Cystinosis Research Foundation SCIENCE REPORT

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CYSTINOSIS RESEARCH FOUNDATION

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Welcome to the fourth edition of our *Science Report*

This edition of the Cystinosis Research Foundation Science Report contains research updates on studies underway and lists the 2009 funded studies with the Abstract/Summary of each new award grant. With more than 21 research updates submitted in 2009, we have chosen to publish only the most current reports, however, all of the research updates can be found in the research section of our website, www.cystinosisresearch.org/Research-Updates.

The recent cystinosis research advancements are the direct result of the Cystinosis Research Foundation's focused efforts and strategic approach to how resources are used. CRF isssues grants to the best and brightest scientists at world-renown institutions who are focused on better treatments and a cure for cystinosis. We foster collaboration among our researchers, scientists and institutions. With the guidance of our Scientific Review Board, we strategically award grants for basic, translational and clinical research. Our research strategy has resulted in a greater understanding of cystinosis, has moved us closer to clinical trials for a cure for cystinosis and has brought us within reach of FDA approval of a new medication which will greatly improve the quality of life for our children. We have created a research synergy that is dynamic and powerful.

In 2009, the CRF awarded more than $3.3 million in research grants. Since its inception, the Foundation has committed and funded more than $10.8 million for cystinosis research. CRF is now funding more than 40 studies including 10 research fellows. Our researchers are working in seven countries around the world to learn more about how to treat and cure cystinosis. The CRF will continue to fund quality research in our quest for the cure.

2010 Call for Funding Proposals

The Cystinosis Research Foundation will announce *A Call for Research and Fellowship Proposals* in the spring and autumn of 2010. CRF is prepared to fund proposals to improve the immediate care of children and young adults with cystinosis and to develop new understanding and treatment of cystinosis to help these children in the future. In addition, CRF is focused on funding translational research that will result in human clinical trials. The Foundation has over $750,000 available for 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 2010.

Cystinosis Research Foundation Fellowship Program

The Cystinosis Research Foundation has established a postdoctoral research fellowship program to attract qualified, promising investigators to establish careers in cystinosis research. Fellows will be funded for 1–3 years to a maximum of $75,000 per year. Applications will be available in conjunction with the spring and autumn *Call for Funding Proposals.*

Visit www.cystinosisresearch.org for details.

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

2009 RESEARCH STUDIES FUNDED AND COMMITTED $3,331,496

SPRING

Corinne Antignac, MD, PhD, Mentor
Zuzanna Andrzejewska, Research Fellow
Hospital Necker, Paris, France
"Role of Cystinosin in Trafficking and Membrane Fusion"
$126,000 – 3-year study

Stephanie Cherqui, PhD, Principal Investigator
The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis"
$750,691 – 2-year study

Alan J. Davidson, PhD, Principal Investigator
Joseph P. Vacanti, MD, Co-Principal Investigator
Harvard Medical School/Mass. General
"Developing New Models of Cystinosis: Zebrafish"
$75,000 – 1-year study

Ranjan Dohil, MD, Principal Investigator
Betty Cabrera, BS, MPH, Research Assistant
University of California, San Diego
"Various Cystinosis Research Projects"
$228,648 – 3-year study

Miriam Sach, MD, PhD, Principal Investigator
University of California, San Diego
"Neural Correlates of Executive Functioning in Cystinosis; a Structural MRI Study"
$65,725 – 1-year study

Mary L. Taub, PhD, Principal Investigator
State University of New York at Buffalo
"Mechanisms Underlying the Fanconi Syndrome in Cystinosis"
$103,437 – 1-year study

Doris Trauner, MD, Principal Investigator
University of California, San Diego
"Psychological Functioning in Cystinosis: Stress and Coping with Chronic Illness"
$66,624 – 1-year study

Kang Zhang, MD, Principal Investigator
University of California, San Diego
"Eye Targeted Gene Therapy for Cystinosis in the Eye"
$321,346 – 2-year study

AUTUMN

Bruce Barshop, MD, PhD, Principal Investigator
University of California, San Diego
"Improvement of Intracellular Cystine Measurement"
$62,930 – 1-year study

Bruce Barshop, MD, PhD
University of California, San Diego
"Tandem Mass Spectrometer Support"
$160,604 – 1.5-years

Bruce Barshop, MD, PhD, Mentor
Ilya Gertsman, PhD, Research Fellow
University of California, San Diego
"Proteomic Based Identification of Cysteinylated Proteins in Cystinotic Cells"
$124,214 – 2-year fellowship

Stephanie Cherqui, PhD, Principal Investigator
The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis" Supplemental Funding"
$163,340 – 2-year study

Stephanie Cherqui, PhD, Mentor
Brian Yeagy, PhD, Research Fellow
The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis"
$138,485 – 2-year fellowship

Pierre J. Courtoy, MD, PhD, Principal Investigator
de Duve Institute, Brussels, Belgium
"Lessons from Cystinotic Mice: Vital Imaging of Protein Handling and Lysosomal Function, Reciprocal Interactions with Regulatory Kinases, and Regeneration Potential by Transdifferentiation"
$236,000 – 2-year study

Ranjan Dohil, MD, Principal Investigator
University of California, San Diego
"A Study to Evaluate Enteric-Coated Cysteamine Therapy in Patients with Cystinosis"
$27,226 – 1-year study

Paul Goodyer, MD, Principal Investigator
Montreal Children's Hospital, Quebec, Canada
Francesco Emma, MD, Co-Investigator
Bambino Gesù Children's Hospital and Research Institute, Rome, Italy
"Stem Cell Microvesicles Rescue Cystinosis in Vitro"
$308,602 – 2-year study

Elena Levtchenko, MD, PhD, Principal Investigator
University Hospital Leuven, Belgium
Roos Masereeuw, PhD, and
Lambertus van den Huevel, PhD, Co-Investigators
Radboud University, Nijmegen, The Netherlands
"Role of P-glycoprotein Expression and Function in Cystinotic Proximal Tubular Cells"
$83,999 – 1-year study

Robert Mak, MD, PhD, Principal Investigator
University of California, San Diego
"Energy Homeostasis and Muscle Wasting in Nephropathic Cystinosis"
$150,000 – 2-year study

Holger Willenbring, MD, PhD, Principal Investigator
University of California, San Francisco
"Pluripotent Stem Cells as a Source of Immunocompatible Renal Progenitor Cells for Therapy of Nephropathic Cystinosis"
$138,625 – 1-year study

For information about our 2010 Call for Funding Proposals or the Cystinosis Research Foundation Fellowship Program, visit www.cystinosisresearch.org.

CORINNE ANTIGNAC, MD, PHD, MENTOR
ZUZANNA ANDRZEJEWSKA, RESEARCH FELLOW

Hospital Necker, Paris, France
"Role of Cystinosin in Trafficking and Membrane Fusion"
$126,000 – 3-year study

ABSTRACT/SUMMARY

Cystinosis (MIM 21980) is a rare inherited lysosomal storage disorder characterized by a defective lysosomal efflux of cystin. Cystin accumulation is responsible for various symptoms particularly a proximal tubulopathy (de Toni-Debre-Fanconi) that progresses, if untreated, to terminal renal failure in childhood. The cystinosis gene encodes a lysosomal transmembrane protein, cystinosin, which functions as a proton-driven lysosomal cystin transporter. Cells overexpressing a cystinosin-GFP fusion protein displayed aggregation of lysosomes as it was observed after overexpression of proteins involved in membrane fusion (hVam6p, Oa1 and LGP85). These large structures were drastically decreased when the 5th inter-TM loop of cystinosin, and /or its C-terminal tail were altered. Furthermore, the resultant proteins were partially relocated at the plasma membrane.

This suggests that cystinosin might be involved in membrane fusion events and that one of these domains, or both, might be important for its intracellular trafficking. Indeed, recent data obtained in the laboratory identified as cystinosin putative partners two proteins implicated in membrane fusion and vesicle trafficking, i.e.Snf8 and Vps39 (hVamp6). Moreover, the tyrosin-based motif located at the C-terminal tail of cystinosin that we previously identified as a lysosome targeting signal, presents similarities to those contained in proteins interacting with various AP complex sub-units. The possible role of cystinosin in the biogenesis of endolysosomal compartments and in vesicular trafficking will be deeply investigated during this three-year fellowship.

BRUCE BARSHOP, MD, PHD, MENTOR
ILYA GERTSMAN, PHD, RESEARCH FELLOW

University of California, San Diego
"Proteomic Based Identification of Cysteinylated Proteins in Cystinotic Cells"
$124,214 – 2-year fellowship

ABSTRACT/SUMMARY

Cystinosis is a lysosomal storage disease caused by dysfunction of the lysosomal membrane protein cystinosin[1,2]. Various mutations to the gene that codes for cystinosin, CTNS, have been identified, including the most common 57k base pair deletion among other insertion, missense and nonsense mutations[3]. The common result of the many genetic abnormalities in the CTNS gene is the inability to export cystine from the lumen of the lysosome to the cell cytosol. Cystine, a disulfide product of two cysteines, accumulates at high concentration in the lysosome, resulting in crystalline formation. The resulting phenotype includes renal Fanconi syndrome, narrowing of the renal tubules and eventual renal failure, as well as hypothyroidism and photophobia among other symptoms[4].

The link between cystine accumulation and phenotype is still poorly understood, with a variety of causes being implicated in the pathophysiology. The cellular mechanisms implicated have included decreases in energy metabolism linked to observed decreases in intracellular ATP, increased oxidative stress due to decreased glutathione production, as well as disruption of thiol-dependent enzyme function[5–7]. Though all of these proposals are plausible mediators for the disease, much doubt remains as to whether the abnormalities seen from these studies can actually perpetuate the severe symptoms seen in cystinosis. Another interesting effect that has been observed in cystinotic studies is the increased likelihood of apoptosis in cystinotic cells. Abnormal regulation of apoptosis has been linked to many diseased states and may in fact play a significant role in cystinosis pathogenesis.

Recent studies have shown that external apoptotic signals such as TNF and UV exposure may result in 2-3.5 fold increases in the rate of apoptosis in cystinotic fibroblasts compared to normal cells[8]. Increased apoptosis rates have also been characterized in cultured renal tube epithelial cells as well as fibroblasts in response to increased lysosomal cystine concentrations8. When normal cells had their lysosomes loaded with cystine in the form of CDME, their rates of apoptosis also increased[9]. The hypothesized link between increased apoptosis in cystinotic cells is correlated with increased lysosomal membrane permeability in response to apoptotic signaling factors. So far the major protein implicated in this process is protein kinase C delta, a pro-apoptotic protein that is significantly activated in response to cysteinylation[10]. The predisposition of cells to undergo apoptosis hinges on a variety of caspase dependent and caspase independent pathways with pro and anti-apoptotic factors controlling the fate of the cell. Though the cysteinylation of PKC delta may in fact greatly contribute to apoptosis in cystinotic cell, a variety of other apoptotic proteins may also be affected by increased cystine concentrations. Not only does protein cysteinylation appear to have effects on apoptosis and possibly play a role in the pathogenesis of cystinosis , it may also have major biological ramifications on a number of processes, including fatty acid metabolism, immune function, oxidative stress and normal aging[11–14].

BRUCE BARSHOP, MD, PHD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Tandem Mass Spectrometer Support"
$160,604 – 1.5-years

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 BARSHOP, MD, PHD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Improvement of Intracellular Cystine Measurement"
$62,930 – 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[1] and with improved renal outcome[2], and that frequent regular dosing of cysteamine is necessary to maintain low leukocyte cystine levels[3–5]. Until a few years ago, 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[6,7], largely because the sensitivity was greater than ninhydrin-based amino acid detection of cystine and even fluorometric detection[8]. However, tandem mass spectrometry[9], using electrospray ionization and stable isotope dilution with an internal standard of tetra-deuterated cystine, has advantages and is very sensitive and rapid. 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 µmol/L, and have been using it routinely since January 2007. 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.

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 heparin solutions for 24 hours or more, the test results were felt to be unreliable[10]. Once the white cell pellet is acid precipitated, the material may be stored at -20ºC without loss of stability over reasonable time frames[10,11]. 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[12], 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[13]. In another laboratory (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[14]. There is a commercial laboratory in the U.S. which 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 granulocytes (polymorphonuclear leukocytes, PMNs)[15].

Therefore, methods that analyze PMNs specifically are preferable in principle, and indeed, such an assay more 2 reliably distinguishes heterozygotes from normals[13,16]. The intracellular cystine in PMNs compared to mixed leukocyte (ML) preparations[13] was approximately 3.8 fold higher in heterozygotes (0.27±0.17 versus 0.07±0.03 nmol/mg protein) and approximately 6.3 fold higher in homozygotes being treated with cysteamine (0.94±0.58 versus 0.15±0.08 nmol/mg protein). The therapeutic target for intracellular cystine in PMNs has not been very well studied, but it was quite logical to apply a target of 0.5 nmol/mg protein, based on the 90th percentile of heterozygotes[13]. We have not employed PMNs at UCSD, however, because it would either require even more work performed at the point of care, or assurance of stability during the time for transport of whole blood. We had always been concerned that the latter condition could not be assured given the large geographic dimensions of North America.

However, with express mailing and given our recent observations, we are hopeful that we can validate the method of local preparation of PMNs. The need for centralized preparation has come to the fore when multicenter studies have been planned to follow response to new preparations of cysteamine (such as the delayed-release formulation being developed by Raptor Pharmaceuticals). The requirement for numerous local sites which are proficient in leukocyte preparation introduced a significant strategic impediment in study design, and this will continue to complicate the planning of multicenter studies in cystinosis. Furthermore, with the delayed release form of cysteamine, it may be even more important to obtain many cystine assays when starting this preparation, since there may be difference in gastric motility, etc. necessitating individualization of dosage. This is a simple proposal to permit our lab to confirm the adequacy of a simple sampling procedure.

STEPHANIE CHEROUI, PHD, PRINCIPAL INVESTIGATOR

The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis"
$750,691 – 2-year study

ABSTRACT/SUMMARY

The long-term objectives are to develop two strategies for the treatment of cystinosis:

1. A multisystemic strategy: non-myeloablative transplantation of autologous hematopoietic stem cells genetically modified ex vivo to express a functional *CTNS* gene.
2. A kidney-specific strategy: renal vein injection of self-complementary adeno-associated virus (scAAV) expressing a functional *CTNS*. As pre-clinical studies, we will use the *Ctns-/-* murine model for cystinosis. Our preliminary data showed that transplantation of syngeneic whole bone marrow cells (BMC) or purified hematopoietic stem cells (HSC) expressing *Ctns* resulted in engraftment of BMC or HSCderived cells and significant reductions of cystine content in all the tissues tested. In Specific aim 1, we propose to use Sca1+ HSC isolated from *Ctns-/-* mice and scAAV1 for delivering the *CTNS* gene ex vivo. The cells will then be transplanted into *Ctns-/-* mice lethally irradiated or in Specific aim 2 using a non-myeloablative regimen. The efficiency of these strategies will be tested by measuring *Ctns* expression and cystine content in different tissue compartments and by well established functional studies to test the prevention or treatment of the kidney dysfunction, eye anomalies and neurological defects. The immune response and safety of this strategy will be also tested. In Specific aim 3,

we propose to test a renal vein injection of scAAV2-CTNS for treating the Fanconi syndrome and subsequent renal dysfunction in cystinosis by direct gene delivery to the kidney. This work builds the foundations for a future clinical trial. It also represents a proof of concept for a non-myeloablative autotologous HSC transplantation strategy to treat other lysosomal storage disorders and a kidney-specific therapy for hereditary nephropathies.

STEPHANIE CHERQUI, PHD, PRINCIPAL INVESTIGATOR

The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis" Supplemental Funding
$163,340 – 2-years

STEPHANIE CHERQUI, PHD, MENTOR
BRIAN YEAGY, PHD, RESEARCH FELLOW

The Scripps Research Institute, La Jolla, California
"Stem and Gene Therapy for Cystinosis"
$138,485 – 2-year fellowship

ABSTRACT/SUMMARY

The long-term objective is to develop a multisystemic strategy for the treatment of cystinosis: transplantation of autologous hematopoietic stem cells genetically modified ex vivo to express a functional *CTNS* gene. As pre-clinical studies, we will use the *Ctns-/-* murine model for cystinosis. Our preliminary data showed that transplantation of syngeneic whole bone marrow cells (BMC) or purified hematopoietic stem cells (HSC) expressing Ctns resulted in engraftment of BMC or HSC-derived cells and significant reductions of cystine content in all the tissues tested. In Specific aim 1, we propose to use Sca1+ HSC isolated from *Ctns-/-* mice and lentivirus vector for delivering the *CTNS* gene ex vivo. The efficiency of these strategies will be tested by measuring Ctns expression and cystine content in different tissue compartments and by wellestablished functional studies to test the prevention or treatment of the kidney dysfunction, eyeanomalies and neurological defects. The immune response and safety of this strategy will be alsotested. In Specific aim 2, we propose to investigate the mechanism underlying the abundant tissue cell engraftment of bone marrow-derived stem cells. This work builds the foundations for a future clinical trial and has an important fundamental implication for stem cell transplantation technology. It also represents a proof of concept for an autotologous HSC transplantation strategy to treat other lysosomal storage disorders.

PIERRE J. COURTOY, MD, PHD, PRINCIPAL INVESTIGATOR

de Duve Institute, Brussels, Belgium
"Lessons from Cystinotic Mice: Vital Imaging of Protein Handling and Lysosomal Function, Reciprocal Interactions with Regulatory Kinases, and Regeneration Potential by Transdifferentiation"
$236,000 – 2-year study

ABSTRACT/SUMMARY

Why is further basic research on cystinosis still needed ? Cystinosis can be viewed as an exemplary success story of *translational medicine.* The single primary molecular defect is clarified (genetic mutations of the CTNS gene encoding cystinosine prevent cystine export across the lysosomal membrane; e.g. Cherqui et al., MCB, 2002); and various cell biological aspects are being uncovered, in large part by the laboratory of C. Antignac: lysosomal targeting motive (Kalatzis et al., HMG, 2004), coupling with H+ transport (Kalatzis *et al.,* EMBO J, 2001) etc… Furthermore, effective *rational treatment* based on "cysteamine by-pass" (classical review by Gahl, Thoene and Schneider, NEJM, 2002) largely corrects growth defect and confers kidney protection, *yet does not provide a cure.* The prospect of gene therapy for cystinosis is grim (Hippert *et al.,* Mol. Ther., 2008), especially by comparison to cystic fibrosis, in which fewer and more accessible organs are affected. It thus appears that a necessary complementary approach is to *improve global management,* which implies *a better understanding of secondary events.* Yet, how cystinosis (and in fact all other genetic or acquired lysosomal storage disorders) cause cell death and secondary tissue remodelling, as well as adaptative natural mechanisms, remain largely unknown. Accordingly, some priority should be given to research addressing *disease physiopathology,* which has become recently possible with the congenic *CTNS-/-* mice, as an appropriate animal model (Nevo *et al.,* NDT, 2009).

ALAN J. DAVIDSON, PHD, PRINCIPAL INVESTIGATOR
JOSEPH P. VACANTI, MD, CO-PRINCIPAL INVESTIGATOR

Harvard Medical School/Massachusetts General
"Developing New Models of Cystinosis: Zebrafish"
$75,000 – 1-year study

ABSTRACT/SUMMARY

Cystinosis is a rare autosomal recessive disorder caused by mutations in *CTNS,* encoding a cystine transporter. In the severest form of the disease, patients develop renal proximal tubule dysfunction that progresses to end-stage renal failure. While various metabolic alterations have been described in cystinotic cells the link between cystine accumulation in lysosomes and dysfunction of the nephrons (the functional units of the kidney) remain poorly understood. The *Ctns* mutant mouse fails to show the renal abnormalities seen in humans. Thus, there is a pressing need to develop better animal or *in vitro* models of Cystinosis. Our laboratory in the Center for Regenerative Medicine (CRM) at Massachusetts General Hospital/Harvard Medical School uses the zebrafish model to examine the development and function of renal tubules and we have recently shown that zebrafish nephrons are anatomically similar to those in mammals.

In addition, we are situated amongst a number of leading human and mouse stem cell labs in the world with expertise in technology that enables adult somatic cells to be converted into embryonic stem cell-like cells (induced pluripotent stem cells; iPS). In this proposal we request one year of seed funding to explore the development of two new model systems with which to study the pathophysiologoy of Cystinosis. In Aim (1) we propose to create a zebrafish Cystinosis mutant by targeting the *ctns* gene using zinc finger nuclease technology. This model system will provide an additional tool with which to better understand Cystinosis and will provide novel platforms for conducting drug screening to identity new therapeutics.

RANJAN DOHIL, MD, PRINCIPAL INVESTIGATOR
BETTY CABRERA, BS, MPH, RESEARCH ASSISTANT

University of California, San Diego
"Various Cystinosis Research Projects"
$228,648 – 3-year study

ABSTRACT/SUMMARY

Coordinator Betty Cabrera will work with Dr. Jerry Schneider and Dr. Ranjan

Dohil. She will participate in the development, organization and realization of a number of cystinosis and cysteamine related projects. These projects are all in some way related to improving the clinical management of patients with cystinosis. Betty has worked as a lab manager at UCSD for the past eight years and was employed as Staff Research Assistant level II. She has been engaged in a number of basic science studies and is able to perform a number of required assays. She will also perform plasma cysteamine and cystamine assays in conjunction with Dr. Bruce Barshop using the tandem mass spectrometer

RANJAN DOHIL, MD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"A Study to Evaluate Enteric-Coated Cysteamine Therapy in Patients with Cystinosis"
$27,226 – 1-year study

ABSTRACT/SUMMARY

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 recent studies showed that; 1. When cysteamine is administered directly into the small intestine the leukocyte cystine levels-lowering effect of cysteamine is prolonged and 2. When enteric-coated Cystagon™ (EC-Cystagon) capsules are orally administered twice a day that it is as effective at depleting cellular cystine as regular Cystagon™ therapy. Cystagon™ is currently the only commercially available preparation of cysteamine and requires dosing four times a day. We propose to conduct a follow-on study in two cystinosis patients from the original enteric-coated Cystagon™ protocol who will continue treatment twice daily for up to one year. The purpose of this continuation study is to collect longitudinal data on the safety and efficacy of this investigational drug. The effectiveness of this experimental treatment will be assessed by measuring leukocyte cytine levels once every two months; side effects will be recorded once every month by using a gastrointestinal symptom survey tool; and safety will be assessed quarterly by monitoring any potential hepatoxicity effects using hepatic panel blood testing.

PAUL GOODYER, MD, PRINCIPAL INVESTIGATOR

Montreal Children's Hospital, Quebec, Canada

FRANCESCO EMMA, MD, CO-PRINCIPAL INVESTIGATOR

Bambino Gesù Children's Hospital and Research Institute, Rome, Italy
"Stem Cell Microvesicles Rescue Cystinosis in Vitro"
$308,602 – 3-year study

ABSTRACT/SUMMARY

There has been an explosion of interest in the prospect that human stem cells might be useful in treating acute kidney injury in adults. Pluripotent cells can be isolated from bone marrow (BMSC) by fluorescence analysis cell sorting (FACS), using embryonic stem cell markers such as CD133 (prominin1) at the cell surface [1]. Stem cells infused into the circulation appear to home to areas of ischemic tissue injury and can assume relevant differentiated cellular phenotypes [2]. However, there has been considerably less enthusiasm for stem cell therapy of inherited renal disease. The number of BMSC which actually adopt an epithelial phenotype is quantitatively unimportant (often only a few percent of cells within the damaged organ); most BMSC home to the interstitium adjacent to epithelial structures [3]. Thus, at first glance, it seems unlikely that BMSC could repair a hereditary disease such as **Cystinosis** in which every cell bears a mutant lysosomal cystine channel.

Several exciting observations have changed the landscape for stem cell therapy of cystinosis. The first is a report from Syres *et al.* suggesting that hematopoietic stem cells from bone marrow (BMHSC) may ameliorate murine cystinosis without actually replacing mutant renal epithelia [4]. When GFP-tagged mBMHSC were infused into mice lacking the cystinosin gene, the stem cells homed to the kidney but took up an interstitial position and constituted only 13% of all kidney cells. Nevertheless, at 4 months the pathognomonic accumulation of whole-kidney cystine was reduced by 70% and renal function was normalized. *In preliminary experiments from Dr. Goodyer's lab, we have shown that human bone marrow mesenchymal stem cells (hBMMSC) rapidly reduce pathologic cystine levels in fibroblasts from a child with homozygous CTNS deletions.* This implies a powerful "paracrine" effect of stem cells on cystinotic tissue and raises important questions about the mechanism involved.

