine reduction is age-dependent. The next step was to validate these observations *in vivo* by directly targeting the liver in *Ctns-/-* mice. This study is now almost complete and the results are summarised as follows:

RESULTS:

Short-term (1 wk) gene transfer experiments: We targeted the liver of young (2 and 3 mo-old) and older (6 and 9 mo-old) mice with first generation (deleted only in the E1 & E3 viral genes) adenovirus vectors expressing green fluorescent protein (GFP; AdGFP), cystinosin (AdCTNS) or cystinosin fused to GFP (AdCTNSGFP). We evaluated transduction efficiency by IF studies of GFP fluorescence from AdGFP or AdCTNSGFP. Regardless of age, the transduction efficiency of both vectors was on average ~50%. With respect to hepatic cystine levels, significant differences were observed between the experimental groups of the young mice but not between those of older mice. Regardless of age, we observed a ~2-fold reduction in cystine levels one-week post-transduction with the control AdGFP vector, which was not significant ($p > 0.05$). In contrast, transduction of young *Ctns-/-* mice with AdCTNS or AdCTNSGFP resulted in a significant ~5-fold decrease on average ($p < 0.05$) in cystine levels as compared to cystine levels in nontransduced mice. Following transduction of older *Ctns-/-* mice with AdCTNS or AdCTNSGFP, we did not significantly reduce cystine levels beyond that observed with AdGFP.

Taken together, we show that it is feasible to reduce lysosomal cystine levels in vivo by viral-mediated gene transfer. Furthermore, our in vivo observations confirm our *in vitro* data that the efficiency of cystine clearance over a short transduction period is age-dependent.

Long-term (1 mo) gene transfer experiments: A possible explanation for the age-dependent efficiency of cystine clearance is that a longer duration of cystinosin expression is required to reduce the higher cystine levels in older mice (2- to 7-fold higher than those of younger mice). The first generation adenovirus vectors are prone to short-term expression in most immunocompetent rodents, especially in the liver. This is due to T-cell mediated destruction of transduced cells, which normally occurs from day 7. Therefore to inhibit the T-cell response, a mild immunosuppression protocol (using cyclosporin A (CsA) delivered via a subcutaneously implanted osmotic pump) was begun one day prior to transduction and used to extend the post-transduction period from 7 to 28 days. A whole blood immunoassay demonstrated that CsA was continually administered over the 28-day period.

We transduced two groups of immunosuppressed *Ctns-/-* mice, aged 3 and 5 mo, with AdGFP, AdCTNS or AdCTNSGFP. Interestingly, and consistent with our short-term in vivo transduction data, significant differences were observed in the hepatic cystine levels following sacrifice between the experimental groups aged 3 mo but not between those aged 5 mo, regardless of a similar transduction efficiency for both ages (~30%; as estimated by GFP fluorescence from AdGFP). For both age groups, we observed a reduction in cystine levels with the AdGFP vector that was not significant and that was lower (1.2-fold) than that observed in the short-term experiments (2-fold). In contrast, long-term transduction of 3 mo-old *Ctns-/-* mice with AdCTNS or AdCTNSGFP resulted in a significant 2.5-fold decrease ($p < 0.05$) in cystine levels as compared to cystine levels in nontransduced mice. As was the case for AdGFP, the observed 2.5-fold decrease was lower than that obtained with AdCTNS or AdCTNSGFP in the short-term transduction experiments. As a negative control, in the absence of an immunosuppressive protocol, cystine levels were not reduced in 3 mo-old mice 1-mo post-transduction with AdGFP, AdCTNS or AdCTNSGFP. One-month post-transduction of 5 mo-old immunosuppressed *Ctns-/-* mice with AdCTNS or AdCTNSGFP, we did not detect a significant reduction in cystine levels beyond that observed with AdGFP.

Thus our long-term transduction data continue to show an age-dependent reduction of cystine clearance and suggest that this phenomenon is due to a factor other than duration of cystinosin expression.

Kupffer cell regeneration post-transduction non-specifically affects cystine levels: Contrary to our expectations, cystine levels were more efficiently reduced in short-term as opposed to long-term transduction experiments. Furthermore, the non-specific effect of AdGFP on cystine reduction was more pronounced 1-wk versus 1-mo post-transduction. These observations suggest that cystine levels are partly non-specifically increasing over time. Intravenous adenovirus vector entry destroys Kupffer cells, which could account for the decrease in cystine levels observed after short-term AdGFP transduction experiments. In turn, we hypothesised that a long post-transduction period may result in the regeneration of Kupffer cells and hence cause a rise in cystine levels.

To test this hypothesis, we artificially depleted Kupffer cells from 2 and 5 mo-old *Ctns-/-* mice by treating with liposomal clodronate (Clodrolip), a potent antimacrophage agent. Mice were sacrificed 1-wk (at which time there should be > 90% depletion) or 1-mo post-treatment. Regardless of age, hepatic cystine levels were ~25% of control levels 1 wk after Clodrolip administration. In contrast 1 mo later, cystine levels had increased to ~70% of control levels. In parallel, we performed immunostaining studies with an antibody to a macrophage marker to demonstrate the absence and presence of Kupffer cells 1-wk and 1-mo post-treatment, respectively.

Taken together, these data suggest that Kupffer cell regeneration causes a non-specific increase in cystine levels. This correlates with the observation that cystine levels rose to 85% of control levels (from 50% at 1 wk) 1-mo post-transduction with AdGFP. In contrast, cystine levels rose to only 40% of control levels (from ~20% at 1-wk) 1-mo post-transduction with AdCTNS, indicating a continued specific role of CTNS in cystine clearance. Finally, our observations suggest that that the cystine content of the Kupffer cells, which represent only 10% of the liver cell population, accounts for 75% of hepatic cystine levels in the mouse model.

ONGOING WORK:

We are currently complementing this study with some additional experiments:

1) We have not been able to successful use the anti-cystinosin antibody to detect cystinosin expression on liver sections by immunofluorescence studies (hence the use of AdCTNSGFP). We are currently performing immunostaining studies, a more sensitive technique, with the anti-cystinosin antibody to try to detect cystinosin expression in liver sections.
2) As mentioned above we did not detect a reduction in cystine levels 1-mo post-transduction of non-immunosuppressed mice with AdGFP, AdCTNS or AdCTNSGFP. Conversely, we did detect GFP expression from AdGFP (~50% of cells transduced) but not from AdCTNSGFP. These observations suggest that cystinosin or cystinosin-GFP may be more immunogenic than GFP. We are currently performing assays to verify this hypothesis.
3) In parallel, we are preparing a manuscript detailing the first *in vitro* and in vivo gene transfer studies for cystinosis.

II) Ocular gene transfer studies

BACKGROUND:

Our article detailing the ocular anomalies in the cystinosis animal model was accepted in March and published in August 2007:

Kalatzis, V., Serratrice, N., **Hippert, C.,** Payet, O., Arndt, C., Cazevieille, C., Maurice, T., Hamel, C., Malecaze, F., Antignac, C., Müller, A. & Kremer, E.J. (2007) A temporospatial guide to the ocular anomalies in a cystinosis mouse model. ***Pediatr. Res.*** 62: 156-162.

The next step of this project is to perform gene transfer studies to the cornea, one of the first tissues affected in cystinosis patients and in the *Ctns-/-* mice.

RESULTS & ONGOING WORK:

To date, we have used first generation vectors for the *in vitro* and in vivo liver studies. However, if we hope to eventually use gene therapy in the clinic, we need to perform in vivo eye studies with more clinically relevant CTNS-expressing vectors. We chose two such vectors that are devoid of all viral genes and that can readily transduce the cornea: an adeno-associated vector (AAV8) and a state-of-the-art, helper-dependent canine adenovirus vector (HD CAV-2). To facilitate vector production and subsequent analysis we needed to include a marker gene, such as GFP, in our expression cassette in addition to CTNS. Thus we opted for the use of an IRES sequence as opposed to fusing GFP to CTNS. In this way we will

avoid possible hindering cystinosin function, or rendering cystinosin more immunogenic, with a GFP tag.

Firstly, we generated the AAV8 vector containing the CTNS-IRES-GFP expression cassette. We performed the initial subcloning step of inserting the expression cassette into an AAV shuttle plasmid. We then tested the plasmid for correct cystinosin expression, localisation and function. Subsequently, we subcontracted the production of the AAV8 vector to the Vector Production Platform at the Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain). Claire Hippert, the recipient of the CRF Ph.D. fellowship, spent a week in Barcelona learning the AAV production steps. We received a first batch of the AAV-CTNS-IRES-GFP vector in July 2007, however due to a problem during the purification steps, the initial stocks were not sufficient for in vivo experiments production. Thus we re-ordered a second batch, which we received at the end of December 2007. The next step is to test the functionality of the transgenes. Last week, we injected AAV-CTNS-IRES-GFP and AAV-GFP in the tail vein of 2 mo-old *Ctns-/-* mice. We will sacrifice these mice 1-wk post-injection and will assay the liver, kidney, lung, spleen, heart and brain for a reduction in cystine levels by cystinosin and for GFP expression. Once we have verified that the vector is functional, we will begin our cornea transduction experiments.

Secondly, concerning HD CAV-2 vector production, our progress in optimising the transfection of canine cells to begin production has advanced slower than we had hoped. However, in December 2007, Sandy Ibanes, a technician, joined our team and is working on HD CAV-2 vector production fulltime so we hope to advance significantly this year.

III) Characterisation of the CNS anomalies in cystinosis mice

BACKGROUND:

We previously performed behavioural studies of 3 (young) and 13 (middle-aged) mo-old *Ctns-/-* mice and showed that the middle-aged mice have marked spatial and working memory defects, reminiscent of those seen in some patients, which were most likely hippocampal in origin.

RESULTS:

As the memory defects we detected in the *Ctns-/-* mice suggested a hippocampal defect, we wanted to assay the cystine levels in the hippocampus with regards to other brain structures. Thus we dissected the brains of the young and middle-aged mice used in the behavioural studies, as well as additional age-matched controls (92 mice in total), and assayed the cystine levels in the hippocampus, residual forebrain, cerebellum and brainstem. Consistent with the age-related defects we identified, we observed higher cystine levels in all brain structures of middle-aged as compared to young *Ctns-/-* mice (cystine levels were already elevated in all tissues of young *Ctns-/-* mice as compared to age-matched controls). Furthermore, consistent with the memory impairments, the hippocampus, a structure playing a prominent role in spatial and contextual memory encoding, showed the highest cystine levels. This was followed by the cerebellum and brainstem, which are structures involved in particular, motor skilled memories.

In parallel, we performed a histological study to detect the presence of cystine crystals. The cystine levels in the hippocampus were not elevated enough to result in the formation of detectable cystine crystals. However, we did detect crystals in the choroid plexus and clustered around capillaries in the parenchyma. The ensemble of our observations are similar to those reported for cystinosis patients.

Taken together, our work suggests cystinosis-associated CNS anomalies are due to progressive cystine accumulation. The article describing this study was accepted in September and published on-line in November 2007:

Maurice, T., **Hippert, C.**, Serratrice, N., Dubois, G., Jacquet, C., Antignac, C., Kremer, E.J. & **Kalatzis, V. (2007)** Progressive cystine accumulation in the CNS of a cystinosis animal model results in severe age-related memory deficits. ***Neurobiol. Aging*** Epub ahead of print: 10.1016/j.neurobiolaging.2007.09.006

ONGOING WORK:

Having identified the brain regions affected in the *Ctns-/-* mice, the next step is to identify the cell type. Clearly neurons are affected, but we do not know if this due to a direct cystine accumulation within this cell type or due to the indirect destruction of supporting cells. We will set up primary neuron, microglial, astrocyte, and oligodendrocyte cultures from the brains of Ctns+/+ and *Ctns-/-* mice, and assay their respective cystine levels to determine which cell type(s) is predominately involved. Primary cultures can only be obtained from newborn mice that are younger than 7 days. Therefore, it is not certain that the cystine levels will be high enough to detect a difference. However, these cells can be kept in culture for 2 weeks during which time we hypothesise that the *Ctns-/-* cells will accumulate cystine at a faster rate than wild-type cells. However, the caveat of culturing cells is that the *in vitro* culture conditions poorly mimic the redox environment in vivo, therefore the results we obtain may not mirror the in vivo situation. To palliate this problem, we will, in parallel, attempt to isolate individual cell types from the brains of different-aged mice and directly assay cystine levels. We will homogenise different brain regions and isolate single cell populations using fluorescent-labelled cell markers (NGF receptor for neurons, EAAT1 for astrocytes, myelin oligodendrocyte glycoprotein for oligodendrocytes, and CD11b for microglial cells) and fluorescence-activated cell sorting. We will assay the cystine levels in each cell population and compare the data with those obtained by assaying the primary *in vitro* cultures. These complementary approaches will give us a better grasp of the in vivo situation.