We believe the impact of stem cells on cystinotic tissue may be related to a **second** set of observations relevant to stem cell lineages in general. Recently, Dr. Janusz Rak (Montreal Children's Hospital) has shown that cancer stem cells reprogram the biology of nearby host cells by shedding microvesicles into the environment; microvesicles are taken up by neighboring cells, delivering both RNA and specialized plasma membrane proteins that alter their biology [5]. *In preliminary observations from Dr. Goodyer's lab, pathologic cystine levels are rapidly reduced when human cystinotic fibroblasts are exposed to microvesicles from human renal cancer cells (RCC), suggesting a generalizeable cystine-depleting mechanism applicable to a wide range of stem cell "delivery systems".*

Thirdly, *in a recent observation by the co-PI of this proposal (F Emma - Rome, IT), it was shown that there are two isoforms of cystinosin; one which is targeted to the lysosomal membrane and a second (cystinosin-LKG) which has a different c-terminal sequence and is delivered promiscuously to both lysosome and plasma membrane [6]; the latter isoform is readily incorporated into exocytotic microvesicles shed by stem cells.* This observation suggests that genetically manipulated stem cells overexpressing the wildtype LKG isoform might constitute an especially effective therapeutic reagent.

We hypothesize that mutant epithelia can take up cystinosin (and/or cystinosin mRNA) from any nearby stem cell which sheds microvesicles and transfers enough wild-type protein to the mutant lysosome to allow cystine egress. If the mechanism is common to most stem cells, it should be possible to achieve the same effect from bone marrow mesenchymal stem cells which are easily propagated and manipulated *in vitro.* In this project, we propose to: 1) confirm that human BMMSC can reduce cystine accumulation in co-cultured human fibroblasts or renal proximal tubular cells from cystinosis patients; 2) determine whether stem cells rescue cystinotic tissue by microvesicle transfer of wildtype cystinosin protein or mRNA to the mutant cells; 3) determine whether hBMMSC overexpressing the cystinosin-LKG isoform may be an especially effective therapeutic agent for cystinosis.

Firstly, these studies will extend the remarkable work of Cherqui in mice by establishing the first proof-of-principle for an effect of human stem cells on human cystinosis. Furthermore, if we can show that the rescue mechanism involves microvesicle transfer of cystinosin to mutant cells, it will suggest that similar effects might be elicited from any stem cell with a high rate of microvesicle shedding and the ability to home to cystinotic tissue – whether or not it has the capacity to undergo transformation into a resident renal tubular cell. This would immediately widen the range of stem cell options for eventual human

trials. Finally, our studies will establish a rational basis for the specific use of the cystinosin-LKG isoform to engineer cystinotic stem cells from the affected patient.

For this project, the Goodyer and Emma laboratories will work together to combine their expertise. Both research groups are headed by pediatric nephrologists with considerable clinical experience caring for cystinosis patients The Goodyer laboratory has extensive experience with genetic manipulation in mice and has made key preliminary observations regarding microvesicle rescue of human cystinotic cells. Dr. Goodyer is cross-appointed in Human Genetics at McGill University; he has access to a wide variety of clinical samples from cystinotic patients and collaborates closely with Dr. Janusz Rak (Montreal Children's Hospital) who has published pioneering work on cancer stem cell microvesicles. The Emma lab has identified a novel isoform of cystinosin, which may offer important advantages in fashioning a modified stem cell to deliver wildtype protein via plasma-membrane-derived microvesicles. Together, we propose studies that will advance our understanding of the mechanism behind stem cell therapy of cystinosis and, thereby, open the door to a number of practical new therapeutic strategies.

ELENA LEVTCHENKO, MD, PHD, PRINCIPAL INVESTIGATOR

University Hospital Leuven, Belgium

ROOS MASEREEUW, PHD AND LAMBERTUS VAN DEN HEUVEL, PHD, CO-PRINCIPAL INVESTIGATORS

Radboud University, Nijmegen, The Netherlands
"Role of P-glycoprotein Expression and Function in Cystinotic Proximal Tubular Cells"
$83,999 – 1-year study

ABSTRACT/SUMMARY

Renal disease in cystinosis is characterized by the development of generalized proximal tubular dysfunction in the majority of patients. Cystine-depleting drug cysteamine postpones or even prevents the deterioration of the renal function, however, it has little influence on the severity of renal Fanconi syndrome.

P-glycoprotein (P-gp) is an ATP-dependent organic cation transporter localized on the apical membrane of the proximal tubules, which plays a role in the efflux of endogenous waste products and xenobiotics, such as drugs, into urine. Studies in mice genetically deficient for P-gp showed proximal tubular dysfunction and ATP deficiency, resembling the phenotype of patients with cystinosis. Our preliminary data indicates that P-gp expression might be reduced in cystinotic proximal tubular cell lines (PTEC) and that cysteamine incubation decreases P-gp activity in these cells.

In this project we will explore the hypothesis that P-gp deficiency in cystinotic proximal tubular cells contributes to the development of renal Fanconi syndrome in cystinosis. The following key-objectives are formulated:

A. Determine P-gp expression in proximal tubular cells of cystinosis patients with renal Fanconi syndrome compared to controls.

B. Assess the functional activity of P-gp in cystinotic and control proximal tubular cell lines at basal conditions and after incubation with cysteamine.

C. Study the consequences of P-gp dysfunction on cell metabolism and tubular cell integrity.

By addressing these key objectives we will increase our insight in the pathogenesis of renal Fanconi syndrome in cystinosis. Furthermore, altered P-gp activity will have consequences for drug dosing in these patients.

ROBERT MAK, MD, PHD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Energy Homeostasis and Muscle Wasting in Nephropathic Cystinosis"
$150,000 – 2-year study

ABSTRACT/SUMMARY

Cystinosis is an autosomal recessive disorder, caused by mutations of the lysosomal cystine carrier protein, cystinosin, encoded by the *CTNS* gene. The intralysosomal cystine accumulation in cystinosis leads to chronic kidney disease (CKD) and multi-organ damage. Growth retardation and generalized muscle wasting are among the commonest clinical characteristics of the disorder. These complications have profound adverse effects on the quality of life. Children with cystinosis often fail to thrive before the onset of advanced CKD, suggesting that factors other than CKD might contribute to the early onset of growth retardation in cystinosis. The underlying mechanism of this growth retardation is not well understood. We hypothesize that abnormal energy homeostasis is present early in the natural history of cystinosis, before the onset of CKD, and may contribute to the profound growth retardation of these children. The generation of Ctns knockout animal model provides an excellent opportunity to test this hypothesis. Indeed our preliminary results showed that there is profound failure to thrive in *Ctns-/-* mice. Body weight was significantly decreased in 15 month-old *Ctns-/-* mice compared with age-matched wild-type (WT) controls. *Ctns-/-* mice also had significantly less lean mass and fat mass than WT mice. Failure to regulate body weight could be due to anorexia or energy inefficiency. Results of a 2-week study showed that food consumption was comparable in 8 month-old *Ctns-/-* mice versus WT mice. Therefore, it is likely that the poor weight gain may be related to energy inefficiency. Furthermore, we have evidence of inflammation in the form of elevated IL-6 mRNA expression in the skeletal muscle of the Ctns-/- mice. Our hypothesis is that inflammation is an important pathogenetic factor in the abnormal energy homeostasis and muscle wasting in cytinosis. We propose to cross *Ctns-/-* mice with IL-6-/- mice. We will characterize the natural progression of metabolic defects in these mice throughout their first year of life. We will expect a much later onset of abnormal energy homeostasis and muscle wasting as well as CKD in the Ctns-/--IL-6-/- double knockout compared with *Ctns-/-* mice. Characterization of these perturbations in energy homeostasis and the underlying molecular pathways will improve the understanding of the pathophysiology of growth retardation and muscle wasting. We are also interested in the impact of inflammation on CKD progression in cystinosis. This new knowledge may provide the basis of novel therapy for the debilitating complications and may lead to improvement in the quality of life in children with cystinosis.

MIRIAM SACH, MD, PHD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Neural Correlates of Executive Functioning in Cystinosis; a Structural MRI Study"
$65,725 – 1-year study

ABSTRACT/SUMMARY

Nephropathic cystinosis is an autosomal-recessively inherited disease due to defective lysosomal cystine transport leading to accumulation of cystine in the lysosomes throughout the body, including the central nervous system (CNS). CNS involvement mainly affects the visuospatial domain with overall preserved verbal and intellectual abilities. Recently, evidence for impaired executive functioning (EF) in cystinosis was provided from neuropsychological results from our laboratory. Executive functioning is mostly mediated by the prefrontal

cortex. We therefore hypothesize that the volume of the frontal lobe is significantly reduced in cystinosis patients and that focal grey and white matter decreases within the frontal lobe and its connected areas correlate with impairment in specific EF abilities. We also hypothesize that the fiber integrity within the frontal lobe is decreased in relationship to the EF dysfunctions. We will test these hypotheses by a combination of structural magnetic resonance imaging (MRI) and EF performance in 23 patients with cystinosis and 23 age-matched healthy controls. These participants represent a subset of subjects, who were recently tested in our laboratory for other studies, and already have both EF testing and MRI scans. In a first analysis, we will assess the frontal lobe volume with its subdivisions in all subjects with respect to EF performance. In a second analysis, we will identify focal gray and white matter decreases in the entire brain in relationship to impaired EF in cystinosis subjects compared to controls. In a third analysis, the frontal lobe masks will be applied to diffusion-weighted images and we will extract fractional anisotropy indices as a measure for fiber integrity for all frontal lobe partitions and assess correlations between fiber integrity and EF abilities. Cerebral volume and fiber integrity decreases will be assessed both for individuals with cystinosis and the group of cystinosis patients. If our hypotheses are correct, volume and fiber integrity of specific frontal lobe structures as well as connected brain regions will be decreased in patients with cystinosis in relation with EF impairment. Our findings will identify the underlying neural substrates of EF in each individual with cystinosis as well as in the group of cystinosis patients. This should provide the basis for further phenotype-genotype identifications enabling future therapeutic intervention and therapy monitoring with the ultimate goal to increase quality of life and achievement in school and profession for patients with cystinosis.

MARY L. TAUB, PHD, PRINCIPAL INVESTIGATOR

State University of New York at Buffalo
"Mechanisms Underlying the Fanconi Syndrome in Cystinosis"
$103,437 – 1-year study

ABSTRACT/SUMMARY

The proposed project is concerned with the hypothesis that in cystinosis, reduced expression of the lysosomal cystine transporter (cystinosin), affects the cellular redox system such that the cellular response to acute renal injury is altered, affecting autophagy and apoptosis. Subsequently, the Fanconi Syndrome emerges, a consequence of the accumulation of reactive oxygen species. A well characterized rabbit renal proximal tubule (RPT) cell culture system in serum free medium will be studied using siRNA against cystinosin to induce cystinosis.

DORIS TRAUNER, MD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Psychological Functioning in Cystinosis: Stress and Coping with Chronic Illness"
$66,624 – 1-year study

ABSTRACT/SUMMARY

Nephropathic cystinosis is a genetic disorder that causes damage to multiple organs in the body. Brain involvement in this disorder is manifested by mild cognitive impairment with specific difficulty with visual spatial, visual memory, and motor coordination skills. Children with cystinosis may have deficits in areas of executive function as well, including organizing and planning, flexibility of thinking, and attention. Difficulties with social skills have also been documented in individuals with cystinosis. This constellation of cognitive and behavioral difficulties could lead to difficulties with adequately adapting to and coping with a chronic illness, which in turn could lead to problems with compliance with life-saving medical regimens.

The proposed study will assess issues related to quality of life, coping strategies, and adaptation to chronic illness, in adolescents and adults with cystinosis, and compare the results with those of individuals with chronic renal disease of mixed etiologies, as well as with healthy age gender and socio-economically-matched controls. These areas will be examined from both the perspectives of the individuals with cystinosis and their caretakers. The results of this study will provide valuable new information that will allow for planning and developing pro-active approaches to improving quality of life, adherence to medical regimens, and more effective strategies for coping with chronic illness for individuals with cystinosis. Until there is a definitive cure for cystinosis, improving quality of life is a valuable goal.

HOLGER WILLENBRING, MD, PHD, PRINCIPAL INVESTIGATOR

University of California, San Francisco
"Pluripotent Stem Cells as a Source of Immunocompatible Renal Progenitor Cells for Therapy of Nephropathic Cystinosis"
$138,625 – 1-year study

ABSTRACT/SUMMARY

The goal of this project is to establish the feasibility of using pluripotent stem cells to generate immunocompatible cells for therapy of proximal tubulopathy, so that the side effects of chronic immunosuppression can be avoided. Besides parthenogenetic embryonic stem cells (pESC), we will use induced pluripotent stem cells (iPSC) as a cell source. Homozygosity at the major histocompatibility locus allows efficient immunological matching of pESC. Derivation of iPSC from a patient's somatic cells facilitates autologous transplantation. Our results obtained in chimeric mice suggest that both pESC and iPSC can differentiate into renal proximal tubular cells with functional and proliferative capabilities similar to normal cells. Next, we will aim at faithfully recapitulating kidney progenitor differentiation of pESC and iPSC in culture. To test the therapeutic efficacy of these cells and guide the development of an effective in vitro differentiation protocol, we have established a mouse model that recapitulates the early-onset kidney injury characteristic for the severe infantile form of human cystinosis.

KANG ZHANG, MD, PRINCIPAL INVESTIGATOR

University of California, San Diego
"Eye Targeted Gene Therapy for Cystinosis in the Eye"
$321,346 – 2-year study

ABSTRACT/SUMMARY

The objective of this proposal is to test the hypothesis that injection of selfcomplementary adeno-associated virus (scAAV) expressing a functional *CTNS* gene will prevent complications and progression of defects of cystinosis in the eye when delivered very early in the disease course and ameliorate the eye disease when administered to older patients. Intracorneal and subretinal administrations are a minimally invasive procedure and would complement systemic cysteamine therapy. AAV vectors presently represent the safest vehicles for gene therapy because of their low immunogenicity, low toxicity, and absence of integration into the host genome. The success of this project will serve as a proof of concept for other gene therapy trials in other tissues or systemically for cystinosis.

PROGRESS REPORT

CORINNE ANTIGNAC, MD, PHD, PRINCIPAL INVESTIGATOR

"Characterization of the Interaction of Cystinosin with Galectin-3 and Vacuolar H+-ATPase"

Date: 4/1/09

Persons working on the project :

Dr Anne Bailleux (PhD – funded by the Cystinosis Research Foundation)
Dr Véronique Chauvet (MD – PhD – EU funding)
Nathalie Nevo (technician, Inserm funded)

Background and objectives

The global aim of our research project is to characterize cystinosin cellular trafficking and to identify functions of cystinosin other than lysosomal export of cystine. The specific aims of the projects are:

Aim 1: Characterize the interaction of cystinosin and galectin-3,

Aim 2: Characterize the interaction of cystinosin and V-ATPase,

Aim 3: Identify other proteins interacting with the 5th inter- TM loop of cystinosin.

Update on the progress of research plan

SPECIFIC AIM #1: Characterize the interaction of cystinosin and galectin-3

a) Further characterize the interaction

When MDCK cells stably expressing cystinosin-GFP are incubated lactose or thiogalactoside the interaction between cystinosin and galectin-3 is abolished, proving that the interaction proceeds through a carbohydrate-dependent mechanism (fig. 1)

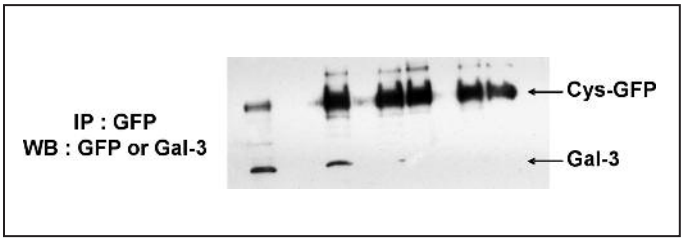

Figure 1. GFP-tagged cystionsin and galectin-3 interact in a carbohydrate dependent manner.ThioDG: thiodigalactoside; Lact: Lactose, Cys-GFP: GFP-tagged cystinosin, Gal-3: galectin-3, IP: immunoprecipitation using antibody against GFP, WB: western blot using antibody against GFP (upper band) or galectin-3 (lower band).

As the glycosylated part of cystinosin is located within the lysosomal lumen, we hypothesized that at least some galectin-3 should be present in the lysosome, which has not be shown as yet. Using digestion by proteinase K experiments (1) we showed that galectin-3 is located inside lysosomes. Indeed galectin-3 is protected from digestion by proteinase K. Permeabilization of lysosomes by Triton X-100 led to the disappearance of galectin-3 after treatment by proteinase K (fig. 2)

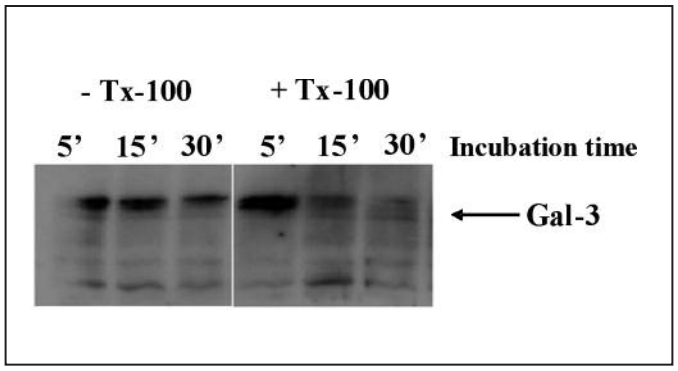

Figure 2. Galectin-3 is located in lysosomes. Purified lysosomes were treated with proteinase K for different time in the presence or absence of Triton X-100 1% (p/v) (Tx-100). In intact lysosomes, galectin-3 in protected from digestion by proteinase K (left panel, in absence of Tx-100). In permeabilized lysosomes, galectin-3 is progessively digested by proteinase K (right panel, in the presence of Tx-100).

The role of the interaction between galectin-3 and cystinosin is not clear. One could be that galectin-3 modulates cystinosin half-life. Based on the N-end rule prediction, cystinosin half-life could vary between 30h (if the signal peptide is conserved, which is probably the case) and 100h (if the signal peptide is cleaved) (3). To address this question, we are now performing metabolic labeling of GFP-tagged cystinosin with ^{35}S-cystin/methionin to evaluate cystinosin half-life expressed in standard MDCK or in MDCK expressing shRNA directed against galectin-3 (4).

b) Perform galectin-3 knock-down studies.

This was achieved through shRNA directed against galectin-3 stably expressed in cells expressing GFP-tagged cytinosin. shRNA were designed from RNAi described in (2), leading to an almost complete absence of galectin-3 expression (fig. 3).

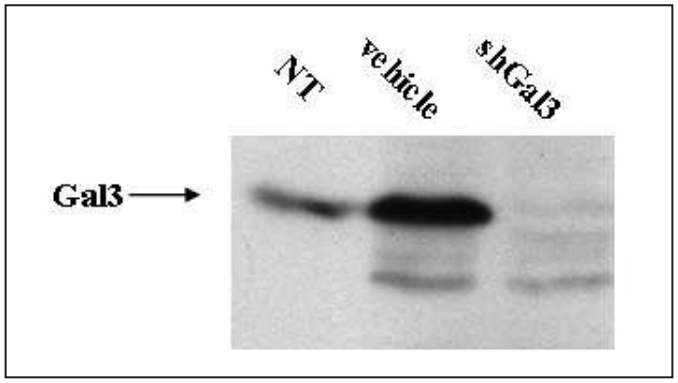

Figure 3. MDCK infected by lentivirus expressing shRNA directed against galectin-3 do not expressed galectin-3 anymore. MDCK cells were infected with lentivirus expressing (shGal3) or not (vehicle) shRNA directed against galectin-3. Cells lysates were subjected to western blot using an antiboby against galectin-3. NT: not tranfected MDCK, Gal3: galectin-3.

GFP-tagged cystinosin localization is now being analyzed by immunofluorescence experiments with labeling of the different intracellular compartments with specific antibodies.

c) Assess the phenotype of cystinosin and galectin-3 double knock-out mice (Ctns-/ - ; Gal3 -/-)

The double KO mice are viable and do not have any obvious phenotype until at least 9 months of age. Groups of 4 mice born on April 1, 2008 have been sacrificed at 9 months and biological and histological studies are underway. Older groups of mice are also being produced at the Scripps Institute.

SPECIFIC AIM #2: Characterize the interaction of cystinosin and V-ATPase

In collaboration with Pierre Courtoy (UCL, Brussels), normal acidification was shown at the single cell and even organelle level by comparing ratiometric pH probing in mock- or rescued cell lines immortalized from two cystinotic patients. Thus, it seems that the interaction between cystinosin and V-ATPase doesn't alter lysosome acidification, but these data need to be verified.

Meanwhile, we are collecting antibodies directed against the various V-ATPase subunits to be able to assess whether there is a direct interaction of cystinosin with one specific subunit.

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PROGRESS REPORT

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

"Treatment of Cystinosis Nephropathy Using Ureteral Injection Aden-Associated Virus Expressing CTNS"

Date: 9/1/2009

This project is done in collaboration with Dr. Samulski, Director of the Gene Therapy Center (University of North Carolina), established AAV expert [1-9]. Several clinical trials for genetic diseases [10, 11] are currently being done using vectors produced by this Center. We are using a scAAV2 as the self-complementary (sc) vectors have significantly higher expression efficiency than the original single strand vectors [12, 13] and AAV2 is currently the most commonly used serotype in clinical trials and it can transduce renal cells [14-16]. However, as explained below, we will determine the most appropriate serotype other than 2 to target the kidney to limit the immune response in human.

SPECIFIC AIM #1: Proof of concept using scAAV2-Luc

We injected scAAV2 expressing luciferase via a retrograde ureteral injection into *Ctns*-/- mice. Luciferase was observed and quantified by the IVIS Imaging System. The results demonstrated that luciferase was detected in the area of the injected kidneys and was stable for 15 months, last time point before sacrifice (data not shown).

Thus the kidney can be successfully transduced by scAAV2 viral particles after retrograde ureteral injection and the transgene expression is stable for at least 15 months.

SPECIFIC AIM #2: Construction and functionality of scAAV2-CTNS and ureteral injection

Construction of scAAV-CTNS and *in vitro* verification:
We subcloned the human *CTNS* gene into a scAAV2 vector backbone provided by Dr. Samulski. *We are working with the human gene in the mouse model studies specifically as the preclinical proof of concept for a human trial.* We verified *in vitro* the functionality of the scAAV2-CTNS vector by transducing *Ctns*-/- fibroblasts. We showed the expression of *CTNS* by RT-qPCR and the significantly reduced level of cystine in AAV-transduced *Ctns*-/- fibroblast compared to controls (data not shown).

Impact of retrograde ureteral injection of scAAV2-CTNS *in vivo*:
We injected $5x10^{10}$ particles of scAAV2-CTNS in the left kidney of *Ctns*-/- mice via ureteral injection. As a first approach, we chose ureteral injection as this would be a minimally invasive route of injection in human. Two months later, RT-qPCR revealed *CTNS* expression in the kidneys (**Figure 1**). However, cystine content was minimally reduced compared to Ctns-/- kidney controls (**Figure 1**). This experiment has been repeated with higher virus particle concentrations, 10^{11} particles, but we obtained the same results. Therefore, retrograde ureteral injection is not the appropriate route of injection to target the cells most impacted by cystine accumulation.

SPECIFIC AIM #3: Other route of injection: renal vein injection

Blue dye injection:
After injection of a blue dye, ureteral injection resulted in blue staining mostly in the medulla of the kidneys (data not shown), whereas renal vein injection resulted in blue staining predominantly in the cortex (**Figure 2**). This area might be the one that needs to be targeted to obtain a significant cystine decrease in the kidney of *Ctns*-/- mice. Therefore, we will use the renal vein as a second potential route for kidney-specific gene therapy for cystinosis.

Renal vein injection of scAAV2-GFP:
We injected 10^{11} particles of scAAV2-GFP in 5 *Ctns*-/- mice via renal vein injection in the left kidney. Two months later, the kidneys were explanted and treated for confocal microscopy analysis. GFP-positive cells were observed in the left kidney for each mouse (**Figure 3**). We are currently analyzing kidney sections to determine the phenotype of the GFP-positive cells by co-immunolocalization with different renal cell-specific antibodies.

Renal vein injection of scAAV2-Luc:
We injected 10^{11} particles of scAAV2-Luc in 8 *Ctns*-/- mice via renal vein injection in the left kidney. Luciferase expression can be observed in live mice using the IVIS imaging system. Luciferase luminescence is observed in the injected kidney at 15 days post-injection in every mouse (**Figure 4**).

The intensity of the expression increases at 1 month and stabilizes at 3 months. These mice are kept alive and will be checked once a month for 1 year to determine the stability of the transgene expression in the kidney after renal vein injection.

Renal vein injection of scAAV2-CTNS:

Our preliminary data using the reporter genes GFP and luciferase indicate that the kidney can be efficiently transduced using the renal vein as a route of injection. We now need to determine if this route will allow the appropriate transduction of the kidney to obtain a decrease of the cystine content in *Ctns*-/- mice.

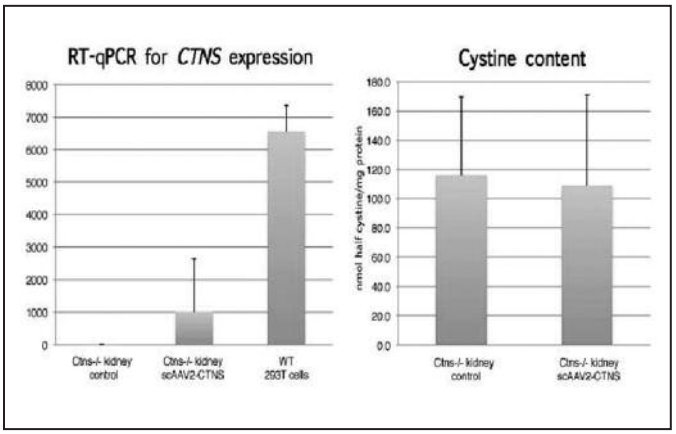

Figure 1. CTNS *expression and cystine content in kidneys after ureteral injection of scAAV2-CTNS compared to controls.*

We injected 10^{11} particles of scAAV2-CTNS in 6 *Ctns*-/- mice via renal vein injection in the left kidney. We will sacrifice these mice 4 months post-injection to quantify *Ctns* expression by RT-qPCR and to measure the cystine content of the left kidney compared to controls. If these preliminary data are positive, we will determine the optimal serotype to target the kidney as described below.

Next step: Determination of the optimal AAV serotype

Few studies have been performed on AAV for gene delivery in the kidney. Most studies used AAV serotype 2 to transduce renal cells *in vivo* and *in vitro* [14-16]. However, the prevalence of neutralizing antibodies in the human population for AAV2 is very high. As pre-existing immunity to AAV2 will probably impact its efficiency for gene delivery, *we will test scAAV serotypes 5, 6, 7 and 8 for gene delivery in our mouse model.* In support of this decision, a recent study showed that AAV4, 5, 7 and 8 could infect murine kidney as well as AAV2 [17].

In contrast, the prevalence of human immunity to AAV5, 7 and 8 is significantly lower than AAV2 [18-21]. Our expert in AAV gene therapy, Dr. Jude Samulski also recommended using either AAV1 or 6 (that use sialic acid as the cellular receptor). As AAV1 has also a high pre-existing immunity in humans [20], we will only add AAV6. Note we will not use AAV serotype 4 based on Dr. Samulski's experience that this serotype is difficult to use and therefore not a good candidate for clinical application.

As reporter genes, we will use GFP and luciferase and the virus particles will be injected via the renal vein if we determine that this is the appropriate route in our cystinosis model as described above. The objectives are to identify the optimal AAV serotype for gene delivery in the kidney with successful transduction of different types of renal cells including proximal tubular and glomerular cells.

In the context of cystinosis, we would like to transduce the proximal tubular cells to avoid the proximal tubulopathy, but also the transduction of a large spectrum of different renal cells including the glomeruli would be important to prevent and/or reverse renal defects as all the cells in the patient kidneys accumulate cystine. A luciferase reporter gene will allow us to follow and quantify the transgene expression in live mice at different time points in the whole kidney using the IVIS imaging system. The GFP reporter gene will allow us determine the phenotype of the transduced cells by fluorescence histology using confocal microscopy. Our first experiments with scAAV2-Luc and scAAV2-GFP, allowed us to conclude that we could obtain a high and stable expression of a transgene in the kidney with $1x10^{11}$ virus particles.

Therefore, we will use $1x10^{11}$ virus particles for this part of the project injected via the left renal vein of 2 month-old *Ctns*-/- mice for each AAV serotype and each reporter.

Figure 2. Blue staining in kidneys after renal vein injection of a blue dye.

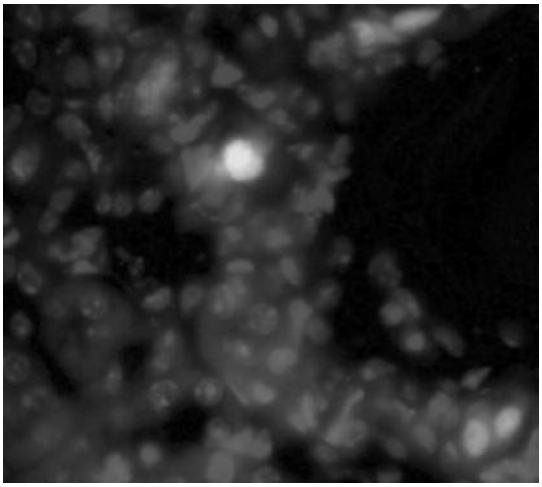

Figure 3. scAAV2-GFP infected kidney section. GFP-positive cells are seen in green and nuclei are seen in blue.

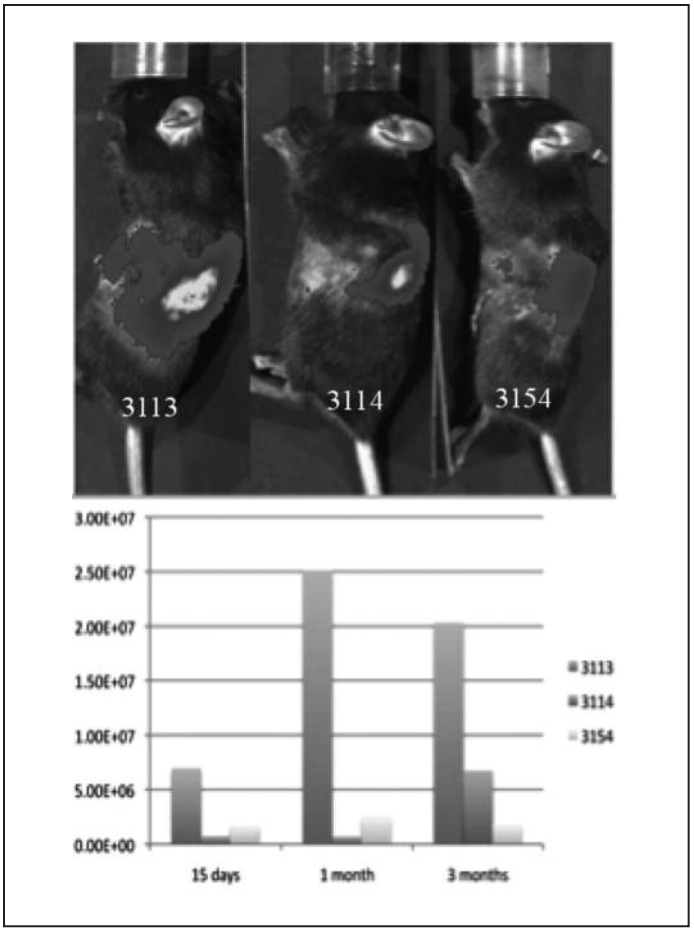

Figure 3. Three representative Ctns*-/- mice injected with scAAV2-Luc via the renal vein.* ***Upper panel:*** *picture taken 3 months post-injection using the IVIS imaging system.* ***Lower panel:*** *histogram of the luciferase luminescence quantification at 15 days, 1 month and 3 months post-injection in the 3 mice.*

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PROGRESS REPORT

STEPHANIE CHERQUI, PHD, PRINCIPAL INVESTIGATOR

"Stem cell and Gene Therapy for Cystinosis"

Date: 1/11/10

C.1.4: Generation of the lentiviral vectors.

The lentiviral vector (LV) has proven its efficacy for long-term HSC transduction in mice but also in some clinical trials with no complications reported to date. Dr. Cherqui has an extensive experience on producing and using lentiviral vectors [1]. We are also collaborating with Dr. Donald Kohn, Professor Microbiology, Immunology and Molecular Genetics and Pediatrics (University of California, Los Angeles). Dr. Kohn's expertise specifically includes using lentiviral vectors for pre-clinical and clinical trials for ADA-deficient SCID, sickle cell disease and cancer immunotherapy. Dr. Kohn provided us with a self inactivated (SIN)-lentiviral vector containing a mutated Woodchuck hepatitis virus Posttranslational Regulatory Element (WPRE) sequence and the ubiquitous human intron-less EF1alpha promoter (EFS) promoter [2, 3]. WPRE that stabilizes the transgene message [4-6], also overlaps with the Woodchuck hepatitis virus X protein, which is a transcriptional activator involved in the development of liver tumors [7]. This potential complication has been avoided by mutating the WPRE sequence, which still induced the long-term expression of the transgene *in vivo* [8]. EFS promoter is potent enough to express clinically relevant genes [3, 9] and greatly decreases the risk of insertional transformation in serially replated HSC [10]. Note that numerous studies show the long-term efficacy of lentiviral-transduced murine bone marrow cells in mouse models for genetic diseases such as Adenosine Deaminase Deficiency (ADA)-deficient mice, Artemis-deficient mice and Wiskott-Alsrich syndrome for recent examples [11-13]. Recently, Dr. Cartier and colleagues published clinical trial data on HSC gene therapy for X-Adrenoleukodystrophy using a SIN-lentiviral vector. These studies showed reconstitution of both immunological and metabolic abnormalities in 2 patients with no signs of clonal dominance 30 months post-transplant [14].

C.1.4.1: Construction and functionality of the lentivirus vectors *in vitro*

We subcloned the GFP (LV-GFP) and luciferase (LV-Luc) reporter genes as well as the human CTNS gene (LV-CTNS) in the SIN-lentiviral vector provided by Dr. Kohn. *We are working with the human gene and using a vector already used in clinical trials in these mouse model studies specifically as the preclinical proof of concept for a human trial.* We verified the integrity of the vectors by restriction enzyme digestions and sequencing.

We produced infectious lentiviral supernatants using a transient expression of 293T cells with three packaging plasmids (*gag-pol*, *VSV-G* and *rev*) and the expression construct, LV-GFP, LV-Luc and LV-CTNS, resulting in assembly of VSV-G (Vesicular Stomatitis Virus-G glycoprotein) pseudotyped viral particles. VSV-G pseudotyping allows a high efficiency transduction with a wide cell target range and is stable enough for viral supernatant freezing and concentration by ultracentrifugation. Moreover, VSV-G efficiently targets HSC [15, 16]. The combination of three separate plasmids to provide the complete viral assembly machinery and the use of an LTR-truncated lentiviral backbone (i.e. SIN vector strategy) essentially eliminates the risk of creating replication competent lentivirus (RCL). Testing for RCL by serial infection has been consistently negative in our experience with this method (data not shown). Titers of concentrated virus were measured by infection of 293T cells after serial dilutions of the virus preparation and determined by flow cytometry for LV-GFP, IVIS imaging system for LV-LUC and RT-qPCR for LV-CTNS as described below. The titers were included between $5x10^8$ to 10^9 vg/ml.

The *in vitro* verification of the functionality of LV-GFP and LV-Luc was determined by measuring transgene expression after transduction of 293T cells. Transgene expression was measured by flow cytometry analysis for LV-GFP and by IVIS imaging system for LV-Luc (Figure 1). A strong GFP expression is observed in 293T cells as well as luciferase expression and the expression is function of the quantity of viral particles added.

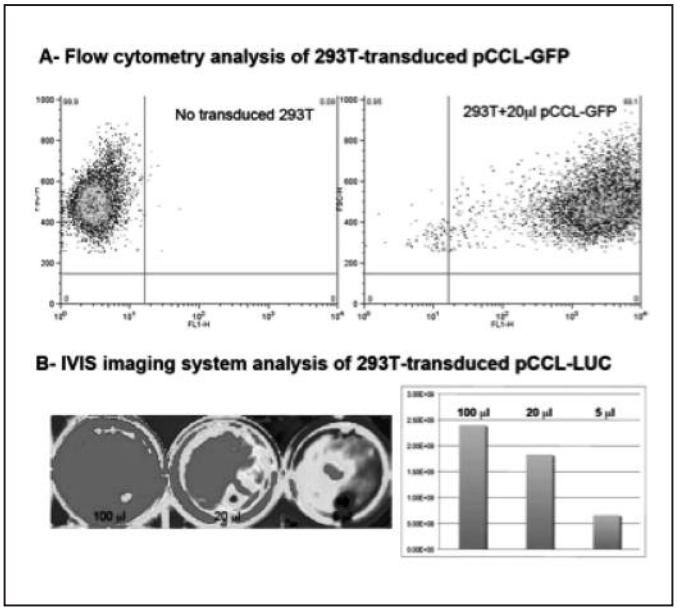

Figure 1. Functionality of LV-GFP and LV-LUC in vitro. ***(A)*** *Flow cytometry analysis of 293T transduced with 20 µl of LV-GFP virus preparation compared to non-transduced cells.* ***(B)*** *IVIS imaging system analysis of 293T transduced with 100, 20 and 5 µl of LV-LUC virus preparation.*

To verify the functionality of LV-CTNS, we transduced *Ctns-/-* fibroblasts generated from skin of a C57BL/6 *Ctns-/-* newborn mouse and control wildtype fibroblasts were generated in the same fashion. Virus particles were added directly to the *Ctns-/-* fibroblasts and the cells collected 48h after. Controls were non-transduced *Ctns-/-* fibroblasts, wildtype fibroblasts and 293T cells.

RNA was extracted from half of the cells. A *CTNS* gene-specific RT-qPCR determined the quantity of CTNS mRNA present in each sample. The titer of virus particles is determined by generating a standard curve of serial dilution of of LV-CTNS plasmid DNA. Figure 2A shows the expression of human *CTNS* in transduced *Ctns-/-* fibroblasts compared to LV-GFP-transduced *Ctns-/-* fibroblasts and 293T. No expression is observed for LV-GFP-transduced *Ctns-/-* fibroblasts as expected. 293T, which are human cells, show the endogenous level of *CTNS*, which is still significantly less than that achieved in the transduced mouse fibroblasts. The other half of the cells was used for

cystine measurements by the UCSD Biochemical Genetics laboratory using mass spectrometry. Figure 2B shows the endogenous level of cystine in wildtype fibroblasts, the high level in LV-GFP transduced-*Ctns*$^{-/-}$ fibroblasts and the significantly reduced level of cystine in LV-CTNS-transduced *Ctns*$^{-/-}$ fibroblasts (p<0.05).

These studies were done three times and proved that the CTNS gene is expressed after transduction of cells in vitro with LV-CTNS and that the human version of CTNS is functional in mouse cells because it leads to the decrease of cystine content in the Ctns$^{-/-}$ *fibroblasts.*

We also performed all the *in vitro* safety tests of the vectors by serial infection of 293T and showed that our lentivirus vector is replication-incompetent (data not shown).

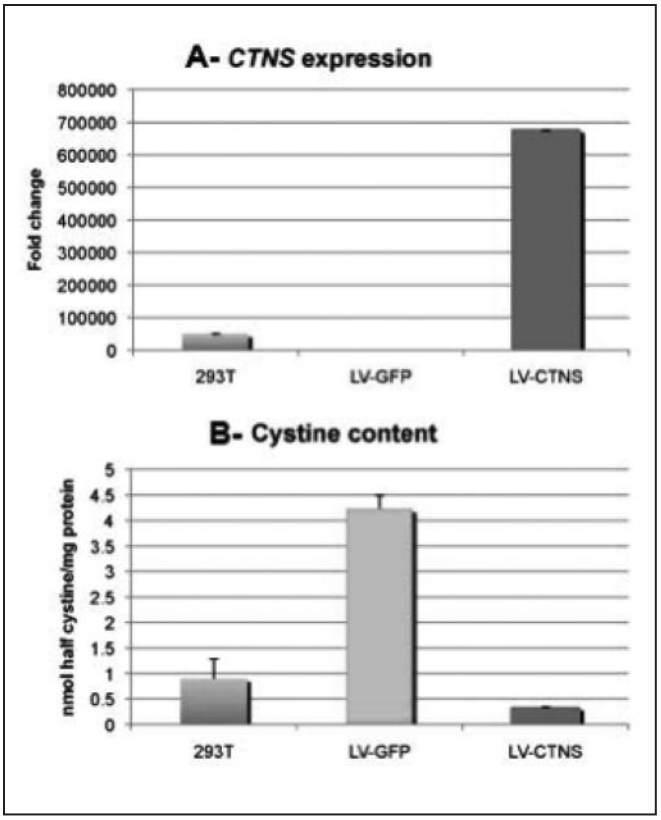

Figure 2. Functionality of LV-CTNS. ***(A)*** *CTNS-specific RT-qPCR showing the expression of CTNS in Ctns-/- fibroblasts after transduction with LV-CTNS.* ***(B)*** *Histogram showing the decrease of cystine level in Ctns-/- fibroblasts transduced with LV-CTNS.*

C.1.4.1: Functionality of the lentivirus vectors *in vivo*

To investigate the feasibility of HSC transduction by our lentiviral vector and optimize our protocol, we performed a preliminary *in vivo* experiment by transducing Sca1^{+} HSC with LV-GFP. Sca1^{+} HSC were cultured *ex vivo* in Stemspan medium (StemCell Technologies) supplemented with murine stem cell factor (mSCF), thrombopoietin (TPO) and murine FMS-like tyrosine kinase 3-ligand (mFlt3L) at a concentration of 100ng/ml and murine interleukin-6 (mIL-6) at a concentration of 20ng/ml. Cells are cultured in plate coated with recombinant fibronectin fragment. Lentiviral vector particles were added at a MOI=10 in presence of polybrene for 16 hours at 37ºC. Cells were then collected, washed twice in PBS and resuspended in appropriate volume of PBS. Tail vein injection of 100µl of non-tranduced or LV-GFP-transduced Sca1+ cells was performed in 6 lethally irradiated *Ctns*$^{-/-}$ mice (3 mice each). One mouse transplanted with LV-GFP-transduced HSC died because of the procedure.

We measured the quantity of GFP^{+} cells in the red blood cell-lysed peripheral blood of the transplanted mice by flow cytometry at 4 weeks after transplantation (**Figure 3**). The quantity of GFP^{+} cells ranged from 50% to 81% depending on the mouse 1-month post-transplant and over 80% 2 months post-transplant.

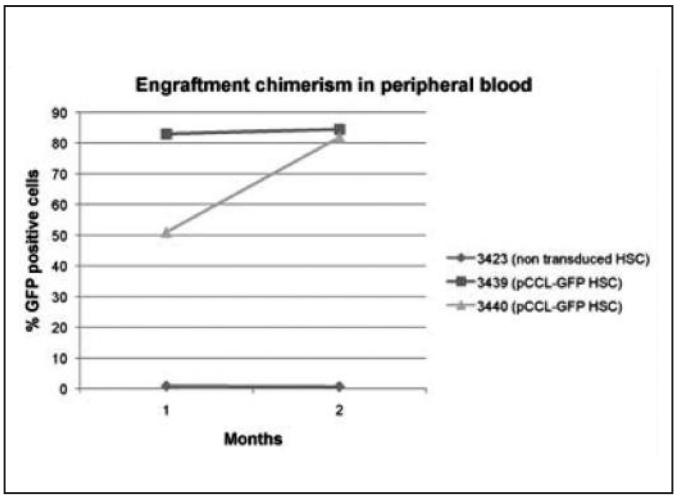

Figure 3. Percentage of GFP-expressing cells in the peripheral blood of 2 Ctns-/- mice transplanted with Sca1+ HSC transduced with LV-GFP compared to 1 negative control.

Conclusions: Our preliminary data with the lentiviral vector showed a strong transgene expression and a great efficiency for HSC transduction. We will perform a dose titration of lentiviral vector using LV-GFP and LV-luciferase in order to use the minimal quantity of viral particles for optimal HSC transduction. The follow up of the mice transplanted will also allow us to determine the fate of the transduced cells and the stability of the transgene expression with time. Once the optimal conditions are determined, we will transplant *Ctns-/-* mice with Ctns-/- HSC transduced with LV-CTNS. The analysis of the transplanted mice will show if transduced HSC have still the potential to engraft in tissues and lead to cystine decrease.

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PROGRESS REPORT

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

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

Date: 12/31/09

OVERVIEW

The project is progressing according to the original research plan. The three mouse lines are all actively breeding (*Ctns-/-*, *vanin-/-* and *Ctns-/-vanin-/-* double knockouts). The animal work for the first cysteamine in vivo study has been completed and tissue analyses continue. We now have a cohort of 10 *Ctns-/-vanin-/-* double knockout mice who are 6 months of age. Although data are still preliminary, it appears that the males are polyuric compared to the *Ctns+/-vanin+/-* double heterozygous sibling controls.

SPECIFIC AIM #1: To perform preliminary studies to develop an optimal model and drug delivery strategy to investigate the effect of cysteamine in a mouse chronic kidney disease model.

Searching for a mouse model of cystinosis-associated nephropathy. Based on our hypothesis that *Ctns-/-* mice have an essentially normal phenotype due to the expression of endogenous cysteamine synthesized via an enzymatic pathway that is encoded by the vanin gene, we have generated a colony of *Ctns-/- vanin-/-* double knock-out mice. We are currently following 26 double knockouts and 16 heterozygous sibling controls. Initial findings based on 24h urine samples collected in individual mouse metabolic cages (n = 2-5 mice per time-point) suggest that the double knockout mice have higher urine volumes, especially in the males (Figure 1). We plan to collect 24h urines every 3 months. As the sample sizes increase, the findings will hopefully reach statistical significant (P values for males at 3 months is already 0.01). Serum BUN levels wil be measured at 9 months. At this point we plan to follow the mice until at least 1 year of age before they are sacrificed to examine kidney histology. Two cages of double heterozygous mice are actively breeding to expand the number of mice in this study.

SPECIFIC AIM #2: To investigate the efficacy of cysteamine therapy for interstitial renal matrix protein reduction in chronic kidney disease and to determine its mechanism of anti-fibrotic action.

Based on the findings from a pilot study, the effect of cysteamine (administered as Cystagon® added to the drinking water that was freshly made every 24h) on the degree of renal fibrosis was investigated using two doses: 400 and 600 mg/kg/day. Groups of mice (n = 8 at each time-point) were studied 3, 7 and 14 days after the onset of chronic injury induced by UUO. Both doses were shown to significantly reduce kidney collagen levels

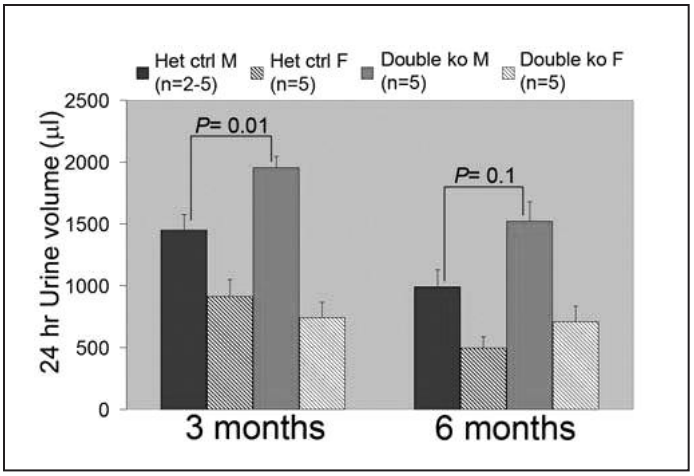


Figure 1. Initial data on 24h urine volumes show larger volumes in male (M) Ctns-/-vanin-/- *double knockouts (Double ko) compared to* Ctns+/-vanin+/- *heterozygous controls (Het ctrl). Additional mice will be entered intothis study.*

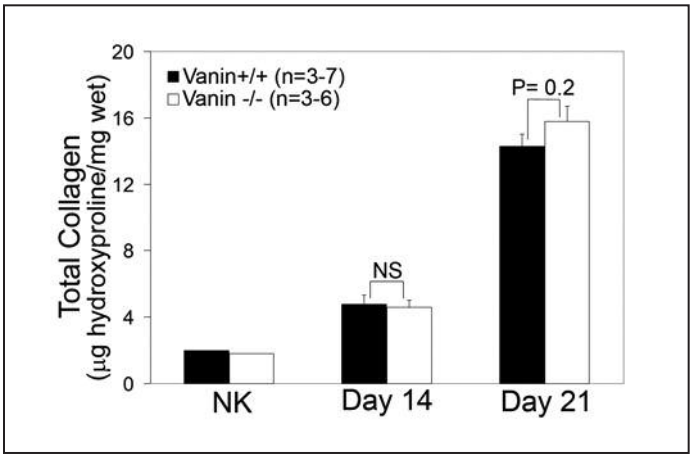


Figure 2. Total kidney collagen levels between vanin+/+ *and* vanin-/- *mice 14 and 21 days after UUO shows no significant differences.*

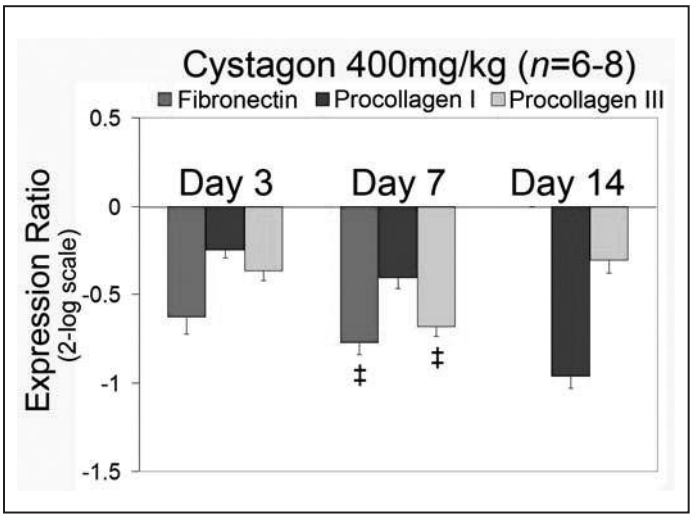


Figure 3. ***Cystagon 400 mg/kg group.*** *Kidney extracellular matrix protein mRNA levels measured by real-time RT-PCR are significantly lower in Cystagon®- treated mice 7 days after UUO. Data are shown as means of the relative expression ratios (treated versus untreated) (n=8/group).*

by day 14 by ~20% compared to the untreated group (shown on last process report). We plan to extend these observations to a 21-day UUO study.