If we are able to culture neurons and micro- and macro-glial cells from young *Ctns-/-* mice as detailed above and detect differences in cystine levels as compared to wild-type cells, we will initially attempt to reduce cystine levels *in vitro* in these cultures using the AAV-CTNS-IRES-GFP vector. These studies will be a first step to our long-term goal of targeted gene transfer to the brain in vivo using stereotactic injection.

CONCLUSION:

We have made significant progress in the characterisation of the anomalies of the *Ctns-/-* mice, resulting in **2 published papers,** and in providing the proof-in-principal that gene transfer can be used for reducing lysosomal cystine levels due to defective cystine efflux **(manuscript in preparation)**. We have now laid the necessary foundations for the more challenging eye and brain-targeted gene transfer studies that we will undertake in the second year of our two-year grant.

PROGRESS REPORT

E.N. LEVTCHENKO, MD, PhD, PRINCIPAL INVESTIGATOR
UMC ST. RADBOUD, THE NETHERLANDS

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

Six-Month Progress Report: 12/1/06 – 5/1/07

SUMMARY OF THE PROJECT

Nephropathic cystinosis is characterized by the lysosomal accumulation of cystine and is generally leading to Fanconi syndrome in the first year of life. This autosomal recessive disorder frequently progresses to end stage renal disease, which is the leading cause of morbidity in patients with cystinosis.

In the current project funded by the Cystinosis Research Foundation we have addressed two 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 development of a conditionally immortalized proximal tubular cell (ci-PTC) model of cystinosis phenotype by our group allows us to investigate the cytokine/chemokine production *in vitro*. In cells cultured on Transwell chambers, the polarity of secretion can be evaluated and provides information about the cause of the progressive development of tubulointerstitial lesions observed in cystinosis patients.

2) *Study of intracellular glutathione status, transport and generation of reactive oxygen species (ROS) in a conditionally immortalized proximal tubular cell line.*

   The altered status of intracellular glutathione observed in cystinosis tissue, points to the role of oxidative stress in the pathogenesis of cystinosis. The *in vitro* cell model mentioned above allows us to investigate the glutathione status and ROS production. Furthermore, we can evaluate the effects of anti-oxidants and cystine depleting agent cysteamine. This might lead to improvements in the therapy of patients with cystinosis.

In this first progress report we describe the preliminary data that we have obtained for both key objectives in the first six months.

PRELIMINARY RESULTS

Key objective 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.

A. Mature ci-PTC's will be cultured until confluent monolayers are obtained

We had already succeeded in culturing ci-PTC's of 3 healthy controls and 3 cystinotic patients, by culturing urine sediment and transfection using SV40T antigen, containing both tsA58 and U19 mutations, and hTERT, containing the essential catalytic subunit of human telomerase. This resulted in cells that proliferate at 33°C and mature at 37°C. To obtain a homogenous cell line, we now have subcloned cell lines of 2 healthy donors and 3 cystinotic patients.

In previous studies we have shown the cobblestone morphology, presence of aminopeptidase N and alkaline phosphatase activity, all characteristic for proximal tubular cells. To underscore this proximal phenotype in the subclones, we have performed additional characterization studies, indicating the presence of dipeptidyl-peptidase IV (dpp-IV, CD26), aquaporin-1 (AQP1), p-glycoprotein (pGP) and organic cation transporter 2 (OCT2) by immunoblot analysis after culturing at 33°C and after 10 days maturation at 37°C (figure 1).

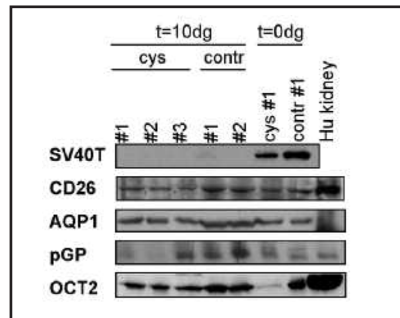

Figure 1. Immunoblots of cell homogenates of conditionally immortalized PTEC of 3 cystinosis (cys) and 2 control (contr) clones cultured at 37°C for 10 days. Cells of cystinosis #1 and control #1 cell line are additionally harvested without maturation at 37°C. Results are compared to total human kidney homogenates.

Furthermore, the expression of SV40T disappeared after 10 days maturation. By immunohistochemistry, the presence of zona occludens 1 (ZO-1) tight junction protein was detected in cells, indicating their epithelial origin (figure 2). The presence of tight junctions in the ci-PTC contributes to an impermeable cell monolayer, which is important for the transport experiments using Transwell chambers.

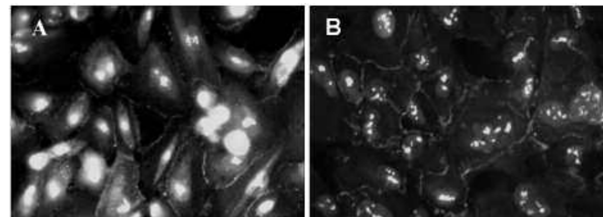

Figure 2. Immunofluorescence image of conditionally immortalized control (A) and cystinosis (B) PTEC cultured for 10 days at 37°C and incubated with antibodies against tight junction marker ZO-1.

According to this additional characterization of the proximal tubular phenotype of the subcloned cell lines, we have selected one clone of each donor for further experiments.

To study the cytokine production, one control and one cystinotic ci-PTC subclone was cultured in triplo in 24 well plates and after 7 days maturation at 37°C, medium was changed to serum free medium. At day 8 of maturation, cells were stimulated with IL1α for 48hr and supernatant tissue culture medium was harvested at day 10. For this preliminary report, we have measured IL-8 in the supernatants in the presence or absence of IL1α using DuoSet ELISA development system (R&D systems, USA). The results show an increase in IL1α–stimulated IL-8 production in both control and cystinotic cells. To perform statistical analysis, we will perform this test in all selected ci-PTC subclones in triplo.

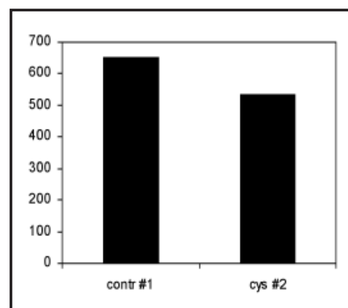

Figure 3. Secretion of IL-8 is measured in culture medium of ciPTC with and without IL1-a stimulation for 48hr after 10days maturation at 37°C.

B. Examination of polarity of secretion

The polarity of secretion will be examined in a Transwell chamber system. To study the polarity of ci-PTC cultured on Transwells, we have performed electron-microscopy (EM). Cells were seeded on Transwells and after 16hr incubation at 33°C to allow the cells to attach, Transwells were transferred to 37°C for 10 days. Subsequently, cells were fixed in situ and further processed suitable for EM (fig. 4). The image shows clearly some apical microvilli indicating polarization of the ci-PTC. Secondly, the EM pictures show some clathrin coated pits, indicating that the endocytic apparatus is functioning.

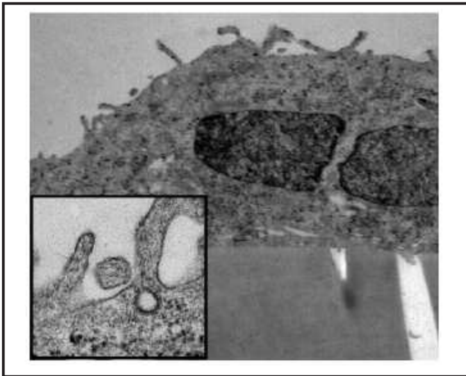

Figure 4. EM picture of a conditionally immortalized control PTEC cultured on transwell membrane (Costar #3412) for 10 days at 37°C showing polarization. Inset: clathrin coated pit, indicating endocytic activity

C. Application of a new technique

When we have analyzed more cytokines, we can evaluate the results and select an appropriate cytokine antibody array. By doing this, we can highly sensitively detect a broad range of cytokines excreted by cystinosis ci-PTC and compare the results with control ci-PTC.

D. Identifying intracellular signaling mechanism

To investigate whether albumin is reabsorbed in proximal tubules via endocytosis in cystinosis we examined the expression of megalin/cubilin in cystinotic renal tissue. These receptors are cooperating in the proximal reabsorption of proteins such as albumin in clathrin-coated pits via the endocytic pathway. The results show megalin and cubilin expression at the brush border of proximal tubule cells in cystinotic tissue (figure 5).

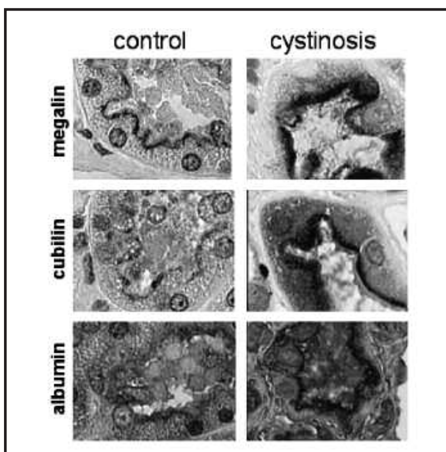

Figure 5. Immunohistochemistry of human kidney tissue of healthy control and cystinotic patient, showing apical megalin, cubilin expression and albumin uptake in proximal tubules.

Presence of albumin in endocytic vesicles indicates that albumin is reabsorbed in cystinotic proximal tubule cells. This finding might be of importance in studying the effects of albumin on cytokine production and the development of interstitial fibrosis.

A detailed mechanism involved in the progressive development of tubulointerstitial lesion found in cystinosis patients might be found after the evaluation of the cytokine antibody array.

Key objective 2)

Study of intracellular glutathione status, transport and generation of reactive oxygen species (ROS) in a conditionally immortalized proximal tubular cell line.

A. Study of proximal tubular GSH transport in conditionally immortalized PTC

So far, we do not have conclusive data on GSH transport in ci-PTC.

B. Study of intracellular glutathione status

The investigation of total and oxidized GSH in ci-PTC provides us with information about the oxidative status in cystinosis.

For this purpose, cells were cultured for 14 days at 37°C and harvested at different stages of maturation. Subsequently, levels of total GSH, oxidized GSSH and intracellular cystine levels were determined using HPLC methods. The data are presented as means of two control and two cystinosis ci-PTC (figure 6). These data confirm our published data in fibroblasts, polymorphonuclear granulocytes and immortalized PTC using HPV E6/E7 gene, in which we have shown increased oxidized GSSH in cystinotic cells (Levtchenko et al, NDT (20), 2005; Wilmer et al, BBRC (337), 2005).

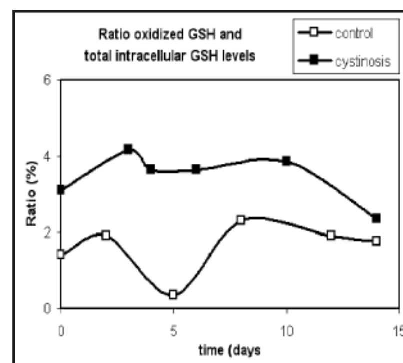

Figure 6. Ratio between oxidized GSH and total intracellular GSH in cultured ci-PTEC during 14 days of maturation at 37C. Levels are measured by HPLC technique. Data are mean valus of 2 subclones.

C. Study of intracellular ROS production, ROS-induced cellular damage and effects of the anti-oxidants.

So far, we have not studied this part of the project.

ONGOING RESEARCH

Key objective 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.

A. Mature ci-PTC's will be cultured until confluent monolayers are obtained

Cytokine IL-8 productions in confluent ci-PTC monolayers have been studied so far in one control and one cystinotic cell line. These studies will be extended in 2 control and 3 cystinotic subclones and will be performed

in triplo to obtain statistically relevant data. Additionally, the production of MCP1, TGF-β, RANTES and ET-1 will be studied in these cell lines.

In addition, we want to investigate the influence of cystine depleting agent cysteamine cytokine production. This part might be scheduled for the second year, if CRF decides to approve this part of the grant.

B. Examination of polarity of secretion

We have successfully investigated the culture of ci-PTC on Transwell chambers. We want to extend these experiments in the next 6 months in addition of albumin at the apical side of the cells and serum free medium at the basal compartment. Subsequently, we can measure cytokine production in both compartments.

C. Application of a new technique

When we evaluate the production of selected cytokines mentioned above, we have planned to extend this research in the second year of the grant using a cytokine array (RayBio human Cytokine Antibody Array). This will provide us with detailed information on cytokine production in cystinotic PTC.

D. Identifying intracellular signaling mechanism

According to the results mentioned under (C), we can extend our research in this field, such as the activation of the transcription factor NFκ-B. This has become of more importance, since we have shown albumin reabsorption in cystinotic kidney tissue.