Kidney tissue studies are now focusing on elucidating the mechanism by which Cystagon® reduces kidney fibrosis. One potential pathway may be its ability to reduce collagen synthesis rates.

Since the last progress report matrix gene mRNA levels have now been measured by real-time RTPCR (qRT-PCR) in all mice 3, 7 and 14 days after UUO (Figures 3 and 4). The peak effect of Cystagon® was observed at 7 days at both treatment doses. Additional mechanisms must also contribute to the anti-fibrotic actions of Cystagon®. Computed-assisted image analysis of immunohistochemically stained kidney sections at day 14 UUO show significantly less interstitial inflammation as measured by the number of F4/80+ interstitial macrophages and significantly fewer interstitial myofibroblasts as measured by the number of αSMA+ cells, in the 600 mg/kg treatment groups (Figures 5 and 6 respectively). Additional studies are ongoing.

SPECIFIC AIM #3: To investigate the effect of cysteamine on apoptosis of renal tubular epithelial cells, oxidant stress, and other novel target pathways of chronic kidney disease.

As originally proposed, the effects on apoptosis, glutathione activity and novel cysteamine targets will be the primary focus of these studies. We have begun to investigate possible effects on transglutaminase (TG2) expression and activity. By Western blotting total kidenyTG2 protein levels were unaffected by Cystagon® treatment (Figure 7). Assessment of TG2 activityis planned next.

In summary, early data from the first Cystagon® treatment study establishes its significant anti-fibrotic effects. Ongoing studies are planned to identify how this effect is achieved and to determine if it is sustained when chronic kidney damage persists for longer periods of time. Over the next 6 months we will be able to determine if the early observations on the renal phenotype of the Ctns-/- vanin-/- double knock-out mice (polyuria) are sustained as the mice age.

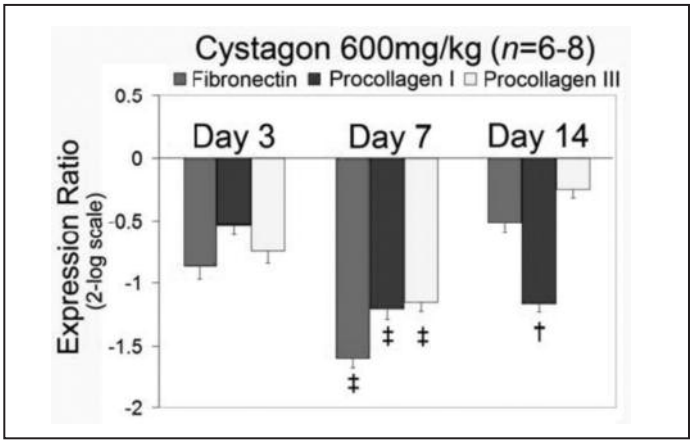


Figure 4. ***Cystagon 600 mg/kg group.*** *Kidney extracellular matrix protein mRNA levels measured by real-time RT-PCR are significantly lower after Cystagon®-treated mice 7 days after UUO. Data are shown as means of the relative expression ratios (treated versus untreated) (n=8/group).*

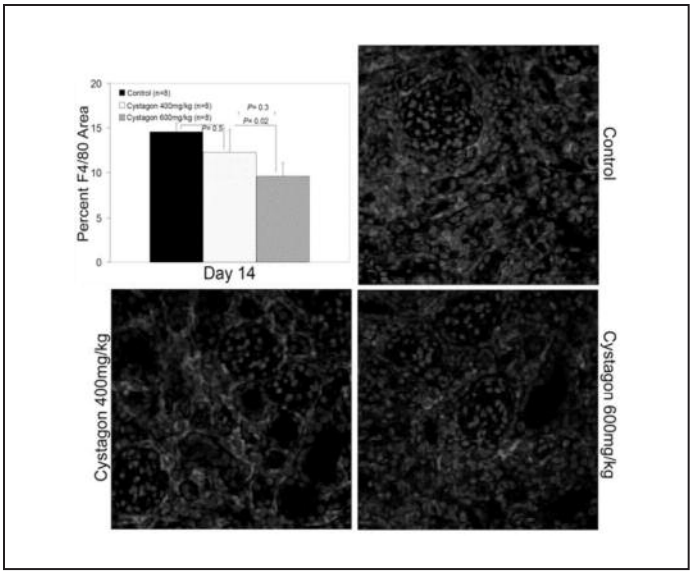


Figure 5. ***Cystagon® reduces interstitial inflammation after UUO.*** *Quantified using computer-assist image analysis, the numbers of F4/80+ interstitial macrophages were significantly reduced by high dose Cystagon® treatment.*

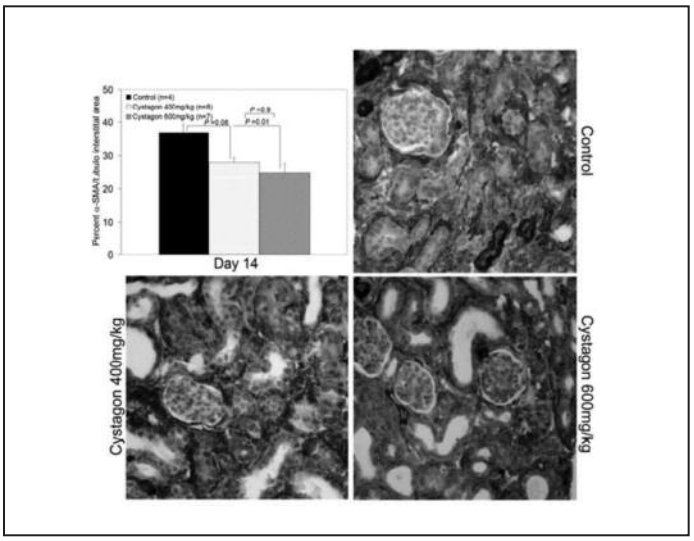


Figure 6. ***Cystagon® reduces interstitial myofibroblasts after UUO.*** *Quantified using computer-assist image analysis the numbers of αSMA+ interstitial myofibroblasts were significantly reduced by high dose Cystagon® treatment.*

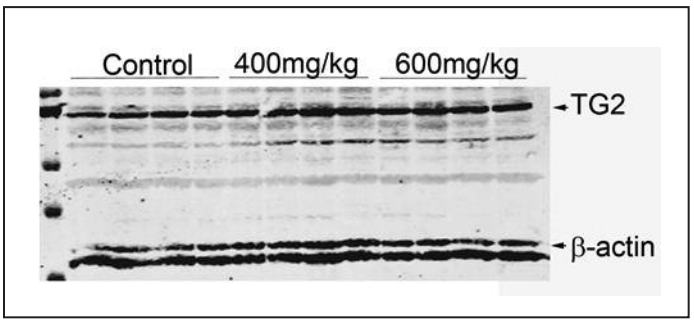


Figure 7. Transglutaminase (TG2) Western blot shows similar kidney protein levels 14 days after UUO between untreated controls and Cystagon® treatment mice. Not shown, band densities after correction for protein loading using β-actin band densities were similar between the three groups..

PROGRESS REPORT

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

"Identification and Analysis of Cis-and Trans-Acting Elements that Activate the CTNS Gene"

Report 4/1/ 09 – 10/30/09

Our previous studies have showed an up-regulation of CTNS mRNA when cell was shifted from a standard DMEM/F12 medium to a cystine-free DMEM medium. Furthermore, we have observed that this up-regulation is mediated simultaneously by increased activity of the CTNS promoter and stabilization of its mRNA. The first specific aim was to identify transcriptional factors that regulate CTNS gene expression and receptive promoter segments to low cystine level.

We have cloned five segments of CTNS promoter described in 2001 by Phornphutkul C. et al., into PGL4 vector carrying luciferase gene. Proximal HK2 cells have been transfected with PGL4 vectors and cultured in DMEM or DMEM with low cystine level (10%) for 24 and 48 hours. Luciferase activity was increased about 2,5 folds for long promoter segments (–769, –348, –316 bp) and about 2 folds for short promoter segments (–283, –81 bp) after cystine deprivation. Under basal condition, the long segments showed a similar and higher luciferase activity than the short segments (figure 1).

An analysis *in silico* on transcriptional factors binding CTNS promoter has identified in the – 769 segment 3 potential sites (-730/-690; -436/-405; -294/-260) which have consensus sequences for the Sp1 transcription factor. We have focused our attention on this transcription factor because in the potential binding site (–294/–260) has been described a mutation (–295 G>C, Phornphutkul C. et al., 2001) causing cystinosis and a polymorphism (–294 C/T, our data) decreasing 30% promoter activity.

To study the DNA-binding capacity of Sp1 under cystine deprivation we have prepared nuclear extracts which were obtained from HK2 cells grown in DMEM or DMEM with low cystine level and measured DNA- binding capacity by using an ELISA assay (transAM Sp1 Flexi Kit) to detect and quantify transcription factor activation in substitution of classic electrophoretic mobility shift assay.

Under these conditions, we have not observed an increase of Sp1 binding activity for all three binding sites. Further investigation are in progress for confirming these data.

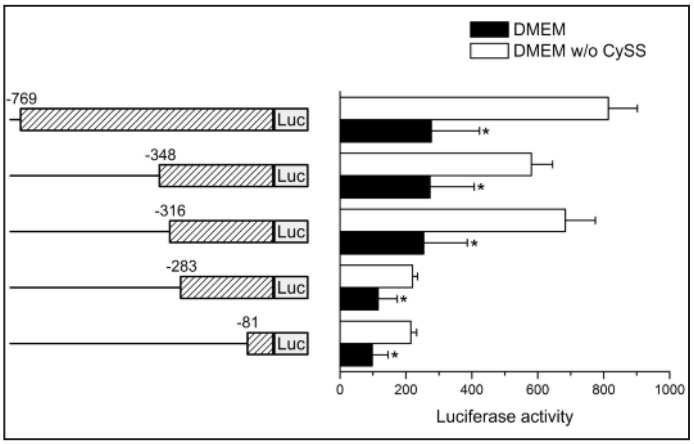


Figure 1

PROGRESS REPORT

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

"Molecular Anatomy and Physiology of Human Cystinosin"

Report 10/1/09

1. Topology study of cystinosin

For the topological study, cDNAs encoding the fusion proteins depicted in Figure 1 were constructed.

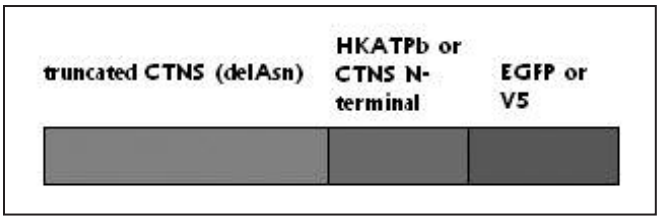


Figure 1. Scheme of cystinosin fusion proteins made for the topology study.

As mentioned in the previous report, a series of C-terminally truncated fragments of human cystinosin (pink segment) lacking endogenous N-glycosylation sites was fused to a glycosylation-based topology reporter (in blue) followed by an expression reporter (in green). The glycosylation state of the topology reporter (glycosylated when it is translocated into the ER lumen) indicates the topology of the truncation site tested for each construct.

Most constructs fused with the glycosylated domain of H^+, K^+-ATPase β subunit and EGFP did not express well in Xenopus oocytes. The reason might be the excessive size of the reporters (> 400 aa together), which were larger than cystinosin itself. The resulting constructs are presumably degraded by the protein quality control system of Xenopus oocytes.

To circumvent this difficulty, we tried to express them in vitro by coupled transcription/translation in the presence of microsomes. On SDS-PAGE, the size of the resulting translation products was in good accordance with those obtained in vivo, in Xenopus oocytes, after deglycosylation. However, we could not observe any glycosylation of the reporter in these in vitro products for a reason that remains unclear.

EGFP was thus replaced by a much smaller tag, the V5 epitope. The resulting fused genes were all well expressed in Xenopus oocytes. Nevertheless, the level of expression was largely time-dependent and construct-dependent. In SDS-PAGE experiments, some of the truncated constructs underwent a ~12-kDa decrease in apparent molecular mass after treatment with PNGase-F or other N-deglycosydases, thus bringing topological information.

With the information gathered from these experiments, we propose a novel topological model of human cystinosin which significantly differs from the current model predicted by topology softwares. The major difference lays in TM1 and TM6. This difference was supported by a similar experiment, where a N-terminal segment of cystinosin was used as topology reporter instead of H^+, K^+-ATPase β.

To verify this novel topological model, we are now performing cysteine-scanning experiments on full-length, active cystinosin constructs. A cystinosin mutant lacking endogenous cysteines was constructed and shown to be active for cystine transport. Ectopic cysteine residues will then be substituted for endogenous residues at chosen sites to test their topological orientation in the full-length context. After a functional assay aimed at confirming that the single cysteine mutants studied are properly folded, biotinylation with impermeant thiol reagents will be performed to get a *validated* topological map of cystinosin.

Our first priority is to check the relative location of the 2 PQ motifs. According to the current topology model, both motifs are on the same side of the membrane whereas, in our revised model, they should sit on opposite sides.

2. Functional analysis of cystinosin

In preliminary experiments of this project, we developed an electrophysiological assay of cystinosin-mediated cystine transport and proposed, based on voltage-dependence properties, that cystine binding is coupled to protonation from the luminal compartment of a residue buried in the membrane. Site-directed mutagenesis of several conserved residues revealed mutants with strongly altered voltage dependence properties that corroborated our working hypothesis. However, these experiments failed to unveil the identity of the proton binding site.

To address this issue, we used a different approach consisting in recording transient currents elicited by applying voltage jumps to cystinosin-expressing oocytes. The underlying rationale was that if cystine-laden cystinosin is protonated at a site buried in the membrane, and therefore located in the voltage gradient, voltage steps should challenge the protonation equilibrium and induce capacitive currents associated to proton transfer.

In good agreement with this model, we observed that, in the presence of cystine, voltage jumps induced transient currents in wild-type cystinosin, with time constants of 4 to 6 ms. 'Titration' of this electrogenic transition by applying a wide range of voltage values indicated a mean transient charge moved of ~8 nC per oocyte. This value, which is proportional to the number of cystinosin molecules, in turn allowed deriving a maximal transport rate of 28 cystine molecules per second per cystinosin molecule.

To identify the protonatable residue coupled to cystine binding, we next repeated this analysis on site-directed mutants. Interestingly, mutation of a single aspartate could abolish the cystine-dependent transient currents and thereby unveil the identity of the proton binding site. Because of its geometric properties (proximity to the cytosol but accessibility from the lysosomal lumen), we propose that this aspartate residue represents a relay site for the translocated H^+ that actively drives cystine out of the lysosome.

A manuscript describing this electrophysiological study is currently in progress.

We hope that these deep biochemical and biophysical analyses of human cystinosin may provide an accurate framework for a better understanding of the cellular roles of cystinosin.

PROGRESS REPORT

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

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

Date: 7/1/2009

RESEARCH UPDATE FROM THE PARIS GROUP

Persons working on the project:

Christine Anne, Inserm scientist

Cécile Debacker, CNRS technician

Karolina Marciniak undergraduate student

Bruno Gasnier, PI

Azita Sharifi (graduate student) participated in some experiments.

BACKGROUND AND OBJECTIVES

Biochemical studies have previously shown that cysteamine depletes accumulated cystine from cystinotic lysosomes by reacting with cystine to form a mixed cysteine-cysteamine disulfide, which then exits lysosomes through an unidentified lysosomal arginine/lysine transporter named 'system c'.

Our aim is to identify this transporter to help improving current cysteamine treatments.

We previously identified several members of the SLC7 transporter family as potential system c transporters based on immunofluorescence data and, for functionally characterized members, on cysteamine-cysteine competition experiments. CAT2A, CAT2B, an orphan CAT-like protein named SLC7A14 and, to a lesser extent, CAT1 appeared as good system c candidates. However, it is still unknown which of these transporters is (or are) actually involved in cysteaminemediated cystine lysosomal depletion.

Our efforts during the past 6 months were devoted to developing a reliable in vitro assay to address this issue. The next step would be to decrease or increase expression of the candidates in the in vitro model (using siRNA-mediated silencing or cDNA transfection) to examine whether these manipulations alter the kinetics of lysosomal cystine depletion.

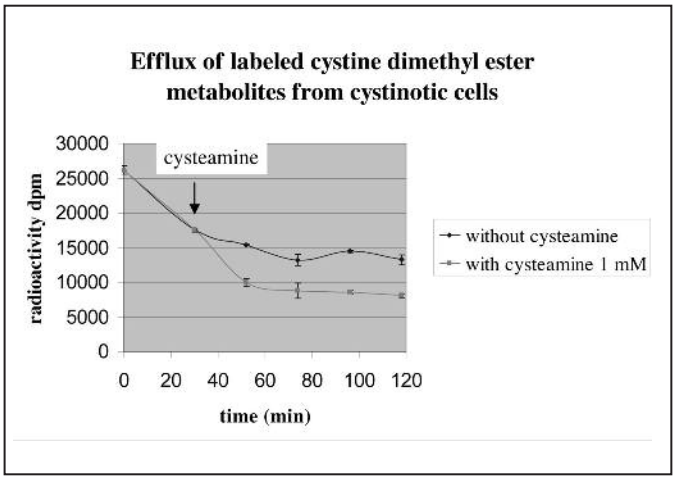
RESULTS

1. Studies on whole cells

As mentioned in our previous report, we first tried to develop a cystine depletion assay in whole cells using readily transfectable cell lines (HeLa, HEK293). Conditions mimicking cystinosis were artificially created by overloading lysosomes from intact cells with labeled cystine using a tritiated ester precursor ([^{3}H]cystine dimethylester). Subsequently cells were treated with cysteamine to deplete the lysosomal [^{3}H]cystine pool. This approach was abandoned because it proved difficult to get a reproducible cysteamine depletion effect from one experiment to another, presumably because the lysosomal [^{3}H]cystine pool was often masked by other 3H-labeled metabolites.

We thus decided to pursue this approach on cystinotic fibroblasts from patients. In these experiments, the [^{3}H]cystine dimethylester precursor was used merely as a radiotracer to follow the fate of lysosomal cystine, as previously done in the seminal experiments from J.A. Schneider and W.A. Gahl and their co-workers. It should be mentioned that, in contrast with these early studies, we could not use 35S to label the lysosomal cystine pool because of the impossibility to perform radioactive esterification reactions in an academic laboratory and of the prohibitive cost of ordering repeated custom syntheses of a compound labelled with a short half-lived isotope. We thus ordered a custom-made tritiated precursor ([^{3}H]cystine dimethylester) from a company. Cystinotic fibroblasts were kindly provided by C. Antignac.

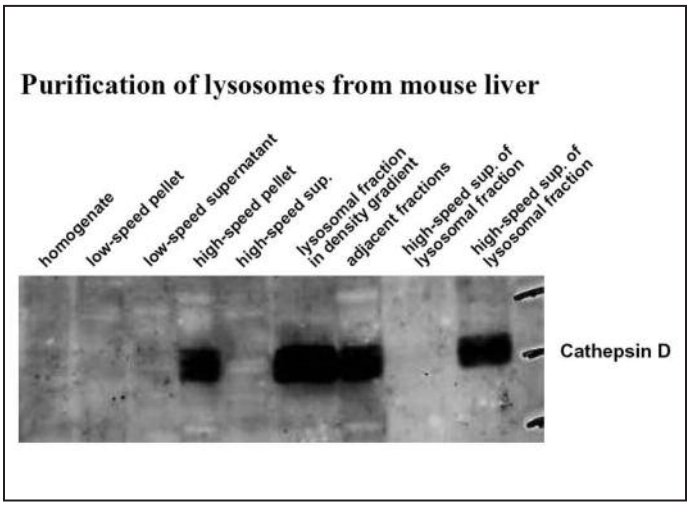
A representative experiment is shown in the Figure below. As expected, cysteamine triggered efflux of radioactivity from cystinotic fibroblasts pretreated with [^{3}H]cystine dimethylester. However, on one hand, this effect was limited to ~20% of the total amount radioactivity and, on the other hand, substantial efflux of radioactivity was observed in the absence of cysteamine. These disappointing findings presumably reflect the fact that the pool of labeled metabolite is much broader and complex when cystine dimethyl ester is labeled with 3H rather than 35S. For instance, the spontaneous efflux of radioactivity observed in the absence of cysteamine may reflect an efflux of [3H]methanol molecules generated in the lysosome after cleavage of the ester bonds (such a process would be 'silent' if we could have used a 35S-labeled precursor).



We tried ordering a custom-made methyl ester precursor exclusively tritiated on the cystine moiety to increase the selectivity of lysosomal cystine metabolic labeling. However, only one cysteamine company committed itself to doing so, but failed and thus ended up synthesizing a generallylabeled tritiated precursor. It should be mentioned that the partial masking of the lysosomal [^{3}H]cystine pool with other [G-^{3}H]cystine dimethylester metabolites not only reduced the relative amount of radioactivity sensitive to the cysteamine treatment, but also hindered measurement of the time course of cysteamine-induced radioactivity depletion (see above Figure), a parameter needed to determine accurately the effect of system c candidate silencing or overexpression in subsequent experiments. For these reasons, the whole cell approach was definitely abandoned.

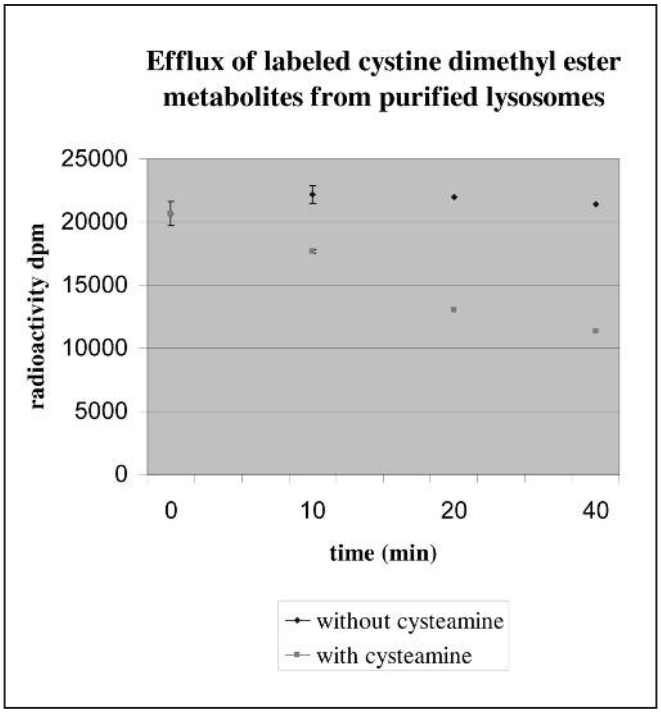
2. Studies on purified lysosomes

To overcome these difficulties, we decided to simplify our assay and measure cystine depletion on purified lysosomes. Because higher amounts and purer lysosomes can be purified from tissue rather than cultured cells, this approach was initiated using mouse liver as starting material. The purification protocol was defined after a 3-day stay of C. Anne in the laboratory of Prof. M. Jadot in Namur (Belgium), an known expert in lysosome purification. The figure bellow illustrates the enrichment of a lysosomal marker, cathepsin D, during a representative purification made in Paris.



We then used these purified lysosomes to assay the cystine-depleting effect of cysteamine by radiotracer flux experiments. Lysosomes were incubated with [G-^{3}H]cystine dimethylester for 60 min, washed, and the radioactivity egress was measured in the presence or absence of 1 mM cysteamine using a filtration assay. It should be mentioned that we used liver tissue from wildtype mice or rats in this set of experiments. However, the concentration of [G-^{3}H]cystine dimethylester was adjusted to overload endogenous cystinosin without triggering an osmotic burden to the lysosome (cleavable permeant esters are sometimes used to disrupt lysosomes).

Representative data are shown below for an experiment performed on wild-type mouse liver lysosomes. As illustrated, in the absence of cysteamine, the amount of radioactivity present in lysosomes remained stable over a time span of 40 min whereas, in contrast, the presence of cysteamine induced a clear efflux of radioactivity with a half-life of ~15 min. After longer incubations, cysteamine depleted radioactivity with a maximal extent of 50% the initial amount (not shown), presumably because the [^{3}H]methanol generated in the lysosome by cleavage of the methyl ester bonds (and representing half of all H atoms) remained associated with the lysosomal membrane in our experimental conditions.