Key objective 2)

Study of intracellular glutathione status, transport and generation of reactive oxygen species (ROS) in a conditionally immortalized proximal tubular cell line.

A. Study of proximal tubular GSH transport in conditionally immortalized PTC

At this point, we are investigating the presence of GSH transporters (OAT1/3 and SDCT2) on ci-PTC by immunoblotting. When we have established these transporters in the next six months, we will perform transport experiments to compare GSH influx and efflux in cystinotic cells with control cells.

B. Study of intracellular glutathione status

We have shown increase of oxidized GSSH in cystinotic ci-PTC. We will confirm these results by a second approach using a colorimetric assay (Cayman Chemical).

C. Study of intracellular ROS production, ROS-induced cellular damage and effects of the anti-oxidants

In the second part of this study, we will investigate the effect of cysteamine exposure on GSH, GSSH and ROS levels using fluorescent reporter molecules and video-imaging microscopy.

PROGRESS REPORT

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

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

Six-Month Progress Report: 5/1/07 – 10/30/07

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 variants in our Mexican-American population. Initially PCR reactions were optimized then PCR amplicons, of approximately 700 bp, were generated then sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction kit. The products were analyzed on an Applied Biosystems 3730 DNA Analyzer. An overview of the experimental progress to date for each gene is presented in Table 1.

	Number of DNA samples	Number of primer pairs	Number of PCR reactions	Number of Sequencing reactions
STUB1	189	8	1,512	3,024
VPS13A	189	4	756	1,512

Table 1: An overview of the PCR and sequencing reactions for the genes STUB1 and VPS13A

The sequences generated from the re-sequencing were analyzed in the SeqScape program to identify single nucleotide polymorphisms (SNPs). The SNPs identified were then compared to the known SNPs in the NCBI dbSNP database. SNPs found in our population, which were not found in the public SNP database, were designated as novel SNPs. All of *STUB1* was sequenced, including 1.5 kb of the 5' promoter region. The results for *STUB1* from the re-sequencing effort are presented in Table 2.

SNPs	5' UTR	Intronic	Exonic	3'UTR	Total number of SNPs identified
Novel	20	4	5	3	32
Known		3	1		4

Table 2: The number and genomic location of the SNPs identified by re-sequencing STUB1

VPS13A is a large gene spanning 240 kb. In the first instance, we re-sequenced 1.5 kb of the 5' promoter region. The data generated so far for *VPS13A* from the re-sequencing effort are presented in Table 3.

SNPs	5' UTR	Intronic	Exonic	3'UTR	Total number of SNPs identified
Novel	5		1		6
Known	9		1		10

Table 3: The number and genomic location of the SNPs identified by re-sequencing VPS13A

The next phase of the project is to analyze SNPs in *STUB1* and *VPS13A* in 1,240 individuals derived from the Mexican-American population. This will be done by genotyping DNA samples using the Illumina BeadExpress system. The results of this genotyping will be presented in the next progress report.

PROGRESS REPORT

JESS THOENE, MD, PRINCIPAL INVESTIGATOR
UNIVERSITY OF MICHIGAN

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

Twelve-Month Progress Report: 12/1/06 – 11/30/07

HYPOTHESIS:

Based on our earlier published work, we hypothesize that proteins in the apoptotic cascade will be cysteinylated by lysosomal cystine during the early stages of apoptosis, thus increasing the rate of apoptosis in cystinotic cells. We have demonstrated for PKC δ that such modification can increase enzymatic activity, thus enhancing the aoptotic cascade, leading to inappropriate cell death, and hence the cystinotic phenotype.

STRATEGY:

As described in the original application, we expose cultures of cystinotic and normal cells to an apoptotic stimulus (TNF α), harvest the cells at appropriate intervals, and analyze the cell proteins by MALDI mass spectrometry. We are assisted in this portion of the work by the University of Michigan Proteome Consortium. To identify which proteins have been cysteinylated by released lysosomal cystine, we first block existing thiols at the time of harvest, either with iodoacetamide, or ICAT, a reagent from Applied Biosystems that has a thiol-specific reactive group adjacent to an alkyl linker, which contains either nine [12C] or nine [13C] atoms - thus resulting in a mass difference of 9 daltons between the control versus the corresponding experimental version of the same tryptic peptide. The cell proteins are then reduced with phosphine and labeled with ICAT heavy or light. The resulting protein pool is then digested, ICAT-labelled proteins isolated via an avidin column, and then analyzed by mass spectrometry. A difference in proteins in normal versus cystinotic cells labeled after native thiols are blocked should identify those proteins cysteinylated by lysosomal cystine after an apoptotic stimulus.

RESULTS:

At this point we have identified 3 candidate proteins that meet the listed criteria in at least one trial. One of the three, MFGE8 has been identified in two independent experiments. Gratifyingly, all three contain cyste(i)ne residues, and are thought to have some role in apoptosis. Two, calmegin and calreticulin may work together in the apoptotic cascade. A brief synopsis of the three proteins, listing their aminoacid sequence, and a summary of their biologic function is given below:

1) Calreticulin:

1) ER Calcium and ER Chaperones: New Players in Apoptosis? Calreticulin-Second Edition

Edited by: Paul Eggleton and Marek Michalak ISBN: 0-306-47845-5

Chapter authors: Nicolas Demaurex, Maud Frieden and Serge Arnaudeau

By using calcium ions as an intracellular messenger, cells walk a tight rope between life and death. Because critical cellular functions depend on the precise delivery of Ca2+ at the right time and place, calcium ions must navigate at all times between intracellular calcium stores and target proteins located in the cytosol, the mitochondria, or the nucleus. Due to the toxicity of high Ca2+ concentrations, even slight disruption of the elaborate calcium signaling machinery can have devastating consequences on cell functions: too much or too little calcium at the wrong time and place might lead to rapid cell death by necrosis, or to the induction of the cell death program of apoptosis. ER chaperones, and most notably calreticulin, play a key role in the making and decoding of both normal and pathological calcium signals. Calreticulin is the main Ca2+-binding protein residing in the ER, and as such contributes most of the ER Ca2+ buffering capacity. Calreticulin also acts as a chaperone for several ER Ca2+ transport proteins, and thus indirectly modulates Ca2+ fluxes across the ER membrane. Accordingly, over- or underexpression of calreticulin leads to rapid and severe alterations in ER Ca2+ homeostasis. Calreticulin expression levels are controlled by the ER Ca2+ levels, thus enabling cells to mount an appropriate response during long-term perturbations in ER Ca2+ storage. **However, calreticulin levels are also increased by a variety of cellular stress conditions, and this upregulation might contribute to the Ca2+ signaling defects leading to apoptosis.** In this chapter, we will review the role of calreticulin and of other ER chaperones in the control of Ca2+-mediated apoptosis.

Sequence of Calreticulin: 3 cys residues

1 mllsvplllg llglavaepa vyfkeqfldg dgwtsrwies khksdfgkfv lssgkfygde
61 ekdkglqtsq darfyalsas fepfsnkgqt lvvqftvkhe qnidcgggyv klfpnsldqt
121 dmhgdseyni mfgpdicgpg tkkvhvifny kgknvlinkd irckddefth lytlivrpdn
181 tyevkidnsq vesgsleddw dflppkkikd pdaskpedwd erakiddptd skpedwdkpe
241 hipdpdakkp edwdeemdge weppviqnpe ykgewkprqi dnpdykgtwi hpeidnpeys
301 pdpsiyaydn fgvlgldlwq vksgtifdnf litndeayae efgnetwgvt kaaekqmkdk
361 qdeeqrlkee eedkkrkeee eaedkedded kdedeedeed keedeeedvp gqakdel

2) Calmegin:

1) Molecular Chaperone Calmegin Localization to the Endoplasmic Reticulum of Meiotic and Post-meiotic Germ Cells in the Mouse Testis
Kazuya YOSHINAGA1), Ichiro TANII1) and Kiyotaka TOSHIMORI1)
Archives of Histology and Cytology Vol. 62 (1999) , No. 3 p.283-293

Department of Anatomy and Reproductive Cell Biology, Miyazaki Medical College

Summary: Calmegin is a testis-specific Ca2+-binding protein that is homologous to calnexin. Recently, sperm from transgenic mice lacking calmegin have been shown to be infertile. To further characterize calmegin, we analyzed the precise stage of expression and the intracellular localization of this protein in germ cells during mouse spermatogenesis by an immunoperoxidase technique using the anti-calmegin monoclonal antibody TRA369. Light microscopic immunocytochemistry showed that calmegin appeared in early pachytene

spermatocytes, with the highest expression in round and elongating spermatids, and disappeared in the maturation phase of spematids at step 15. Immunoelectron microscopy showed that selective localization was found at the endoplasmic reticulum membrane and the nuclear envelope of spermatogenic cells. During the maturation phase, a dramatic reduction in calmegin occurred in the endoplasmic reticulum of the spermatids, suggesting that the major function of calmegin has been completed by the time spermatids reach step 14. In addition, although the immunoreactivity was completely absent in the calmegin-deficient mutant mouse testis, ultrastructural analysis showed that mature sperm from the knockout mice were normal. This suggests that calmegin is not required for the morphogenesis of male germ cells. Thus, our results suggest that **calmegin has a major role in mouse spermatogenesis,** and also indicate that this protein would be useful as a maker molecule to study the functional role of the endoplasmic reticulum in the process of spermatid differentiation.

Sequence of human calmegin: 8 cys residues

1 mhfqafwlcl gllfisinae fmdddveted feenseeidv neselsseik yktpqpigev
61 yfaetfdsgr lagwvlskak kddmdeeisi ydgrweieel kenqvpgdrg lvlksrakhh
121 aisavlakpf ifadkplivq yevnfqdgid cggayiklla dtddlilenf ydktsyiimf
181 gpdkcgedyk lhfifrhkhp ktgvfeekha kppdvdlkkf ftdrkthlyt lvmnpddtfe
241 vlvdqtvvnk gslledvvpp ikppkeiedp ndkkpeewde rakipdpsav kpedwdesep
301 aqiedssvvk pagwlddepk fipdpnaekp ddwnedtdge weapqilnpa crigcgewkp
361 pmidnpkykg vwrpplvdnp nyqgiwsprk ipnpdyfedd hpflltsfsa lglelwsmts
421 diyfdnfiic sekevadhwa adgwrwkimi anankpgvlk qlmaaaeghp wlwliylvta
481 gvpialitsf cwprkvkkkh kdteykktdi cipqtkgvle qeekeekaal ekpmdleeek
541 kqndgemlek eeesepeeks eeeieiiegq eesnqsnksg sedemkeadestgsgdgpik
601 svrkrrvrkd

3) **MFGE8:**

1)Identification of a factor that links apoptotic cells to phagocytes. Hanayama R, Tanaka M, Miwa K, Shinohara A, Iwamatsu A, Nagata S. Nature. 2002 May 9; 417(6885): 182-7 Department of Genetics, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan.

Apoptotic cells are rapidly engulfed by phagocytes to prevent the release of potentially noxious or immunogenic intracellular materials from the dying cells, thereby preserving the integrity and function of the surrounding tissue. Phagocytes engulf apoptotic but not healthy cells, indicating that the apoptotic cells present a signal to the phagocytes, and the phagocytes recognize the signal using a specific receptor. Here, we report a factor that links apoptotic cells to phagocytes. We found that milk fat globule-EGF-factor 8 (MFG-E8), a secreted glycoprotein, was produced by thioglycollate-elicited macrophages. MFG-E8 specifically bound to apoptotic cells by recognizing aminophospholipids such as phosphatidylserine. **MFG-E8, when engaged by phospholipids, bound to cells via its RGD (arginine-glycine-aspartate) motif--it bound particularly strongly to cells expressing alpha(v)beta(3) integrin.** The NIH3T3 cell transformants that expressed a high level of alpha(v)beta(3) integrin were found to engulf apoptotic cells when MFG-E8 was added. MFG-E8 carrying a point mutation in the RGD motif behaved as a dominant-negative form, and inhibited the phagocytosis of apoptotic cells by peritoneal macrophages *in vitro* and in vivo. These results indicate that MFG-E8 secreted from activated macrophages binds to apoptotic cells, and brings them to phagocytes for engulfment.

MFGE8 Protein sequence : 14 cys residues

1 mprprllaal cgallcapsl lvaldicskn pchngglcee isqevrgdvf psytctclkg
61 yagnhcetkc veplgmengn iansqiaass vrvtflglqh wvpelarlnr agmvnawtps
121 snddnpwiqv nllrrmwvtg vvtqgasrla sheylkafkv ayslnghefd fihdvnkkhk
181 efvgnwnkna vhvnlfetpv eaqyvrlypt schtactlrf ellgcelngc anplglknns
241 ipdkqitass syktwglhlf swnpsyarld kqgnfnawva gsygndqwlq ifpgnwdnhs
301 hkknlfetpi laryvrilpv awhnrialrl ellgc

Preliminary conclusions:

The method seems to effectively identify candidate proteins cysteinylated only in cystinotic cells after an apoptotic stimulus. The three proteins identified so far posses cystine residues, thus are able to be cysteinylated, and they are involved, at least to some extent, in apoptosis. Calreticulin is involved in spermatogenesis. We speculate that abnormal activity of calreticulin in the testes of adolescent cystinotic males could be an explanation for the male infertility seen in this disease.