NEXT OBJECTIVES

We now have a clear, and reproducible (not shown), biochemical assay to measure the cystinedepleting effect of cysteamine. Our next goal is to repeat to apply this assay on lysosomes purified from CAT-defective mice to identify the transporter involved in this process. Because the above assay requires a high number (20) of 12-week old mice for a single experiment, we are currently trying to scale down our protocol.

PROGRESS REPORT MAINZ GROUP

Persons working on the project:

(Ellen Closs, Jean-Paul Boissel and Isabel Jaenecke)

In the third grant period, we

- further characterized the transport properties of the chimera between the human cationic amino acid transporter hCAT-2A and the putative lysosomal "system c" transporter SLC7A14, referred to as hCAT2/A14-BK (see scheme in Fig. 1).
- investigated the subcellular distribution of the chimera
- made new constructs to localize protein regions responsible for lysosomal localization
- made new constructs to try to force the protein into the plasma membrane

Characterization of the transport properties of the hCAT2/A14-BK chimera

Our preliminary results had shown that the hCAT2/A14-BK chimera (Fig. 1), containing the domain of SLC7A14 that corresponds to the so called "functional domain" of the CAT proteins, exhibits transport properties similar to the lysosomal transport system c in human skin fibroblasts. In addition, we found that SLC7A14 is prominently expressed in these cells (data not shown). This supports the notion that SLC7A14 may indeed represent the lysosomal system c.

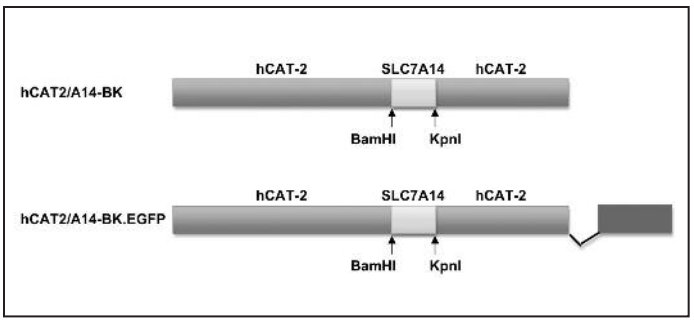


Figure 1. ***Scheme of the chimera between hCAT-2 and SLC7A14.*** *The upper scheme shows the protein domain of SLC7A14 (red) introduced into the hCAT-2A backbone (blue). The lower scheme shows the same chimera fused to EGFP at its C-terminus.*

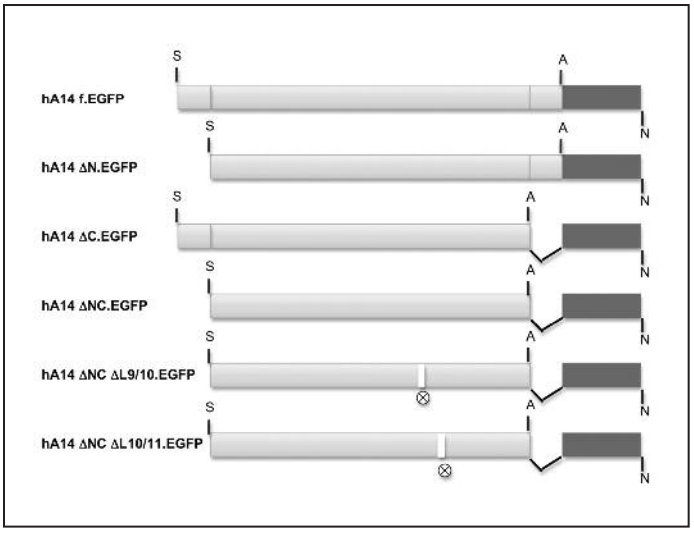


Figure 2. ***Schemes of SLC7A14-EGFP-fusion proteins to be tested.*** *The schemes show fusion proteins between EGFP (green) and SLC7A14 (red) with truncated N- and C-termini as well as partial deletion of two large loops between predicted transmembrane domains 9/10 and 10/11 as indicated. These will be tested for plasma membrane localization and transport activity.*

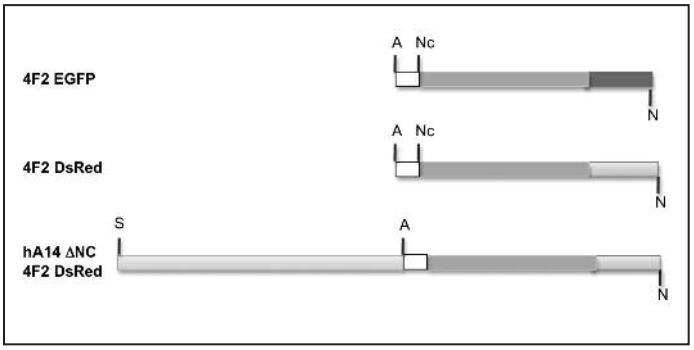


Figure 3. ***Schemes of SLC7A14-4F2-EGFP-fusion proteins to be tested.*** *The schemes show fusion proteins between the membrane protein 4F2 (CD98, gray) and either EGFP (green) or DSRed (dark red) that will be used as marker for membrane localization. A fusion protein between SLC7A14 with truncated N- and C-termini (red), 4F2 (gray) and DsRed (dark red) was constructed to try to force SLC7A14 into the plasma membrane.*

Further experiments revealed the following transport properties of the chimera:

1) In agreement with system c, the chimera exhibited a high apparent KM for L-arginine that was similar to the apparent KM of hCAT-2A and significantly higher than the apparent KM of hCAT-1.

2) Transport by the chimera was moderately trans-stimulated, in contrast to hCAT-2A which showed no trans-stimulation and hCAT-1 which exhibited a stronger trans-stimulation.

3) Transport by the chimera was pH-dependent, similar to hCAT-2A and different from hCAT-1, which was pH-independent. We further showed, that the corresponding chimeras hCAT-2/1 and hCAT-1/2A (containing the "functional domain" of hCAT-1 in the hCAT-2 Progress Report Mainz Group backbone and vice versa) exhibit pH-independence and -dependence, respectively. This provides evidence that the pH-dependence is indeed determined by the "functional domain". SLC7A14 is thus likely to be also pH-dependent. As discussed by Pisoni et al. (1), the observed pH-dependence (lower activity at pH 5 compared to pH 7.5) points rather to a function of SLC7A14 as an import then an export transporter for cationic amino acids in lysosomes. We wondered if the pH-dependence might be altered in the absence of transsubstrate. However, in efflux experiments, the pH-dependence of the chimera as well as of hCAT-1 and hCAT-2A were the same in the absence or presence of trans-substrate.

4) Pisoni et al. identified cationic amino acid derivatives that interfere with transport by system c, but not sytem y^+ (1). In accordance with these observations, transport of the chimera, but not of hCAT-1, hCAT-2B and hCAT-3 (the system y^+ transporter expressed in skin fibroblasts) was inhibited by ϵ–trimethyl-L-lysine. However, the inhibition of the chimera was less pronounced than reported for system c (34% versus 50%). Transport by hCAT-2A (which is not expressed in skin fibroblasts) was inhibited to a similar extend as the chimera. The strongest inhibitor of system c identified by Pisoni et al. (1), α-N-methyl-Larginine (synthesized for our experiments by Tocris Bioscience, Bristol, UK), did not inhibit the chimera nor CAT-1 or CAT-2A. Further experiments are necessary to find out if inhibition by α-N-methyl-L-arginine occurs at a protein region outside the functional domain.

Protein expression and plasma membrane localization of the hCAT2/A14-BK chimera

We found that the transport activity of the chimera was very variable in different experiments. Thus, the expression period (between cRNA injection and experiment) was extended to 3 days, instead of 2 days usually used for the hCAT proteins. To assess the amount of total and cell surface protein expressed in the oocytes, EGFP was fused to the C-terminus of the chimera (see Fig. 1) and the expression of this fusion protein was compared to the expression of a corresponding hCAT-2A.EGFP fusion protein. Fluorescent micrographs showed exclusive plasma membrane localization of both fusion proteins. In addition, Western blot analysis of total and biotinylated cell surface proteins revealed similar expression of both fusion proteins in the plasma membrane.

Protein regions responsible for lysosomal localization

Our preliminary results showed that the hCAT-2/SLC7A14-BK chimera was the only chimera that mediated transport of cationic amino acids across the plasma membrane of oocytes. To assess protein expression and plasma membrane localization, a series of EGFP fusion proteins was constructed that will be characterized in the next funding period (Fig. 2). These include SLC7A14 truncated at the N- and C-termini (which are extended compared to the hCAT proteins), as well as partial deletion of two large loops between predicted transmembrane domains 9/10 and 10/11 (to mimic the structural architecture of hCAT-2A,and the other CAT proteins). Furthermore, fusion constructs between SLC7A14, 4F2, and DsRed were constructed to try to force plasma membrane targeting of SLC7A14 (Fig. 3).

RESEARCH FOCUS FOR THE NEXT 6 MONTHS:

Characterization of new SLC7A14-EGFP and SLC7A14-4F2-EGFP fusion proteins

The new fusion proteins will be tested in the oocyte expression system for membrane localization (using either fluorescent microscopy or biotinylation of surface proteins) and transport activity.

Measurements of lysosomal transport

In collaboration with the group of Bruno Gasnier in Paris, we are in the process to set up a protocol to measure lysosomal transport directly. Using human cell lines with stable overexpression of SLC7A14.EGFP, this will enable us to study the function of the protein in is native environment.

REFERENCE

1. Pisoni, R.L., J.G. Thoene, R.M. Lemons, and H.N. Christensen. 1987. Important differences in cationic amino acid transport by lysosomal system c and system y+ of the human fibroblast. *J Biol Chem* 262:15011-15018.

FINAL REPORT

TAOSHENG HUANG, MD, PHD, MENTOR
SHA TANG, PHD, RESEARCH FELLOW

"Molecular and Pathogenesis Study of Cystinosis"

Date: 6/04/09

HYPOTHESIS

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

ACCOMPLISHED WORK

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

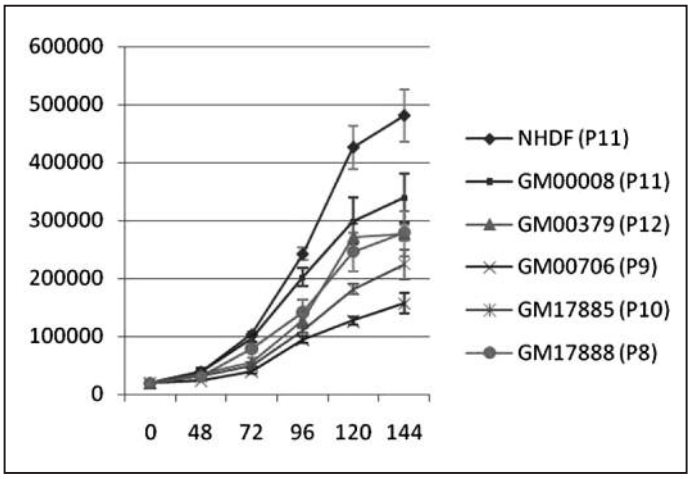
1. Cell cultures and intracellular cystine levels:

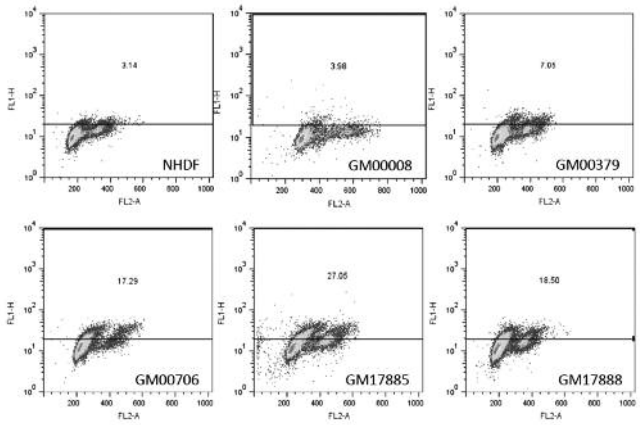
Cultures of normal and cystinotic human fibroblasts were obtained from the Coriell Cell Repositories and are maintained in the laboratory. The cystinotic lines represent different genotypes and total intracellular cystine levels of these fibroblasts were determined in Dr. Schneider's laboratory at UCSD.

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

2. Apoptosis /cell cycle analysis:

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





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

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

3. Gene expression analysis:

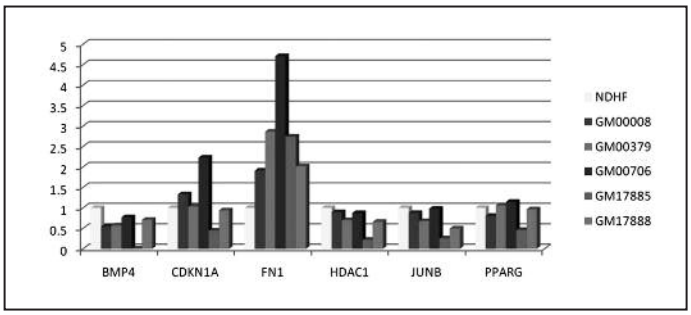
To investigate if cystine accumulation in the lysosomes affects gene expression levels in cystinosis, we used Affymetrix Human Genome U133 Plus 2.0 Array to identify differentially expressed genes in cystinotic cell. We used cystinotic GM00706 (homozygous for 57 kb deletion) and NHDF as control. These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

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

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

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

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



4. Redox status and energy metabolism analysis:

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

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

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

5. Problems and updates:

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

Molecular Analysis of CTNS Mutations from an Indian Cystinotic Boy

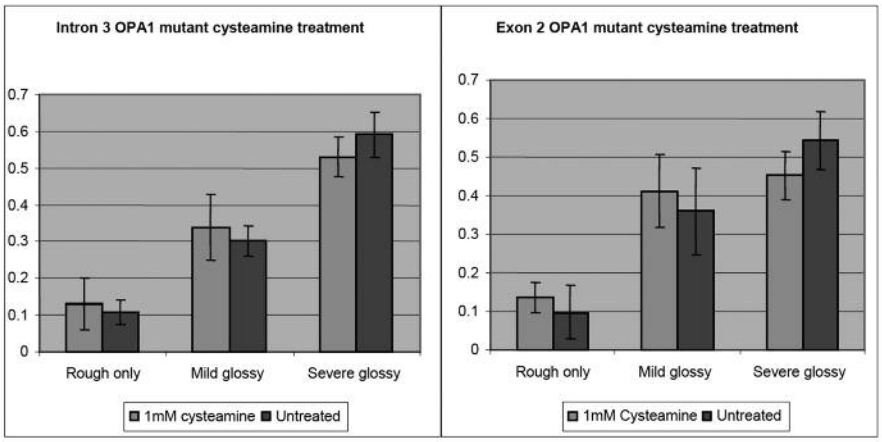
We performed the first molecular characterization of cystinotic patients from India. The proband was born to consanguineous parents and has an older brother sibling dying with a diagnosis of cystinosis. The proband also presents with certain phenotypes not associated with cystinosis previously. The boy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

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

We demonstrated that heterozygous mutation in Optic atrophy 1 (OPA1), a mitochondrial protein, resulted in shortened lifespan, increased susceptibility to oxidative stress and elevated production of Reactive Oxygen Species (ROS) in *Drosophila.* Heterozygous *dOpa*1 mutation also caused an impairment of respiratory chain complex activities, especially complexes II and III, and reversible decreased aconitase activity. A manuscript summarizing the findings has been accepted by PloS ONE (http://www.plosone.org/article/info:doi/10.1371/journal.pone.0004492) and CRF is acknowledged for supporting the work. Although these data are not directly related to cystinosis research, through the OPA1 work we now have the expertise in the techniques necessary for our proposed mitochondrial dysfunction-cystinosis correlation study. These important methods include mitochondrial respiration analysis, mitochondrial complex enzymatic activity measurements, ROS generation characterization and mitochondrial aconitase activity assays.

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

OPA1 is a dynamin-like GTPase located in the inner mitochondrial membrane and mutations in OPA1 are associated with autosomal dominant optic atrophy (DOA). In our Drosophila model, homozygous mutation of dOpa1 in the eyes (large clone) caused rough (mispatterning) and glossy (decreased lens and pigment deposition) eye phenotype. Cysteamine, the FDA-approved drug for standard treatment of cystinosis, has neuroprotective effects and is also in clinical trials for therapy of Huntington's disease. Cysteamine has been shown to increase the level of BDNF in treated striatal nerve cells and prevent mitochondrial depolarization. We tested the effects of cysteamine to rescue the eye phenotype of large clone progenies. As shown in the figure, treatment of cysteamine (1 mM) did not result in significant improvements in either the glossy or rough eye phenotypes.



PROGRESS REPORT

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

"Gene Transfer Studies for Cystinosis"

Date: 7/24/09

INITIAL SPECIFIC AIMS

1. Generate a HD CAV-2 vector containing a CTNS-IRES-GFP cassette.

2. Optimise ocular injections *in vivo* to reach the corneal stroma of *Ctns-/-* mice using ΔE1ΔE3 CAV-2 and AAV8 vectors expressing GFP. Subsequently, perform ocular injections with *CTNS*-expressing HD CAV-2 vectors and AAV8 vectors to test for phenotypic correction in the cornea.

3. Refine the characterisation of the CNS anomalies in *Ctns-/-* mice at the cellular level by fluorescenceactivated cell sorting (FACS) of different CNS cell types and assaying their respective cystine levels. The long-term goal of this project is to evaluate the feasibility of *CTNS* gene transfer to the CNS to prevent deterioration of this organ.

ABBREVIATIONS

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

1. GENERATION OF A HD CAV-2 VECTOR CONTAINING A CTNS-IRES-GFP CASSETTE

Background:

At the time of my fellowship application, we had optimised and finished the production of a control helper-dependent (HD) CAV-2 vector (devoid of all viral genes) containing a GFP cassette (HD CAV-GFP) and were ready to begin the production of a HD CAV-2 vector containing a CTNS-IRES-GFP cassette (HD CAV-CIG).

Results:

Sandy Ibanes (research assistant) and I successfully produced HD CAV-CIG land verified that the expression cassette was functional *in vitro*: we detected both cystinosin and GFP expression by immunoand epifluorescence studies of transduced cells as well as by western blot analyses, and reduced cystine levels by 70% in *CTNS-/-* fibroblasts. However production of HD CAV-CIG was not straightforward. The size of HD CAV-CIG (32 kb) is similar to that of the helper vector (33 kb) thus precluding an efficient separation between the two vectors by cesium chloride (CsCl) gradient. Thus, there was a high percentage of helper contamination in our first stock of HD CAV-CIG. To address this technical problem, we reproduced HD CAVCIG by varying the centrifugation times to improve the separation from the helper vector. After production, we estimated a helper contamination of ~6% by quantitative PCR (qPCR). This contamination rate, although acceptable for *in vivo* experiments in mice, needed to be further reduced.

Thus, I returned to our initial 32-kb HD CAV-CIG plasmid to remove 2 kb of sequence by restriction enzyme digestion. Our rationale was that this smaller vector (which I will refer to as HD CAV-CIG-30 kb) would separate more efficiently from the helper than HD CAV-CIG-32 kb. In parallel, Sandy produced a new helper vector (CAV-attB-Cherry) that has a delayed packaging time as compared to the HD vector, which should thus decrease the rate of helper contamination. Furthermore, CAV-attB-Cherry expresses a red fluorescent protein, as opposed to the original helper that expressed a non-fluorescent protein, which will allow us to more efficiently sort and collect cells, thus further minimising the rate of helper contamination. We established the fluorescence-activated cell sorting (FACS) parameters necessary to isolate a double population of red- and green-positive cells. Sandy is currently preparing a second stock of CAV-attB-Cherry, which should be finished within a week at which point we will begin production of HD CAV-CIG-30 kb.

2. *IN VIVO* CORNEAL-TARGETED GENE TRANSFER STUDIES

Background:

My gene transfer studies using E1/E3-deleted (ΔE1ΔE3) adenovirus vectors to the liver provided the proof-of-concept that viral vector-mediated gene transfer could reduce lysosomal cystine levels *in vivo*. My next goal was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis, using the more stable HD CAV and AAV viral vectors.

Results:

Nicolas Serratrice (Ph.D. student) and I showed that intra-stromal injection of ΔE1ΔE3 CAV-2 as well as HD CAV-2 vectors *ex vivo* in human cornea and *in vivo* in mouse cornea resulted in a strong transgene expression from 24 h, which was short-lived (4 weeks *ex vivo*, 2 weeks *in vivo*). Thus we think that the nonintegrating CAV-2 vectors are likely eliminated from the cornea due to an apoptosis/proliferation repair mechanism following injection.

In contrast, my results with the AAV8 vectors are more encouraging as well as intriguing. I injected mouse corneas with AAV-GFP and followed expression by *in vivo* microscopy and histological studies:

i) Forty-eight h after AAV-GFP injection, I detected expression in the corneal epithelium, which disappeared by 1 wk post-injection (p.i.). I began to see GFP expression in the corneal stroma around 4 wk p.i., which persisted until at least 6 mo p.i. (longest time-point tested). Like CAV-2, AAV vectors are theoretically "non-integrating" thus we don't know why they are able to escape the fate of CAV-2 vectors following corneal repair.

ii) I made the interesting observation that if I re-injected mouse corneas with PBS 1 wk after the initial AAV-GFP injection (i.e. when GFP is barely expressed), I provoked GFP expression in the stroma. This expression rapidly decreased after 24 h. The same kinetics was observed when I re-injected with PBS 1 mo after the initial AAV-GFP injection (i.e. when GFP is already expressed). I am currently performing qPCR experiments on injected corneas to determine whether PBS re-injection results in an increase in genome copies or mRNA expression.

iii) I performed similar experiments with AAV-CIG. In a first experiment, I assayed cystine levels in *Ctns-/-* mice 2.5 mo after AAV-CIG injection but did not detect a reduction in cystine levels. However, I did not detect a significant GFP expression in this experiment so it is possible that *CTNS* gene expression was also too low to allow cystine clearance. I am currently repeating these experiments. To increase transgene expression levels, I assayed cystine levels in mice that I re-injected with PBS 1 wk after the initial AAV injection. I detected a 50% decrease in cystine levels but with both AAV-GFP and AAV-CIG. Thus, for an as yet unknown reason, injection of AAV caused a non-specific decrease in cystine levels. Finally, I will repeat this experiment (i.e. re-injection PBS 1 wk after AAV injection) but this time we will assay cystine levels 3 weeks later). In this way, I will test whether the non-specific reduction due to AAV-GFP disappears to reveal a specific *CTNS* effect similar to my recent observations in the liver (Hippert *et al.* 2008 Mol. Ther.).

Finally, to complement this study, I will compare the tropism of three other AAV vectors, serotypes AAV1, -2 and -5, to AAV8 in human corneas. In this way, I will evaluate the best vector for long-term expression following intra-stromal injection.

3. REFINE CHARACTERISATION OF THE CNS ANOMALIES IN *CTNS-/-* MICE

Background:

Our previous work suggested that *Ctns-/-* mice have age-related learning and memory defects likely due to cystine accumulation in the hippocampus. Having identified the brain regions affected, the next step is to identify the cell type(s) as this will also dictate the choice of vector for subsequent gene transfer studies. Our strategy was to dissociate the brain, label individual cell types with fluorescent-labelled cell markers, isolate these cells via FACS, and assay each cell type for their respective cystine levels.

Results:

I first followed our initial strategy of isolating the different cell types by FACS to assay cystine levels but this resulted in two major problems: the lack of specificity of the antibodies and the recovery of only a small number of cells precluding a cystine assay. I tried using an Optiprep gradient to isolate cell types into different fractions, which were then individually collected. Lastly, I performed multiple technical modifications to improve the purity of the fractions. My preliminary results indicate that the microglia have the highest cystine content; the microglia are the resident macrophages of the brain. These results are thus consistent with our previous observations (Hippert *et al.* 2008) where we showed that the Kupffer cells, the macrophages of the liver, have the highest cystine content in this tissue. The high cystine levels in *Ctns-/-* macrophages are likely due to the high metabolic activity of this cell type.

Finally, in parallel, I have been performing stereotaxic injections in the brain of wild-type mice to determine the correct coordinates to consistently reach the hippocampus. My next goal is to inject HD CAVCIG (targets neurons) and AAV8-CIG (targets glial cells) in *Ctns-/-* mice and assay cystine levels posttransduction.

PROGRESS REPORT

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

"Gene Transfer Studies for Cystinosis"

Date: 7/24/09

INITIAL SPECIFIC AIMS

1. Generate a HD CAV-2 vector containing a CTNS-IRES-GFP cassette.
2. Optimise ocular injections *in vivo* to reach the corneal stroma of *Ctns-/-* mice using ΔE1ΔE3 CAV-2 and AAV8 vectors expressing GFP. Subsequently, perform ocular injections with *CTNS*-expressing HD CAV-2 vectors and AAV8 vectors to test for phenotypic correction in the cornea.
3. Refine the characterisation of the CNS anomalies in *Ctns-/-* mice at the cellular level by fluorescenceactivated cell sorting (FACS) of different CNS cell types and assaying their respective cystine levels. The long-term goal of this project is to evaluate the feasibility of *CTNS* gene transfer to the CNS to prevent deterioration of this organ.
4. Begin kidney gene transfer using novel, recently characterised, kidney-tropic Ad vectors.