Next Steps:

The major concern we have had is the lack of sensitivity of MALDI to enable detection of the scarce, but powerful regulatory proteins involved in the apoptotic cascade.

We plan to address this issue in two ways:

1) Increase the number of cells harvested at each point: Previously we have harvested about 10*6 cells per condition and time point. We will increase this by a factor of 5 in the next series.
2) Alter the method of harvesting: We have used scraping in order to avoid partial digestion of proteins at harvest which would cloud the MALDI results. However, it is possible that scraping is lysing a substantial proportion of cells, thus releasing the cystosolic proteins into the harvest buffer , where they are lost to analysis. We will compare the identity of proteins obtained from cells harvested by trypsinization with that obtained by scraping in order to determine if this will enhance the yield of identifiable proteins.

PROGRESS REPORT

JESS THOENE, MD
UNIVERSITY OF MICHIGAN

"Application to the Cystinosis Research Foundation for a Grant Supplement to Establish a Cystinosis Research Lab"

Twelve-Month Progress Report: 12/1/06 – 12/30/07

The bulk of progress which has occurred in study of cyteinylated proteins which amplify apoptosis in cystinotic cells was reported to you in the progress report for the parent grant sent to you last month.

At that time we noted:

"The major concern we have had is the lack of sensitivity of MALDI to enable detection of the scarce, but powerful regulatory proteins involved in the apoptotic cascade.

We plan to address this issue in two ways:

*1) Increase the number of cells harvested at each point: Previously we have harvested about 10*6 cells per condition and time point. We will increase this by a factor of 5 in the next series.*

2) *Alter the method of harvesting: We have used scraping in order to avoid partial digestion of proteins at harvest which would cloud the MALDI results. However, it is possible that scraping is lysing a substantial proportion of cells, thus releasing the cystosolic proteins into the harvest buffer , where they are lost to analysis. We will compare the identity of proteins obtained from cells harvested by trypsinization with that obtained by scraping in order to determine if this will enhance the yield of identifiable proteins.*"

We have since made contact with Dr Ursula Jakob, associate professor of Biochemistry, Cell Biology, and Microbiology at the University of Michigan, and a highly regarded scientist in the field of protein redox biology. Some of her recent publications are:

Ilbert, M., Horst, J., Ahrens, S., Winter, J., Graf, P.C., Lilie, H., **Jakob, U.** (2007). "The redox-switch domain of Hsp33 functions as dual stress sensor." Nat Struct Mol Biol 556-563.

Leichert, L.I. and **Jakob, U** (2006). "Global Methods to Monitor the Thiol-Disulfide State of Proteins in vivo." Antioxid Redox Signal 8: in press.

Mufti, A.R., Burstein, E., Csomos, R.A., Graf, P.C.F., Wilkinson, J.C., Dick, R.D., Challa, M., Son, J.K., Bratton, S.B., Su, G.L., Brewer, G.J., **Jakob, U.** and C.S. Duckett (2006). "XIAP Is a copper binding protein deregulated in Wilson's disease and other copper toxicosis disorders." Mol Cell 21: 775-785.

Liu, X.I., Korde, N., **Jakob, U.,** and Leichert, L.I.O (2006). "CoSMoS: Conserved Sequence Motif Search in the proteome." BMC Bioinformatics 7: 37.

Leichert, L.I.O. and **Jakob, U** (2005). "Protein Thiol Modifications in vivo." Antioxidant and Redox Signaling invited review.

My lab staff met with her and colleagues and together we have devised a new way to harvest cystinotic proteins to improve the yield and hopefully increase the recovery of relevant apoptotic proteins from these cells under these conditions.

We are in the process of growing stocks of cells and obtaining the needed reagents and expect to have proteins ready for MALDI analysis by February.

FINAL REPORT

DORIS A. TRAUNER, MD, PRINCIPAL INVESTIGATOR

"Mitchondrial Dysfunction in Cystinosis Myopathy"

A. HYPOTHESIS:

The myopathy associated with nephropathic cystinosis is the result of mitochondrial dysfunction with resultant deficiency in respiratory transport chain function.

B. SPECIFIC AIMS:

To test the above hypothesis by studying respiratory transport chain activity 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.

C. METHODS:

Eleven adolescents and adults with nephropathic cystinosis participated in the study. Some had evidence of myopathy by history and by neurological examination, and confirmed by electromyographic studies; others had no clinical evidence of myopathic changes. All underwent muscle biopsies. One-half of the muscle specimen was sent to pathology and studied histologically and by electron microscopy. The other half was sent to Dr. Richard Haas's lab at UCSD to undergo metabolic studies, including carnitine levels, cytochrome oxidase activity, and activities of other enzymes of the electron transport chain (the pathway by which energy is produced by the muscle cells). In addition, all subjects underwent tests of respiratory function during exercise, as well as a neurological exam including detailed testing of strength.

In the second phase of the study, 5 of the cystinotic patients received a combination of carnitine, Coenzyme Q10, biotin, riboflavin and thiamine on a daily basis for 3 months. The other 5 received a placebo. After 3 months, strength was assessed by a neurologist unaware of the treatment status, using objective measures such as grip strength in addition to subjective muscle testing. Each patient underwent a second EMG following 3 months of treatment. After 3 months, the control group was offered the same treatment as the experimental group, and all 10 individuals were re-tested at the end of 6 months and 1 year.

D. RESULTS:

Eleven patients (age range 16-43 years) were enrolled in the study. Six were aged 18 to 25 and had little to no clinical evidence of weakness; five were aged 36 to 43 and all had myopathic symptoms. 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 study. All subjects participated in comprehensive baseline exercise testing by Dr. Paul Phillips which included ergometry and respiratory exchange ratio measures after which the subjects underwent a muscle biopsy under light sedation and local anesthesia performed on the UCSD CRC by Dr. Richard Haas.; 5 females and 6 males underwent muscle biopsy. All have completed the 12 month study.

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 had 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 who completed the testing. 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.