1. GENERATION OF A HD CAV-2 VECTOR CONTAINING A CTNS-IRES-GFP CASSETTE

Background:

At the time of our grant application, we had optimised and finished the production of a control helperdependent (HD) CAV-2 vector (devoid of all viral genes) containing a GFP cassette (HD CAV-GFP). This viral stock was of high titre and the contamination with the helper vector, which provides the viral proteins necessary for HD production in trans, was < 0.01%. We showed that HD CAV-GFP was functional in vitro and in vivo. Following this technical advancement, we were ready to begin the production of a HD CAV-2 vector containing a CTNS-IRES-GFP cassette (HD CAV-CIG).

Results:

We successfully produced HD CAV-CIG and verified that the expression cassette was functional *in vitro:* we detected both cystinosin and GFP expression by immuno- and epifluorescence studies of transduced cells as well as by western blot analyses, and reduced cystine levels by 70% in CTNS-/- fibroblasts. However production of HD CAV-CIG was less straightforward than that of HD CAV-GFP. The size of HD CAV-CIG (32 kb) is similar to that of the helper vector (33 kb) thus precluding an efficient separation between the two vectors by cesium chloride (CsCl) gradient (Fig. 1). By comparison, the size of HD CAV-GFP is 30 kb, which allows a better separation from the helper and hence resulted in the low contamination rate. Thus, there was a high percentage of helper contamination in our first stock of HD CAVCIG. To address this technical problem, we reproduced HD CAV-CIG by varying the centrifugation times to improve the separation from the helper vector. After production, we estimated a helper contamination of ~6% by quantitative PCR (qPCR). This contamination rate, although acceptable for *in vivo* experiments in mice, needed to be further reduced.

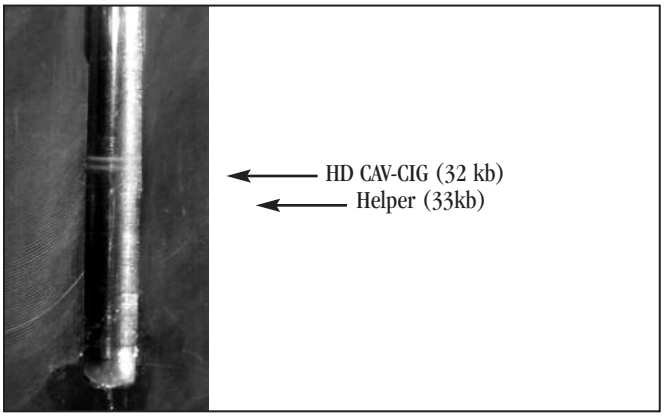


Figure 1: ***CsCl separation of HD CAV-CIG from the helper vector.*** *The banded vectors are too close to allow retrieval of only HD CAV-CIG.*

Thus, we returned to our initial 32-kb HD CAV-CIG plasmid to remove 2 kb of sequence by restriction enzyme digestion. Our rationale was that this smaller vector (which I will refer to as HD CAV-CIG-30 kb) would separate more efficiently from the helper than HD CAV-CIG-32 kb. In parallel, we produced a new helper vector (CAV-attB-Cherry) that has a delayed packaging time as compared to the HD vector, which Figure 1: CsCl separation of HD CAV-CIG from the helper vector. The banded vectors are too close to allow retrieval of only HD CAV-CIG. HD CAV-CIG (32 kb) Helper (33 kb) PI: V. Kalatzis 4 should thus decrease the rate of helper contamination. Furthermore, CAV-attB-Cherry expresses a red fluorescent protein, as opposed to the original helper that expressed a non-fluorescent protein, which will allow us to more efficiently sort and collect cells, thus further minimising the rate of helper contamination. We established the fluorescence-activated cell sorting (FACS) parameters necessary to isolate a double population of red- and green-positive cells (Fig. 2). We are currently preparing a second stock of CAV-attBCherry, which should be finished within a week at which point we will begin production of HD CAV-CIG-30 kb.

2. *IN VIVO* CORNEAL-TARGETED GENE TRANSFER STUDIES

Background:

Our gene transfer studies using E1/E3-deleted (ΔE1ΔE3) adenovirus vectors to the liver provided theproof-of-concept that viral vector-mediated gene transfer could reduce lysosomal cystine levels in vivo. Our next goal was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis, using the more stable HD CAV and AAV viral vectors.

Results:

Our first results showed that intra-stromal injection of ΔE1ΔE3 CAV-2 as well as HD CAV-2 vectors *ex vivo* in human cornea and *in vivo* in mouse cornea resulted in a strong transgene expression from 24 h, which was short-lived (4 weeks *ex vivo*, 2 weeks *in vivo*). Thus we think that the non-integrating CAV-2 vectors are likely eliminated from the cornea due to an apoptosis/ proliferation repair mechanism following injection.

In contrast, our results with the AAV8 vectors are more encouraging as well as intriguing. We injected mouse corneas with AAV-GFP and followed expression by in vivo microscopy and histological studies:

i) Forty-eight h after AAV-GFP injection, we detected expression in the corneal epithelium likely due to the needle traversing this tissue (Figs. 3A and B). This expression disappeared by 1 wk post-injection (p.i.), probably due to the 7-day turnover of this tissue. We began to see GFP expression in the corneal stroma around 4 wk p.i. (data not shown) This expression persists until at least 6 mo p.i. (longest time point tested to date; Figs. 3C and D). Like CAV-2, AAV vectors are theoretically "non-integrating" thus we don't know why they are able to escape the fate of CAV-2 vectors following corneal repair. It is possible that the AAV vector genome i) integrates into stromal keratocytes, ii) remains encapsidated and capsid disassembly only occurs after cell division or iii) extra-chromosomally forms >50 kb concatamers that are not lost during cell division.

ii) We made the interesting observation that if we re-injected mouse corneas with PBS 1 wk after the initial AAV-GFP injection (i.e. when GFP is barely expressed; Figs. 4A and B), we provoked GFP expression in the stroma (Figs. 4C and D). This expression rapidly decreased after 24 h. The same kinetics was observed when we re-injected with PBS 1 mo after the initial AAV-GFP injection (i.e. when GFP is already expressed). We are currently performing qPCR experiments on injected corneas to determine whether PBS re-injection results in an increase in genome copies or mRNA expression.

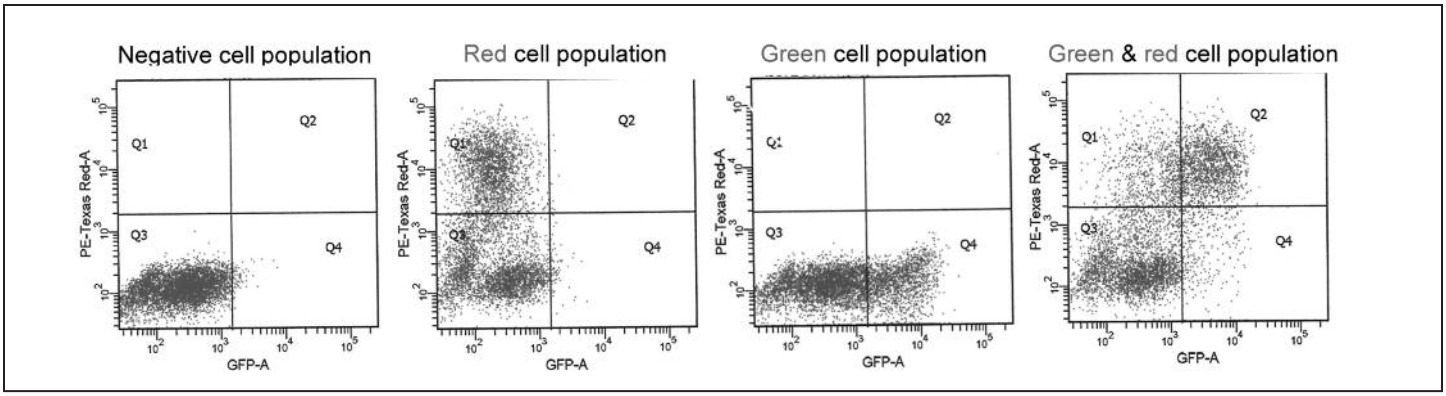


Figure 2: ***Establishing FACS parameters.*** *Cells were incubated with the HD CAV-CIG-32 kb and the helper CAV-attB-Cherry vectors.* ***Panel 1:*** *Control cell population that shows no green or red fluorescence (Quadrant (Q3).* ***Panel 2:*** *Cells transduced with only CAV-attB-Cherry and showing red fluorescence (Q1).* ***Panel 3:*** *Cells transduced with only HD CAV-CIG-32 kb and showing green fluorescence (Q4). Panel 4: Cells simultaneously transduced with HD CAV-CIG-32 kb and CAV-attB-Cherry showing green and red fluorescence (Q2). During production, it is this double-positive cell population that is collected and amplified to generate the corresponding vector stock.*

iii) We performed similar experiments with AAV-CIG and detected the same tropism and profile of GFP expression. In a first experiment, we assayed cystine levels in Ctns-/- mice 2.5 mo after AAV-CIG injection. We did not detect a reduction in cystine levels. However, we did not detect a significant GFP expression in this experiment so it is possible that CTNS gene expression was also too low to allow cystine clearance. We are currently repeating these experiments. To increase transgene expression levels, we assayed cystine levels in mice that were re-injected with PBS 1 wk after the initial AAV injection. We detected a 50% decrease in cystine levels but with both AAV-GFP and AAV-CIG (Fig. 5). Thus, for an as yet unknown reason, injection of AAV caused a non-specific decrease in cystine levels. Finally, we will repeat this experiment (i.e. re-injection PBS 1 wk after AAV injection) but this time we will assay cystine levels 3 weeks later). In this way, we will test whether the non-specific reduction due to AAV-GFP disappears to reveal a specific CTNS effect similar to our recent observations in the liver (Hippert et al. 2008 Mol. Ther. 16: 1372-81).

Finally, to complement this study, we will compare the tropism of three other AAV vectors, serotypes AAV1, -2 and -5, to AAV8 in human corneas. In this way, we will evaluate the best vector for long-term expression following intra-stromal injection. Our rationale is as follows: if injection into the cornea allows gene expression for 1 yr (or more), then corneal gene therapy may be a feasible alternative to the application of cysteamine eye drops every waking hour.

3. REFINE CHARACTERISATION OF THE CNS ANOMALIES IN *CTNS-/-* MICE

Background:

Our previous work suggested that Ctns-/- mice have age-related learning and memory defects likely due to cystine accumulation in the hippocampus. Having identified the brain regions affected, the next step is to identify the cell type(s) as this will also dictate the choice of vector for subsequent gene transfer studies. Our strategy was to dissociate the brain, label individual cell types with fluorescent-labelled cell markers, isolate these cells via FACS, and assay each cell type for their respective cystine levels.

Results:

This project is challenging. We first followed our initial strategy of isolating the different cell types by FACS to assay cystine levels but this resulted in two major problems: the lack of specificity of the antibodies and the recovery of only a small number of cells precluding a cystine assay. We tried using an Optiprep gradient to isolate cell types into different fractions, which were then individually collected. We performed multiple technical modifications to improve the purity

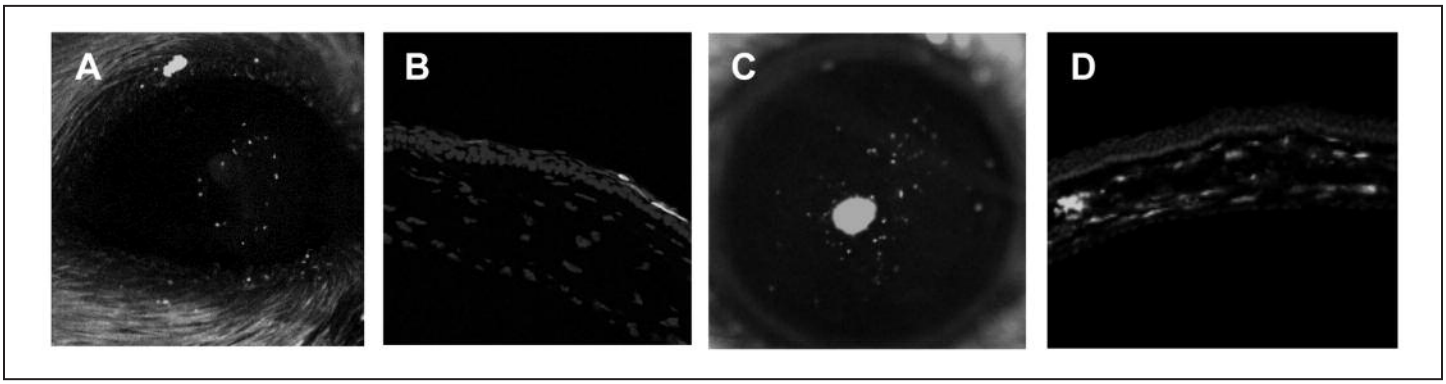


Figure 3: ***GFP expression following intra-stromal AAV-GFP injection by in vivo microscopy (A and C) and histological studies (B and D). (A and B).*** *GFP expression is observed in the corneal epithelium 48 h p.i. (C andD) GFP expression persists in the corneal stroma 6 mo p.i.*

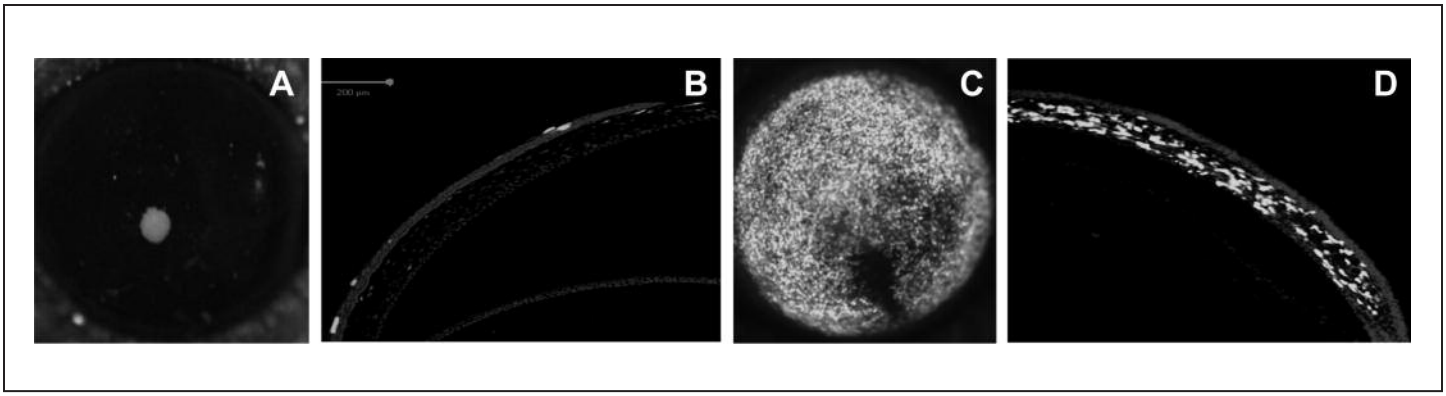


Figure 4: ***GFP expression from AAV-GFP without PBS re-injection (panels A and B) or 24 h after PBS reinjection (panels C and D).*** *When the mouse eye is not re-injected with PBS, a faint GFP expression is observed 1 wk post-AAV-GFP injection by* in vivo *microscopy (A) that is localised to both the corneal epithelium and stroma on histological sections (B). In contrast, PBS re-injection results in a strong GFP expression throughout the corneal stroma as seen by in vivo microscopy (C), which is confirmed by histological analysis (D).*

of the fractions. Our preliminary results indicate that the microglia have the highest cystine content; the microglia are the resident macrophages of the brain. These results are thus consistent with our previous observations (Hippert et al. 2008) where we showed that the Kupffer cells, the macrophages of the liver, have the highest cystine content in this tissue. The high cystine levels in Ctns-/- macrophages are likely due to the high metabolic activity of this cell type.

Finally, in parallel, we have been performing stereotaxic injections in the brain of wild-type mice to determine the correct coordinates to consistently reach the hippocampus. Our next goal is to inject HD CAVCIG (targets neurons) and AAV8-CIG (targets glial cells) in Ctns-/- mice and assay cystine levels posttransduction. This work should validate our in vitro data as to the cell type most affected in cystinosis.

4. BEGIN KIDNEY GENE TRANSFER EXPERIMENTS

Background:

Until now we have been reticent to perform kidney gene transfer studies as there were no suitable vector systems for non-invasive delivery to this organ. Further complicating the matter is that for cystinosis, renal gene delivery should reach both the proximal tubule epithelium and the glomeruli. Although human adenovirus (hAd) vectors transduce these tissues, systemic administration has been difficult due to sequestration by the liver.

Results:

To circumvent sequestration of adenoviruses by the liver following systemic administration, we tried a liver-bypass technique for direct renal delivery. This technique involves clamping of the hepatic triad (comprising the hepatic vein, hepatic artery and portal vein) for 30 min, which temporarily prevents blood from reaching the liver without clinical consequences. During clamping, adenovirus vectors are administered into the circulation via injection in the retro-orbital venous plexus. As a pilot experiment, we used a hAd vector expressing GFP (hAd-GFP). When we injected 5 x 10^{10} pp of hAd-GFP in non-clamped mice we detect a strong GFP expression in the liver but not in the lung, pancreas and kidney. In contrast, in clamped mice, we detected GFP expression in the lung and pancreas but we did not detect GFP expression in the kidney. Administration of a ten-fold higher quantity of Ad-GFP (5 x 10^{11} pp) resulted in a low GFP expression in kidney, mainly located in the glomeruli. Transduction with AAV-GFP did not result in GFP expression in any tissue, most likely due to the lower titre of this vector, which limits the amount of particles in relation to the maximum volume that can be administered. As we don't feel that the level of kidney transduction with hAd-GFP is sufficient to restore a cellular phenotype, we are currently looking into other routes of administration to more efficiently transduce this structure.

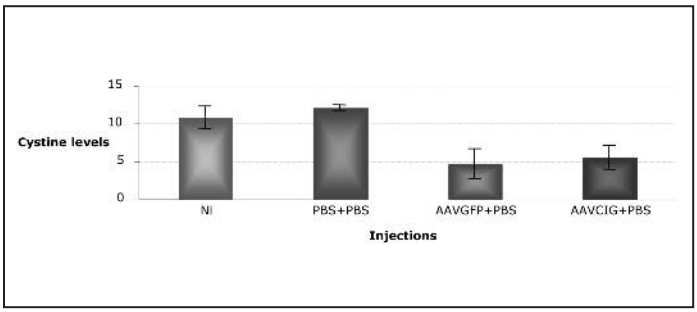


Figure 5: ***Assay of cystine levels 24 h after PBS re-injection.*** *AAV injection followed by PBS re-injection caused a 50% decrease in cystine levels (expressed in nmol half-cystine/mg protein), irrespective of transgene, when compared to non-injected or PBS-injected controls.*

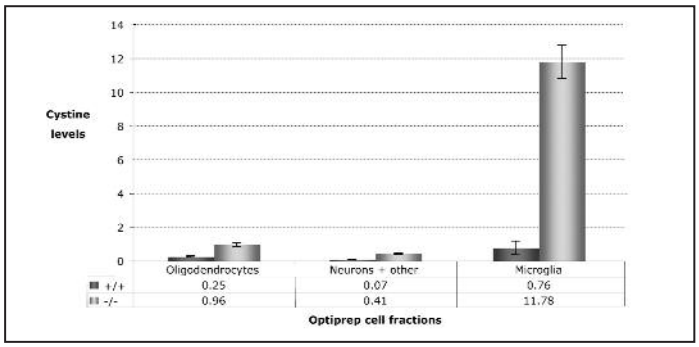


Figure 5: ***Assay of cystine levels in cell types of the brain of wild-type and Ctns-/- mice.*** *The microglia have the highest cystine levels, followed by the oligodendrocytes and, lastly, neurons.*

PROGRESS REPORT

ELENA LEVTCHENKO, MD, PHD, PRINCIPAL INVESTIGATOR

"Unraveling the Mechanisms of Cysteamine Toxicity in Patients with Cystinosis"

Date: 11/9/09

Background

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

Specific aims

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

Patient	Sex	GFR (mL/min/ 1.73 m^2)	CTNS mutation	Age at onset cystinosis treatment	Concomitant medications	
1	Male	87	Unknown	2 years, 1 month	L-thyroxine	Indomethacin
					L-carnitine	Somatotropin
					Potassium citrate	
2	Male	161	Unknown	1 year, 1 month	Unknown	
3	Female	25	Unknown	9 months	Potassium	Somatotropin
					Folic acid	Riboflavin (Vit B2)
					Iron	Pyridoxine (Vit B6)
					Epoietin beta	
4	Male	62	c.18-21 del GACT c.18-21 del GACT	11 months	L-thyroxine	Somatotropin
					L-carnitine	Calcitriol
					Sodium chloride	Epoietin beta
					Phosphate	Folic acid
					Sodium bicarbonate citrate	Hydrochlorothiazide
5	Male	30	Unknown	1 year, 1 month	Phosphate	Citrate
					Potassium chloride	Esomeprazol
					Alphacalcidiol	
6	Male	101	57kb del 57kb del	7 years, 8 months	Alphacalcidol	Somatotropin
					Potassium bicarbonate	Iron
					Sodium potassium phosphate	
7	Male	67	Unknown	2 years, 2 months	L-carnitine	Indomethacin
					Potassium	Alphacalcidiol
					Phosphoric acid	Nicardipine
					Sodium bicarbonate	
8	Male	73	Unknown	9 months	Potassium chloride bicarbonate	Alphacalcidiol
					Sodium bicarbonate	

Table I. Clinical information of all reported patients with Ehlers-Danlos like syndrome

Key-objectives/work plan

A. To collect full information on patients reported with the above-mentioned symptoms.

B. To investigate whether or not there are differences in cysteamine plasma levels between patients with and patients without severe adverse events.

C. To analyze genetic variations in genes involved in classical EDS.

D. To investigate the effects of a range of cysteamine concentrations on different human cell lines.

Results

A. Clinical information was obtained from the patients' physicians and is summarized in table I. A manuscript is currently being drafted.

B. Cysteamine plasma curves were made in 2 of the above-mentioned patients (patient 3 and 6) and 5 patients without similar events after the intake of 15 mg/kg of cysteamine base (figure 1). So far no difference was found between these 2 types of patients.

C. Blood samples were obtained from 3 patients with and 5 patients without Ehlers-Danlos like syndrome . They will be screened for aberrations in the genes involved in classical EDS (COL5A1, COL5A2, COL1A1, COL1A2, TNXB). The DNA and fibroblasts samples of the other patients are required.

D. We investigated the response of human endothelial cells (HUVEC, human umbilical vein endothelial cell line, commercially available at Lonza Group Ltd, Basel, Switzerland) to different concentrations of cysteamine. Cysteamine was added to the medium in concentrations of 0.03 mM, 0.1 mM, 0.3 mM, 1.0 mM, 3.0 mM and 10 mM. One group of control cells received no cysteamine. All these seven concentrations were administered during 1, 6 and 24 hours. After that time, the viability and proliferation of the cells was investigated in order to estimate the effects of cysteamine on normal human endothelial cells using the commercial available WST1 Rapid Cell Proliferation Kit (Clontech). The results are pending.

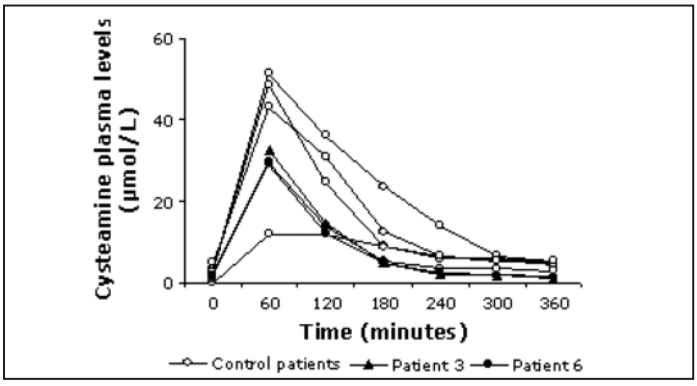


Figure 1: Cysteamine plasma curves of 5 patients without events (control patients) and 2 patients with Ehlers-Danlos like syndrome (patient 3 and 6).

PROGRESS REPORT

ELENA LEVTCHENKO, MD, PHD, LAMBERTUS VAN DEN HEUVEL, PHD

FRANCESCO EMMA, MD, MENTORS

MARTIJN WILMER, RESEARCH FELLOW

"Pathogensis of Renal Disease in Nephropathic Cystinosis"

Date: 12/30/09

Lysosomal cystine accumulation might lead to alterations in the metabolism of antioxidant glutathione (GSH) resulting in decreased cellular capacity to deal with oxidative stress. In response to oxidative stress, cystinotic cells might have increased production of cytokine and chemokines. These mechanism can contribute to the development of interstitial fibrosis. In this project, we aim to study oxidative status and albumin-mediated production of pro-inflammatory and pro-fibrotic substances in a conditionally immortalized proximal tubular cell model (ciPTEC). The influence of different mutations in CTNS, leading to lysosomal cystine accumulation, and the additional deletion of CARKL gene, which is mutually deleted in the common 57kb deletion, are subject of this research project. Additionally, we aim to develop podocytes with the cystinotic genotype to evaluate morphological or metabolic changes.