EMG/NCV: Two patients refused the EMG study. Two patients had normal studies (20 and 21.5 years old). Three showed myopathic changes, with decreased amplitude and duration of motor unit action potentials, and numerous polyphasic potentials. Two patients had neurogenic changes, one in addition to myopathic changes and one without myopathic changes.

Muscle pathology: Muscle biopsy histochemistry and electron microscopy (EM) was quite variable. Only one (21 year old patient) was entirely normal. Fiber size variation was noted in 4 biopsies and 2 had marked type 2 fiber predominance. Two of the older patients had ring fibers typical of cystinotic myopathy (Figure 1) with scattered atrophic muscle fibers.

Filamentous bodies were seen in 3 biopsies on EM and finger print bodies in one. Three were reported with prominent lipid staining and 2 had prominent glycogen on EM. In 3 biopsies trichrome staining was prominent suggesting mitochondrial accumulation, in 2 this was seen at the EM level. One subject had increased COX negative fibers and 2 subjects had scattered degenerating mitochondria on EM. The prominent lipid, glycogen, COX negative fibers and moth eaten fibers are changes reported in mitochondrial disease. (In mitochondrial disease patients, particularly in children, histochemistry and electron microscopy frequently shows minimal changes or is normal).

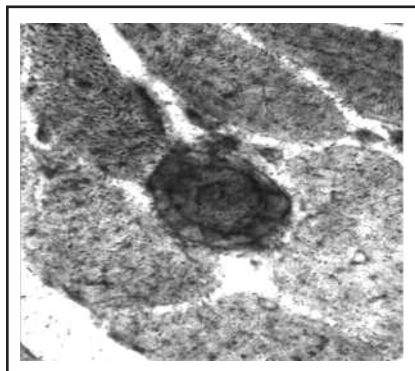

Figure 1. Ring fiber in muscle biopsy of adult with cystinosis.

Biochemical measures: Baseline plasma CoQ10 levels were measured and results in the ten subjects are shown in table 1.

Coenzyme Q levels in 10 Cystinotic Subjects		
Subject Controls	**Plasma CoQ (0.55-1.64 umol/L)**	Muscle CoQ (14.4-31.6 ug/g wet weight)
1	**0.54**	13.65
2	**0.52/0.42**	27.35
3	**0.54**	25.53
4	**0.5**	31.67
5	**0.46/0.45**	15.95
6	**0.55/0.57**	29.9
7	**NA**	26.37
8	**0.62/0.69**	30.94
9	**0.55/0.59**	39.45
10	**0.52/0.47**	9.12

Table 1. Pretreatment or placebo data was available in 9 of 10 subjects. In 8 of these plasma CoQ10 levels were at (2) or below (6) the lower limit of the normal range. Muscle CoQ10 levels were reduced in 2 of 10 subjects.

Carnitine levels in plasma and muscle were well in the normal range in 9/9 subjects tested and only one subject was confirmed to be taking carnitine at baseline. In 6 of 9 subjects plasma acyl carnitine profiles were abnormal with increased levels of predominantly short and medium chain species – compatible with some impairment of mitochondrial beta oxidation.

Muscle biochemistry: Electron transport studies on isolated mitochondria revealed normal citrate synthetase [CS] (a mitochondrial marker), normal complex I/CS and II-III/CS but the complex IV/CS activity of the cystinotic group was significantly reduced (with a tight standard deviation) compared to a large disease control group and a smaller group of normal samples (Table 2).

MUSCLE MITOCHONDRIA COMPLEX IV/ CITRATE SYNTHETASE RATIO		
Disease Controls (A) n=87	**Normal Controls (B) n=14**	Cystinosis (C) n=10
Mean 0.62	**Mean 0.66**	Mean 0.44
SD 0.23	**SD 0.31**	SD 0.09
Unpaired T test C vs A p = 0.0119	**C vs B p = 0.0115**	

Table 2. Cystinotic Muscle Mitochondrial Complex IV (COX) activity expressed as a ratio to citrate synthetase. Disease controls are a series of adult and pediatric patients found to have normal electron transport activities. Normal controls are orthopedic surgery patients.

Clinical trial: There was no clear evidence of improvement after 6 or 12 months of treatment with a "mitochondrial cocktail" consisting of l-carnitine, Co-enzyme Q, biotin, riboflavin and thiamine supplementation. However, it was apparent that at least some of the participants were not taking the medications, since their repeat Co-enzyme Q levels were not elevated.

E. CONCLUSIONS:

Co-enzyme Q10 deficiency in nephropathic cystinosis is suggested by the finding of low or borderline CoQ10 plasma in 8 of 9 patients with data. Two had quite low muscle CoQ10 levels. Muscle histochemistry and EM was variable but in several patients changes compatible with mitochondrial dysfunction were seen. The muscle COX activity results are significant and although not very low do differ markedly from the normal mean. COX deficiency suggesting secondary mitochondrial impairment has been reported in other myopathic conditions. Carnitine levels in plasma and muscle were uniformly normal despite the fact that only one individual was supplemented at baseline. Results of the exercise testing revealed baseline abnormalities but no noticeable improvement after either 6 months or 1 year of treatment with a 'mitochondrial cocktail'.

Taken as a whole the results of this study support the hypothesis that there is mitochondrial dysfunction in nephropathic cystinosis. Coenzyme Q is an important antioxidant and electron transport chain component. Low levels are worrisome, and may contribute to myopathic symptoms in older patients as well as to contribute to the cause of the cardiomyopathy. If this finding is confirmed in a larger study it would seem prudent to consider CoQ10 supplementation at least in older patients as Q levels fall in aging normally. The lack of effect of the 'mitochondrial cocktail' which included 1200 mg/day of CoQ10 is not unexpected. The effect of treatment might best be viewed as a long term protectant rather than an agent capable of producing a symptomatic benefit in most subjects. This dose of CoQ10 was well tolerated.

F. FUTURE PLANS:

These data suggest that CoQ10 deficiency may be widespread in nephropathic cystinosis and mitochondrial dysfunction is an accompanying problem. Further study is indicated as chronic treatment with CoQ10 supplementation may be beneficial for nephropathic cystinosis patients and in particular for myopathy and cardiomyopathy symptoms. Based on this study, the next logical step would be to study levels of CoQ in platelets and plasma of a larger cohort of children, adolescents and adults with cystinosis, as well as to conduct a larger study of CoQ10 treatment in adolescents and young adults with cystinosis. The ultimate goal is to prevent the progressive myopathy and cardiomyopathy that is occurring in so many adults with cystinosis.

2/11/2008