This 2nd progress report describes the findings of the Fellowship Grant funded by the Cystinosis Research Foundation in the first year. Currently, a scientific paper is in preparation for submission, concerning the data of glutathione and oxidation status.

Aim I Evaluation of albumin mediated cytokine and chemokine production in ciPTEC.

a) Transfection of ciPTEC

Currently, we have extended our cell population to 4 healthy controls and 14 cystinotic patients. Moreover, the population of the cystinotic patients have a diverse genotype as listed in table 1, allowing us to differentiate whether observed alterations are due to mutations of CTNS, CARKL or both.

	cell line	age[1] (months)	sex	mutation
PT1	cys1	108	m	c.922_923insG
PT2	cys2	78	m	hom 57kb del
PT4	cys3	152	m	c.198_218del
PT13	cys4	129	m	hom57kb del
PT23	cys5	82	f	[57kb del]+[c.665A>G]
PT24	cys6	82	f	[57kb del]+[c.665A>G]
PT25	cys7	134	f	ND
PT41	cys8	50	m	hom57kb del
PT46	cys9	47	m	hom57kb del
PT47	cys10		m	[57kb del]+[c.696insC]
PT48	cys11	11	f	[57kb del]+[c.927_928insG]
PT53	cys12	78	f	[57kb del]+[c.del18_21GACT]
PT54	cys13	96	f	[57kb del]+[c.665A>G]
PT55	cys14	209	m	hom57kb del

Table 1: Overview of the ciPTEC cell lines developed from urine of patients with cystinosis. Patients with hom 57kb del have both dysfunctional CTNS and CARKL. ND- mutations in CTNS are not detected..

To address the influence of CTNS and CTNS-LKG (a recently described cystinosin isoform), we have currently succeeded in the transfection of both CTNS and CTNS-LKG isoforms in one cystinosis cell line (PT46.2) carrying the homozygously 57kb deletion. Cystine levels were measured using HPLC (figure 1). Although transfection was confirmed by rtPCR (shown in progress report I, June 2009), cystine levels were only moderately decreased in both cells transfected with CTNS and CTNS-LKG. These assays are currently repeated.

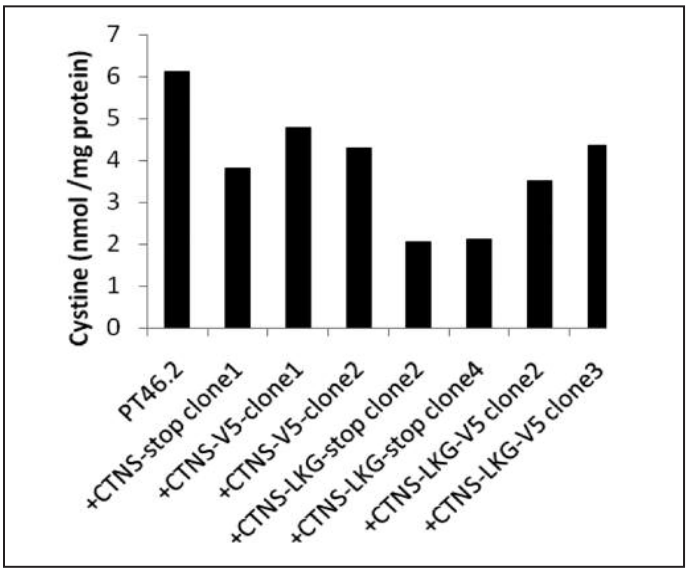


Figure 1. (B) Intracellular cystine levels in ciPTEC after transfection with CTNS or CTNS-LKG measured using HPLC. Used vectors contain either normal stop codon (stop) or V5-tag (V5). Several clones of each construct are obtained and analyzed.

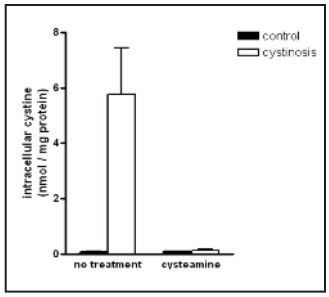
Since cystine levels are moderately decreased and not in the range of levels observed in heterozygous mutated subjects, we are currently evaluating alternative models to study cellular aberrations caused by either mutations in CTNS, CTNS-LKG or CARKL. The current study population as presented in table 1 includes both small mutations in CTNS and large 57kb deletion including CARKL. Using both cell types, we can distinguish effects between mutations only in CTNS versus mutations in both CTNS and CARKL. Alternatively, using siRNA in healthy control cell lines, decreased expression of CTNS, CTNS-LKG or CARKL can be obtained. Furthermore, we will transduce cystinotic cells carrying hom57kb deletion with Lenti viral constructs expressing CTNS, CRNS-LKG or CARKL to establish stable transfected cell lines.

b) Measuring albumin induced production of pro-inflammatory and profibrotic substances in ciPTEC

Pilot experiments indicated that incubation with 1mM cysteamine depleted cystine levels in cystinotic ciPTEC comparable to control levels. Additional experiments revealed that within 8 hours, cystine levels were elevated back to cystinotic levels.

To investigate the long term effect on cystine depletion, we measured cystine and glutathione levels in 4 control and 8 cystinotic ciPTEC (with a homozygous or heterozygous 57kb deletion) after 2 days of treatment with cysteamine, with replacement of the cysteamine containing medium every 6 hours, which is similar to the cysteamine treatment regimen in patients with cystinosis. Using these conditions, cystine levels were in the range of healthy controls (figure 2).

Figure 2. Levels of cystine in cystinosis and control ciPTEC with or without 2 days treatment with cysteamine.



Although we did not observe any significant differences between control and cystinotic levels of total GSH, both in proliferating cystinotic and control cells contain higher GSH levels compared to matured cells ($p<0.05$ in control and $p<0.01$ in cystinotic ciPTEC), indicating cysteamine increases cellular capacity to deal with oxidative stress (figure 3).

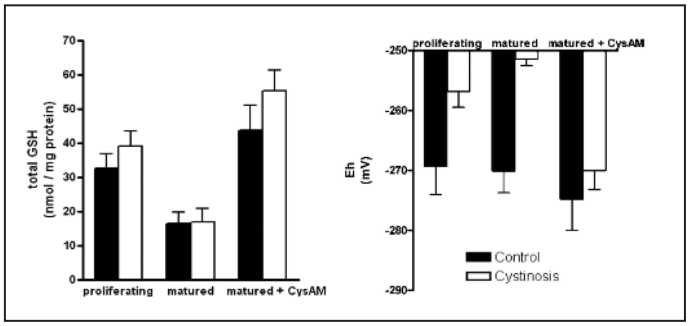


Figure 3. Levels of total GSH were measured using HPLC in cells cultured for 0 (proliferating) or 10 days at 37C (matured). In one set of experiments, cells were treated for 2 days with cysteamine, starting at day 8 of maturation (CysAM; medium was replaced every 6 hours to maintain low cystine levels). Using Nernst equation, redox status was determined by calculating the reduction potential Eh (mV) of the GSSG/2GSH couple. Both in proliferating and matured cystinotic cells, reduction potential was decreased. Upon cysteamine treatment, redox status was comparable to control levels

Despite total GSH levels were not altered in cystinotic ciPTEC, oxidized GSSG levels were significantly increased in proliferating (control 0.26 +/-0.05 vs cystinosis 1.01 +/-0.16; $p<0.01$) and matured (control 0.15 +/-0.04 vs cystinosis 0.66 +/-0.16; $p<0.05$) ciPTEC. Treatment with cysteamine decreased the differences in GSSG levels between control and cystinotic ciPTEC ($p=0.12$). This was further emphasized by the finding that the ratio GSSG/total GSH, an indicator for oxidative stress, was significantly elevated both in proliferated (0.66 +/-0.16 versus 1.96 +/-0.29; $p<0.05$) and matured (0.62 +/-0.09 versus 1.66 +/-0.20; $p<0.01$), but not in cysteamine treated (0.60 +/-0.13 versus 1.51 +/-0.61) cystinotic ciPTEC.

Using Nernst equation as described by Schafer et al. [Schafer Free Radical Biology & Medicine], we could quantitatively estimate the redox state of the GSSG/2GSH couple in ciPTEC. Following this equation, we could demonstrate the decrease of reduction potential in both proliferating (-269 +/-5 mV in control and -256 +/-3 in cystinosis; $p<0.05$) and matured (-270 +/-4 versus -251 +/-1; $p<0.01$) in cystinotic ciPTEC (figure 3). Subsequent to cysteamine treatment, reduction potential was restored in cystinotic ciPTEC (-269 +/-3; $p=0.43$), supporting the similar ratio GSSG/total GSH after cysteamine treatment.

These experiments will be repeated with cell lines with small mutations in CTNS to determine whether alterations are a consequence of deletion of the CARKL gene. Furthermore, GSH and GSSG status will be examined in transfected cell lines with CTNS and CTNS-LKG.

In ciPTEC of controls (n=4) and cystinosis (n=8), all carrying the 57kb del, either homo- or heterozygously, we have measured the production of cytokine IL-8 and MCP-1 (figure 4), either in basal conditions or in presence of albumin (BSA, 20-500 µg/ml). The albumin concentration was adapted to the calculated albumin concentration at the end of the proximal tubule in patients with cystinosis (range 11-45 µg/ml). The results indicate that both production of IL-8 and MCP-1 is increased in cystinotic ciPTEC, although there is some overlap. Moreover, in both control and cystinotic ciPTEC, production of IL-8 and MCP-1 is correlated to albumin concentration up to 200 µg/ml. This suggests that dysfunctional tubular albumin uptake can be involved in progression of renal interstitial fibrosis in cystinotic patients. The production of RANTES could not be measured in the supernatant of ciPTEC. Experiments to measure TGF-beta production are currently performed.

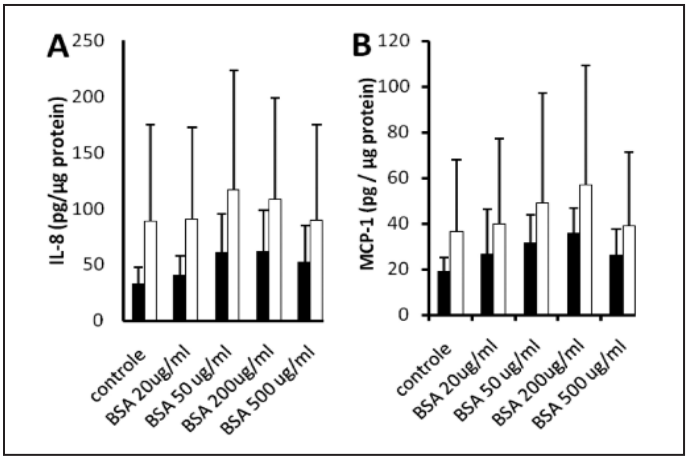


Figure 4. Production of (A) IL-8 and (B) MCP-1 in ciPTEC of controls and cystinotic patients in presence or absence of BSA.

To study the influence of cysteamine on the production of inflammatory and pro-fibrotic substances, we have performed pilot experiments in one control ciPTEC to determine after which period cytokine production can be measured. Since cystine levels are decreased by cysteamine for only 8 hours, this is the maximum time to incubate ciPTEC with cysteamine and to measure cytokine production in the supernatant of ciPTEC. The results of the pilot experiments indicate that after 6 hours IL-8 and MCP-1 can be measured, but are just above the detection limit (figure 5). Further experiments are planned to test whether this is sufficient for measuring the involvement of cysteamine on cytokine production.

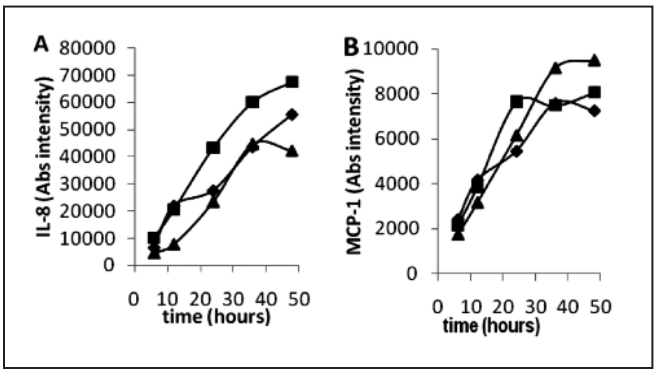


Figure 5. Production of (A) IL-8 and (B) MCP-1 in ciPTEC of controls and cystinotic patients in presence or absence of BSA (200µg/ml) or cysteamine (1mM).

Aim II. Evaluation of ROS production and apoptosis in cystinotic ciPTEC.

a) ROS production

To determine production of reactive oxygen species (ROS) in ciPTEC, experiments have been performed using fluorescent probes hydroethidine (HEt) and DCFDA, of which fluorescence is dependent on ROS. Using HEt, rotenone (1µM) was used as a positive control for superoxide production. After incubation with DCFDA, a range of peroxide (H_2O_2, 10-1000µM) was used as a stimulator of ROS production. Additionally, the influence of cysteamine (1mM, 2.5 hr) on ROS production was evaluated (figure 6). Although we observed a clear trend of increased ROS measured with both DCFDA and hydroethidine, this increase was not significant. Furthermore, cysteamine had no effect on ROS production (data not shown).

Lipid peroxidation was evaluated by measuring F2-isoprostanes in cellular homogenates by HPLC. Pilot experiments (one control and one cystinosis) showed a two fold increase in cystinotic cell line (203 pg /mg protein) compared to control (103 pg/mg protein), indicating increased lipid peroxidation. These results need to be confirmed in other cell lines.

Additionally, we have performed an Oxyblot Detection Kit (Chemicon) to determine carbonyl groups as a result of protein oxidation (see previous progress report I, June 2009). After repeating these experiments, no significant elevation of protein oxidation in cystinotic samples could be detected.

Aim III Evaluation of morphological and metabolic changes in cystinotic podocytes

We have developed and characterized 2 podocytes cell lines derived from cystinotic patients. These lines will be used to investigate the morphology using electron microscopy and immunofluorescence microscopy (localization of neprin, podocin, podocalyxin, synaptopodin). Further ATP metabolism and glucose uptake will be measured in these cell lines.

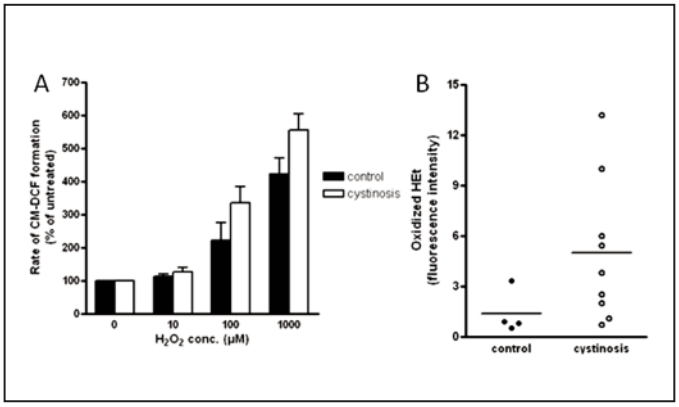


Figure 6. ROS production in ciPTEC. (A) Using DCFDA, formation of CM-DCF was determined as indicator of ROS, in control and cystinotic ciPTEC. As stimulator H2O2 was added to the culture medium. (B) Using Hydroethidine (Het) ROS production was measured using flow cytometry in control and cystinotic ciPTEC.

PROGRESS REPORT

ERIC K. MOSES, PHD, PRINCIPAL INVESTIGATOR
JOHN BLANGERO, PHD, AND KATY FREED, PHD, CO-INVESTIGATORS

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

Date: 11/1/09

SUMMARY OF PROJECT

The Problem

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

The Approach

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

Specific Aims

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

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

PROGRESS TO DATE

In the last progress report, an update on the collection of samples and the re-sequenicng of *CTNS* was described. Briefly, in July 2007 the Cystinosis Research Network Family Conference took place in San Antonio, Texas. With University of Texas Health Science Center San Antonio Institutional Review Board ethics approval, whole blood (5-50 ml) was collected from consenting individuals affected by cystinosis and their first degree relatives. Blood was collected from 40 individuals with cystinosis and 107 samples from unaffected individuals. White blood cells (lymphocytes) were collected and destined for use as a source of DNA, RNA and stable cell lines.

Re-sequencing of CTNS: The DNA has been used to re-sequence CTNS and the distribution of *CTNS* genetic variants in cystinosis families was presented in the last Progress Report. 158 single nucleotide polymorphisms (SNPs) and 5 insertions/deletions have been identified in the cystinosis families. Of these genetic variants, 100 were referenced in the public NCBI database while 58 were novel. Three of the novel SNPs were found in exons.

Generation of cystinotic cell lines: Permanent cystinotic lymphoblastoid cell lines (147) will be generated using Epstein-Barr virus to transform the lymphocytes. Each cell line will represent one cystinosis family member. Unfortunately, there have been a number of unforeseen and unavoidable delays in the transformation of the lymphocytes. The major problems stems from the viral agent used to immortalize the cells in culture. Recently, the monkey cell line used to produce the viral agent was no longer commercially available within America or Great Britain. We tested an old cell line we have previously used here at the SFBR but the transformation of the lymphocytes was slow and variable. We have now sourced a new batch of cells from a University researcher in New Jersey. The new cell lines have now been cultured in our laboratory and the viral agent isolated. Regrettably, the whole process of sourcing new cell lines and the logistics of getting the cells and protocol sent to our laboratory set the work back 6 months.

On a positive note, we are in the process of transforming our white blood cells and to date have generated 40 lymphoblastoid cell lines. These cell lines have been stored in Liquid Nitrogen until required.

Whole genome transcriptional profiling: Originally, we were going to extract RNA from the transformed cell lines and use this material to perform whole genome transcriptional profiling. Given the problems described above we undertook to extract RNA from 147 untransformed lymphocyte samples. We extracted RNA from Ficoll gradient lymphocytes using Trizol (Invitrogen) and RNeasy columns from Qiagen. The quality of the RNA was determined using agarose gel electrophoresis and the quantity determined using a NanoDrop ND-1000 spectrophotomer. A total of 500 ng of RNA was then used as a template to generate biotin-labeled amplified aRNA using the Ambion MessageAmp II Amplification Kit. aRNA total yield (ug) and purity (260 nm:280 nm) were determined spectrophotometrically using the NanoDrop ND-1000 and a total of 1.5 ug aRNA was dried and stored at –20ºC before sample hybridization. Hybridization of aRNA to Illumina Sentrix Human Whole Genome (WG-6) Series I BeadChips and subsequent washing, blocking and detection were carried out using Illumina's BeadChip 6 _ 2 protocol. The WG-6 BeadChips contain >48,000 probes derived from human genes in the NCBI RefSeq and UniGene databases. Samples were scanned on the Illumina BeadArray 500GX Reader using Illumina BeadScan.

We have only recently generated this data and we are now embarking on analyzing this data set to determine which genes are differentially expressed in association with cystinosis. We will then extend our analyzes to test whether the genetic variation identified in the CTNS gene influences the quantitative expression of any other gene(s).

PROGRESS REPORT

DAVID A. PEARCE, PHD, MENTOR
SEASSON P. VITIELLO, PHD, RESEARCH FELLOW

"Yeast as a Model System for Cystinosis: Alterations in V-ATPase function in ers1- "

Date: 11/4/09

The single-celled eukaryote Saccharomyces cerevisiae, or budding yeast, is a useful model system because it is amenable to genetic manipulations and biochemical analyses. The functional ortholog of cystinosin is Ers1p, which is encoded by the ERS1 gene. We aimed to identify and study the cellular defects that occur when ERS1 is deleted in yeast. In our January 2009 progress report, we described a forward genetic screen that used an ERS1 deletion strain, ers1-Δ. This screen revealed cold resistant phenotypes that suggested alterations in vacuolar pH and the vacuolar H^+-ATPase (V-ATPase). The V-ATPase is a complex of proteins consisting of a membrane bound V0 subunit and a soluble, cytosolic V1 subunit. Association of the V1 and V0 subunits results in ATP hydrolysis-driven proton transport, which establishes the acidity and proton-motive force required for transport of various substrates, including cystine. As previously reported, although vacuolar pH is more acidic, there is a 20% decrease in V-ATPase subunit association in ers1-Δ as compared to $ERS1^+$ cells. The association was measured by co-immunoprecipitation of the two subunits, followed by Western blotting of the eluate using antibodies specific for components of the V1 and V0 subunits. Densitometry was performed on the Western blot to calculate percent assembly. Subunit dissociation and re-association after dissociation was measured in a similar manner. We found that subunit dissociation is unchanged and curiously, subunit re-association following dissociation is also unchanged (Figure 1). The decrease in subunit association is not due to decreased V1 or V0 subunit protein levels in the cells or at the vacuole (Figure 2). Two-dimensional blue native gel electrophoresis, a method to separate complexes in enriched organelle fractions, also showed no difference in protein levels in enriched vacuolar fractions (data not shown). We aim to characterize why association, but not re-association, is different in ers1-Δ as compared to $ERS1^+$.

We speculate that the V-ATPase is being down-regulated through decreased subunit association to correct for the decreased vacuolar pH, and this decreased subunit association results in decreased V-ATPase-dependent H+ pumping and ATP hydrolysis in ers1-Δ. Future experiments will measure these activities in ers1-Δ. An ers1-Δcup5-Δ double deletion strain was phenotypically similar to the cup5-Δ single deletion strain, which supports this hypothesis (data not shown), although more experiments need to be performed to verify this. Further analysis of the ers1-Δcup5-Δ double deletion strain may give clues into the relationship between ERS1 and the V-ATPase. Experiments will include measuring vacuolar pH and V-ATPase activity. In addition, two plasmids were recently constructed from the yeast shuttle vector pRS316, a single copy, centromeric plasmid with URA3 auxotrophic selection. The first construct contains 520 base-pairs upstream of ERS1, followed by the ERS1 open reading frame. The second construct contains 520 base-pairs upstream of ERS1, followed by CTNS cDNA. These plasmids will be used to complement the above phenotypes. Alterations in the V-ATPase can affect many cellular processes, including autophagy. Macroautophagy, which is dependent on V-ATPase function, does not appear to be compromised in ers1-Δ as tested by a colony formation assay (data not shown). Cells were incubated in low nitrogen containing media and plated to determine cell viability in those conditions. Carboxypeptidase Y (CPY) trafficking can also be affected by alterations at the vacuole, specifically protein trafficking, where CPY is either incorporated into the vacuole or, if there is a problem with the vacuole, it is secreted into the extracellular area. The amount of CPY that is secreted can be measured by growing cells on a nitrocellulose membrane, washing the cells away, and Western blotting the membrane using an antibody that is specific to CPY. We saw no CPY secretion defect in ers1-Δ, indicating that this particular pathway is not affected by loss of ERS1 (data not shown). Other pathways that may be affected by V-ATPase dysfunction in ers1-Δ are degradation of macromolecules within the vacuole, transport of small molecules across the vacuolar membrane, vesicular trafficking and endocytosis, and mitochondrial function. Our long term goal is to analyze these pathways in ers1-Δ, eventually studying these alterations in higher eukaryotic model systems.

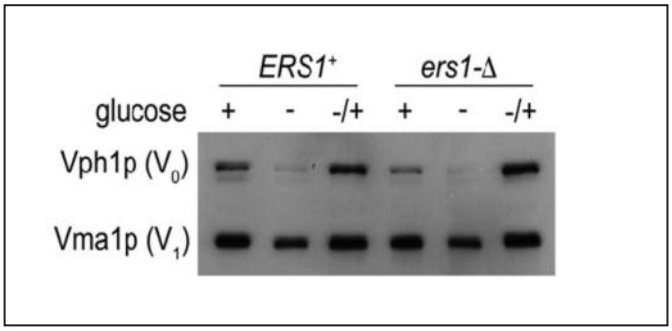


Figure 1: ***V-ATPase association is decreased, but dissociation and re-association is normal in ers1-Δ.*** *Association of the V1 and V0 subunits was measured by co-immunoprecipitation of the subunits from cell lysates. The V1 subunit was immunoprecipitated and the eluate was subjected to SDS-PAGE followed by Western blotting for the V0 and V1 subunit constituents Vph1p and Vma1p, respectively. The co-immunoprecipitation was done in the presence of glucose (+) when the V-ATPase is fully assembled in the ERS1+ strain, in the absence of glucose (-) when most of the subunits are dissociated, and in the absence of glucose followed by addition of glucose (-/+) to monitor re-association of the complex.*

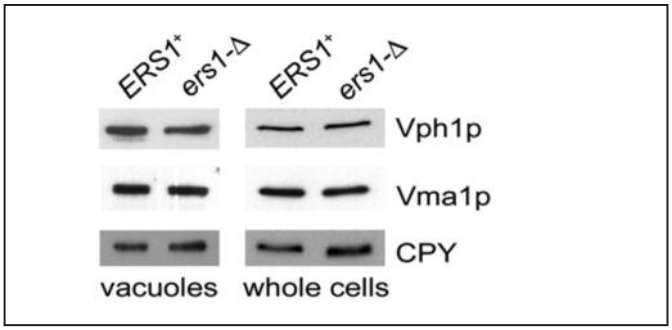


Figure 2: ***V-ATPase subunit protein levels are unchanged in ers1-Δ as compared to ERS1+.*** *V-ATPase subunit levels were measured in cell lysate and enriched vacuole preparations by SDS-PAGE and subsequent Western blotting for Vma1p and Vph1p, constituents of the V1 and V0 subunits, respectively. Carboxypeptidase Y (CPY), a soluble*

This data combined with our previous reports on our studies are currently being assembled into a manuscript for submission. We hope to pass this manuscript on to you once it has been accepted for publication.

PROGRESS REPORT

JENNIFER L. SIMPSON, MD, PRINCIPAL INVESTIGATOR
JAMES V. JESTER, PHD, CO-INVESTIGATOR

"Evaluation of Novel Corneal Imaging and Therapeutics in the CTNS Knockout (Cystinosis) Mouse Model"

Date: 11/24/09

PROJECT OVERVIEW

We proposed two specific aims in our original grant submission, restated below:

Specific Aim I: Develop a screening system for novel therapeutic interventions based on non-invasive, quantitative imaging of cystine crystals in the cystinosin knockout (Ctns-/-) mouse cornea using in vivo confocal microscopy and second harmonic imaging microscopy.

Specific Aim II: To evaluate candidate therapies based on proposed hypotheses and determine their potential effectiveness to intervene in the progression of corneal cystinosis pathophysiology using the screening system developed in Specific Aim I.

We are pleased to report that the project is on schedule and that our team has reached important milestones as summarized in the project status report below.

PROJECT STATUS REPORT

Specific Aim 1A: Establishment of CTNS-/- Mouse Colony
The mouse colony at the UCI Medical Center Animal Facility is established and self-sustaining, with the ability to produce sufficient animals for planned interventional studies.

Specific Aim 1B: Development of Screening System
The natural history has been defined with the ability to assess corneal crystal concentrations quantitatively utilizing in vivo confocal microscopy. This natural history is now being used to define interventional treatment evaluation periods in comparison with control animals.

Specific Aim 2: Interventional Studies
Attempts to transplant human and rabbit mesenchymal stem cells (MSC) into the *CTNS*- mouse model were not successful, with transplanted cells dying within two weeks. We are currently collaborating with Dr Winston Kao, who has successfully transplanted human umbilical stem cells (HUMSC) into the lumican knockout mouse, an experimental model for corneal opacity.

HUMSCs are abundant, can be easily isolated and expanded, and can be stored (in liquid nitrogen), and quickly recovered, making them an excellent candidate for tissue banking. Localized transplantation of human umbilical mesenchymal stem cells (HUMSC) is therefore potentially a promising treatment modality for corneal cystinosis (as well as other hereditary and congenital corneal dystrophies), that can be evaluated in our in-vivo confocal imaging *Ctns*-/- mouse model.

While likely not affecting cystine transport directly in native host cell populations, we hypothesize that successful establishment of a population of normal keratocytes within the corneas of Ctns-/- mice can potentially result in multiple benefits. The removal of damaged cystine-laden host keratocytes, the resolution of inflammatory processes, and the laying down of new stromal elements would all be expected to improve corneal functioning, structure and symptoms. To test this hypothesis, we will transplant human umbilical cord mesenchymal stem cells and evaluate their ability to restore normal corneal architecture in *Ctns*-/- corneas using in-vivo confocal imaging.

As a positive control, we are also evaluating the effect of topical cysteamine in the CTNS- mouse model.

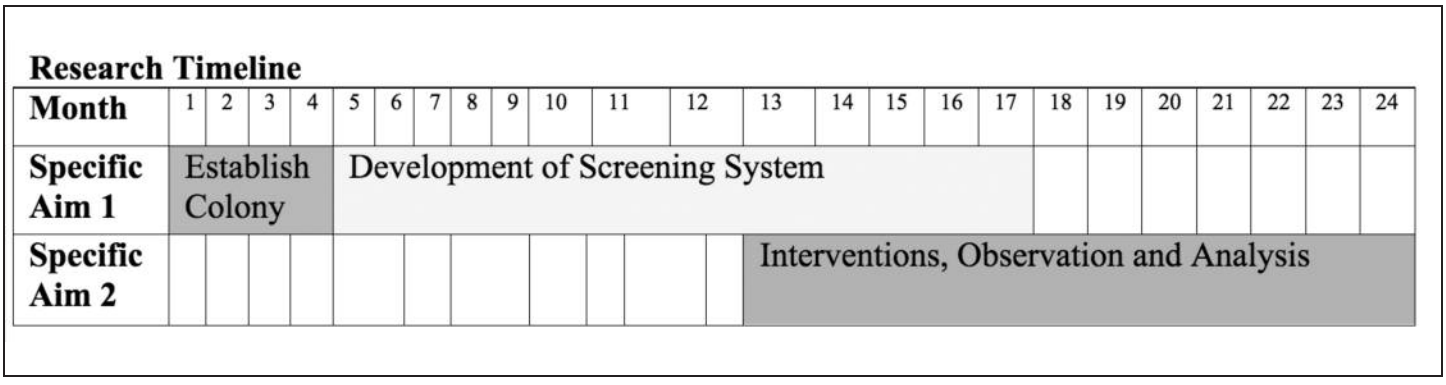
Work has also begun on the synthesis of PLGA nanoparticles, which will be evaluated as a possible alternative drug delivery system for cysteamine.

External Funding
We are actively pursuing external funding to support this research and have submitted an NIH Challenge Grant as well as a core grant proposal to the Guenther Foundation.

Personnel Updates
No changes in personnel have occurred on this grant.

We also proposed a research timeline, which is also restated below:

Research Timeline



PROGRESS REPORT

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

"Characterization of Novel Lysosomal Genes for Immune Regulation and Spermatogenesis in Nephropathic Cystinosis"

Date: 4/1/09 – 9/30/09

Abbreviations used in the Progress Report:
Lipopolysaccharide – LPS; myeloid DC – mDC; plasmacytoid DCs – pDC; Peripheral Blood Mononuclear Cells – PBMC; Renal Proximal Tubule Epithelial Cells – RPTE.

Overview:
The project is currently progressing according to the proposed research plan. Though a 2 year funding was requested, the proposal was funded for one year for Specific Aim 1 only. Preliminary data and further details of the experimental plan were requested to subsequently fund Specific Aim 2. This will be submitted in a separate grant application on November 4, 2009.

Specific Aim 1 is focused on the immune response in patients with nephropathic cystinosis.

In the first 6 months of the funded study, the following progress has been made:

1. IRB approval for the study was submitted and obtained.
2. We have enrolled 8 cystinotic patients (out of total 13 cystinotic patients at Stanford Hospital) with IRB approved written consent.
3. Six matched control participants have been enrolled in the study: 3 healthy volunteers and 3 pediatric non cystinotic transplant patients with stable renal allograft function.
4. Relevant demographic and clinical data were collected on all 14 participants.
5. The following samples have been obtained and preserved on all 14 enrolled participants:
   - ***Serum samples*** *for cytokines measurement;*
   - ***Peripheral Blood*** *samples a) to conduct fresh whole blood myeloid and plasmacytoid dendritic cells (DC) phenotyping and ex vitro stimulation assays to evaluate DC maturation markers and intracellular cytokines responses and signals transduction activation and b) to isolate and cryopreserve peripheral blood mononuclear cells (PBMC) for future monocytes, T-, B- cell subsets phenotyping and functional assays;*
   - ***Urine*** *for Renal Proximal Tubule Epithelial Cells (RPTE) isolation, growth and analysis;*
   - ***Skin biopsies*** *were performed on 5 cystinotic patients by licensed dermatologist at Stanford University in preparation for submission of Specific Aim 2. Skin fibroblasts were isolated and cryopreserved by Dr. Rejo Pera.*

In response to Specific Aim 1, the following selected detailed experiments are outlined for review:

Aim 1: Cystinotic renal transplant recipients can demonstrate reduced allo-responses to donor antigen with intact third party responses to foreign antigens.

Aim (1A) *To investigate whether there is altered HLA class II protein expression in recipient cystinotic peripheral blood mononuclear cells (PBMCs) and renal proximal tubule epithelial (RPTE).*

Aim (1B) *To test if altered antigen-presenting capacity of DC from cystinotic patients is responsible for the donor specific hyporesponsiveness, when compared to healthy DC cells.*

Aim (1C) *To investigate if there is donor specific hyporesponsiveness in cystinotic transplant recipients, when compared to other matched transplant recipients, with intact immune responses against third party antigens.*

Experiments Performed to date: (Experiments are performed within 1hour of blood receipt.)

Design	Goal	FITC or Alexa488	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	V450	Pac Orange
Whole Blood; Ex vitro 24 h LPS	DC phenotype	CD86	CCR7	CD123	HLA-DR	CD40	CD3/14/20	CD11c	CD45
Ex vitro 5h LPS+BFA	DC-intracellular cytokines	TNFa	IL-12	CD123	HLA-DR	IL-10	CD3/14/20	CD11c	CD45
Ex vitro 15 min IL-6	DC-signals transduction	pStat1	pStat5	CD123	HLA-DR	pStat3	CD3/14/20	CD11c	CD45

Table 1: ***Antibodies used for 8-color whole blood Flow Cytometry analysis of dendritic cells phenotype, intracellular cytokine staining and phospho-flow.***

1. Whole blood assays of peripheral blood myeloid and plasmacytoid dendritic cells phenotypes by 8 color flow cytometry.

Preliminary Analysis:

We phenotyped $CD11c^+$ myeloid (mDC) and $CD123^+$ plasmacytoid (pDC) dendritic cells phenotypes using 8-color flow cytometry analysis according to standard protocols {Ida, 2006} for HLA-DR expression, maturation CCR7 and T-cell co-stimulatory CD40 and CD86 molecules expression (Table 1). All antibodies were purchased labeled with fluorochromes from BD Immunocytometry, San Jose, CA. Flow cytometric acquisition was performed on a BD LSRIII Flow Cytometer (Becton Dickinson Immunocytometry Systems, USA) with BD FACSDiva software following by data analysis using FlowJo and CytoBank softwares (Figure 1).

The distributions of mDC and pDC in fresh whole blood were similar between all studied cystinotic and control samples (65-91% of mDC and 6-28% of pDC from $CD45^+$/$HLA\text{-}DR^+$/$CD3^-$/$CD14^-$/$CD20^-$ blood cells). Little if any expression of the CCR7 maturation marker on fresh whole blood mDC and pDC was detected in either of studied samples. We observed that mDC were predominantly $CD86^+$ whereas pDC were predominantly $CD86^-$ in all samples without significant differences between studied groups. However higher percentage of mDC were $CD40^+$ in cystinotic patients (15.3±11.9%) when compared to healthy volunteers (1.03±1.1%) and non cystinotic transplanted patients with stable renal allograft (4.3±4.6%). Thus we may suggest altered maturation and activation status of mDC in cystinotic patients as well as their ability to interact with activated T-cells (Figure 2). All DC are HLA-DR positive, geometrical mean fluorescence intensity of HLA-DR on DC was also similar in cystinotic (14014.3±5081.8 for mDC and 12349.7±5791.3 for pDC) and control (11008.1±1865.2 and 14744.3±5892.5, respectively) samples, suggesting that intensity of HLA-DR expression on peripheral blood DC in cystinotic patients does not differ from healthy individuals or transplanted patients with stable renal allograft.

To summarize, despite the increase in percentage of $CD40^+$/$CD11C^+$ DC in cystinosis, there does not currently appear to be a significant difference in whole blood mDC and pDC phenotypes observed in cystinotic patients versus controls.

2. *Ex vitro* responses to Toll-like receptor stimulation in order to compare antigen-presenting cells in cystinotic patients versus control participants. We are also optimizing *ex vitro* stimulation assays to evaluate DC maturation and co-stimulatory molecules expression, intracellular cytokines production and signals transduction activation responses.

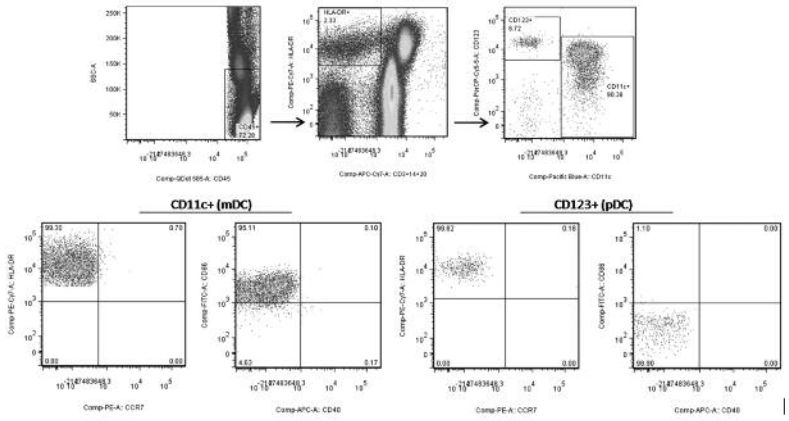


Figure 1: ***Flow cytometry gating strategy to detect expression of maturation and co-stimulatory molecules by DC in fresh whole blood.*** *Initial gate was placed on CD45+ monocytes and lymphocytes. On second plot DC were identified as being HLA-DR+/CD3-/CD14-/CD20-. Third plot shows the distribution of CD11c+ and CD123+ staining of cell within the "DC gate". Further, CD11c+ (mDC) and CD123+ (pDC) were evaluated for CCR7, CD40, CD86, HLA-DR expression as shown on bottom panel. Presented plots were created during analysis of healthy volunteer's staining using FlowJo software.*

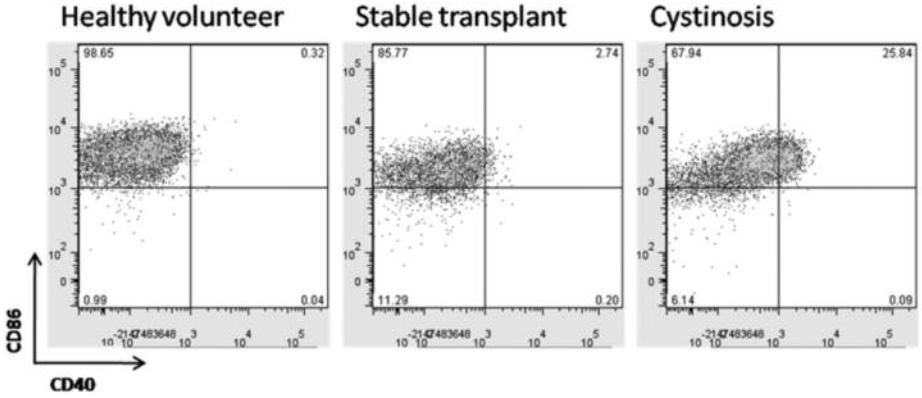


Figure 2: ***Whole blood flow cytometric detection of CD86 and CD40 in CD11c+ mDC.*** *Increased expression of surface CD40 on mDC in cystinotic patients vs controls was observer. Shown plots are from representative healthy volunteer, non cystinotic transplant patient with stable renal graft function and cystinotic patient.*

Whole blood ex vitro assay to assess peripheral blood DC phenotype and function in response to Toll-like receptor stimulation. For ex vitro stimulation, whole blood was aliquoted at 300 ul per 5ml round-bottom 21x75 mm Falcon™ polystyrene tubes and stimulated with 1ug/ml of lipopolysaccharide from *E. coli* (LPS, Sigma, cat#L2762) for 24 h at 37°C in a 5%CO_2 humidified atmosphere at a 5° slant. Unstimulated test tubes received medium and were used for evaluation of spontaneous DC activation. After incubation, samples were treated with 5mM EDTA for 10 min at 37°C to reduce clumping and to detach the cells from tube walls and surface staining was performed as shown on Table 1. Red blood cells were lysed using BD FACS Lysing Solution (cat. No. 349202) and 8-color Flow Cytometry analysis was performed. Analysis of this data is currently pending.

Using whole blood, we also have initiated study to measure ***intracellular cytokine levels*** (TNFa, IL-12 and IL-10) in mDC to compare cytokine production response to LPS stimulation in cystinotic vs. control participants as well as monitor spontaneous pDC response. Whole blood samples were stimulate with 1 ug/mL of LPS in the presence of 1x Brefeldin A (BFA, inhibitor of cytokine secretion; eBioscience, cat#00-4506-51) for 5h. Further, surface staining for CD123, CD11c, HLA-DR, CD45 and CD3/CD14/CD19 was performed as shown on Table 1 and red blood cells were lysed. At this step cells were fixed and frozen at -80°C for future permabilization and intracellular cytokine staining.

In order to compare the ***nuclear transduction signals*** (pSTAT1, pSTAT3, pSTAT5) activation in response to IL-6 stimulation in cystinotic vs. control participants, we perform whole blood surface staining for CD123, CD11c, HLA-DR, CD45 and CD3/CD14/CD19 as shown on Table 1, incubated cells with 50 ng/ml of IL-6 (Becton Dickinson Immunocytometry Systems, USA, Cat.No. 550071) for 15min at 37C, and lysed red blood cells. At this step cells were fixed and frozen at -80C for future permabilization with newest permabilization buffer available from Becton Dickinson and nuclear transduction signals staining.

3. Fresh blood serum and serum from samples incubated for 24 h with/without LPS were frozen for *detection of cytokine levels* to compare *in vivo* cytokines level in cystinotic vs. control participants and to evaluate the whole blood cells response to LPS stimulation.

4. Renal Tubular Epithelial Cells (RPTE) were successfully obtained from urine specimen of cystinotic patients and grown *in vitro.*

Urine specimens (50ml) were obtained from 3 cystinosis patients and processed regarding the protocol described by Racusen et al {Racusen, 1991}. Briefly, urine samples were gently centrifuged (72 x g) for 5 min and the cell pellets were resuspended in 2 ml of culture medium (F12/DMEM medium with 10mM HEPES buffer, 10% fetal calf serum, 50U of penicillin per ml, 50ug of streptomycin per ml and 1.25ug of Fungizone per ml). Further, cells were seeded into six-well plate. Cultures were maintained at 37C in 5% CO_2 atmosphere and were refed every 2 - 3 days with culture medium. Four weeks later no culture contamination was observed and we collected and cryopreserved $0.3x10^6$ RPTE cells (Figure 3). Cryopreserved cystinotic patient RPTE will be used for further HLA-DR4 immuno-staining and LP1-siRNA transfection along with RPTE cells from 5 cystinotic patients available from Dr. Gagl, NIH and commercially available normal RPTE cell lines, as proposed in the grant.

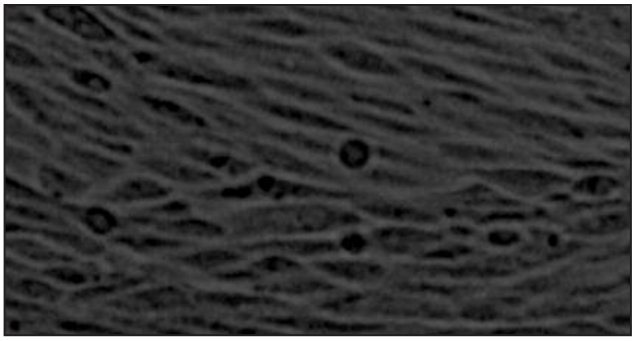


Figure 3: ***Monolayer of in vitro growing RPTE cells from cystinotic patients.*** *Presented phase-contrast micrograph shows RPTE monolayer from urine cells from a cystinosis patient (magnification x200).*

PROGRESS REPORT

HOLGER WILLENBRING, MD, MENTOR
SILVIA ESPEJEL CARBAJAL, PHD, RESEARCH FELLOW

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

Date: 6/1/2009 – Final Report

A. OBJECTIVE AND SPECIFIC AIMS

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

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

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

b) Assessment of the extent of donor BMM reprogramming to renal tubular function as exemplified by de novo expression of the transcription factor HNF4.

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

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

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

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

B. STUDIES AND RESULTS

Low number of fusion events between bone marrow-derived macrophages expressing measles virus H and F protein and renal proximal tubular cells in *CD46+/-, R26R+/-* mice.

We have shown that the tropism of the measles virus (MV) H protein for CD46 in combination with the MV F protein can be used to induce fusion between cells of a human CD46 transgenic mouse model (CD46+/- mice) and cells expressing the MV proteins via plasmid transfection. We also demonstrated that bone marrowderived nuclei are reprogrammed by fusion with renal proximal tubular cells to activate expression of the transcription factor HNF4, suggesting that bone marrow-derived cells can be used to genetically and functionally correct a wide range of tubulopathies, including cystinosis.

In our last report, we showed that fusion can be induced between MV H/F expressing macrophages and CD46/R26R-expressing renal proximal tubular

cells in vivo. In this same report, we also described experiments transplanting BMM armed with a bi-specific antibody (an antibody for the macrophage-specific F4/80 antigen linked to an antibody for the cell surface marker DPPIV present on renal proximal tubular cells) into the kidney. However, we were not able to detect induced fusion events with this strategy. Flow cytometry of BMM mixed with CD46-expressing hepatocytes suggested that this could be due to loss of activity of the conjugated antibodies in the linking process (data not shown). This problem can likely be overcome by new linking attempts or the use of novel cell surface marker antibodies. However, in the interest of time, we focussed on establishing the key principles of targeted and induced cell fusion in the kidney in our model comprised of BMM expressing MV H/F proteins and CD46+/- mice.

The number of induced fusion events between MV H/F expressing macrophages and renal proximal tubular cells observed in CD46+/-, R26R+/- mice was low (2 fused cells/100,000 renal proximal tubular cells). Although BMM and CD46+/-, R26R+/- mice were on the same strain background, loss of fusion products may have occurred due to an immune response against the MV proteins. To rule out acute rejection of H/F expressing cells, we are currently comparing the number of engrafted GFP-expressing macrophages in kidneys of CD46+/-, R26R+/- and immune-deficient mice (Rag2-/-, c-/-). These analyses will reveal whether induction of fusion with H/F proteins will require transient immune suppression.

A mouse model of renal Fanconi syndrome.

In order to assess the efficacy and, to some extent, safety of cell therapies such as induced fusion for therapy of tubulopathies, a mouse model is needed. For this purpose, we proposed to use the fact that FAH, the last enzyme in the tyrosine degradation pathway, is only expressed in hepatocytes and renal proximal tubular cells and that its deficiency causes mild renal injury by an accumulation of the substrate fumarylacetoacetate (FAA). This renal injury aggravates when hepatocytes of mice lacking FAH (Fah-/- mice) are also deficient in homogentisic acid dioxygenase (HGD), an enzyme upstream of FAH (1). Typically, hepatocytes take up and degrade most tyrosine but it they are HGD deficient, the HGD substrate homogentisic acid (HGA) is shifted towards the kidney where it enters the tyrosine degradation pathway, thereby producing large amounts of the toxic metabolite FAA in proximal tubular cells. As a result, we hypothesized that Fah-/- mice repopulated with HGD deficient hepatocytes would develop renal Fanconi syndrome.

To investigate this, we transplanted Hgd-/-, Fah+/+ hepatocytes into the spleen of Fah-/- immune-deficient mice (Fah-/-, Rag2-/-, c-/-). Mice were taken off 2-(2-nitro-4-trifluoro-methylbenzyol)- 1,3 cyclohexanedione (NTBC), a drug protecting Fah-/-mice from FAH deficiency, 4 weeks after hepatocyte transplantation to allow their selective expansion. Two months after transplantation, mice showed repopulation with Hgd-/-, Fah+/+ hepatocytes in the liver (Fig. 1A). As expected, progressive repopulation was associated with development of kidney damage including massive enlargement (Fig. 1B), death of proximal tubular cells (Fig. 1 D) and proteinuria (Fig. 1D-E). Hence, we believe that we have generated a mouse model truthfully recapitulating the kidney injury underlying renal Fanconi syndrome. Since this model can be established in a relatively short period of time and allows the use of NTBC for "tittering" the level of tubulopathy, we hope that it will be helpful for research on the pathology and therapy of cystinosis.

Along these lines, to use this model to test the therapeutic potential of induced fusion with BMM, we will transplant HGD deficient hepatocytes into CD46+/-, Fah-/- (immunodeficient) mice to repopulate their livers and induce chronic renal tubular damage. Then, we will inject BMM expressing both GFP and the measles virus proteins into the kidneys of these mice to assess the regenerative capabilities of renal proximal tubular cells corrected by fusion. We expect that fused FAH expressing renal tubular cells will have a growth advantage over the FAH deficient cells, and therefore, will be able to repopulate and regenerate the damaged proximal tubular epithelium of the recipient mice.

D. REFERENCES

1. Held P.K., Al-Dhalimy M., Willenbring H., Akkari Y., Jiang S., Torimaru Y., Olson S., et al. In vivo genetic selection of renal proximal tubules. Mol Ther 2006;13:49-58

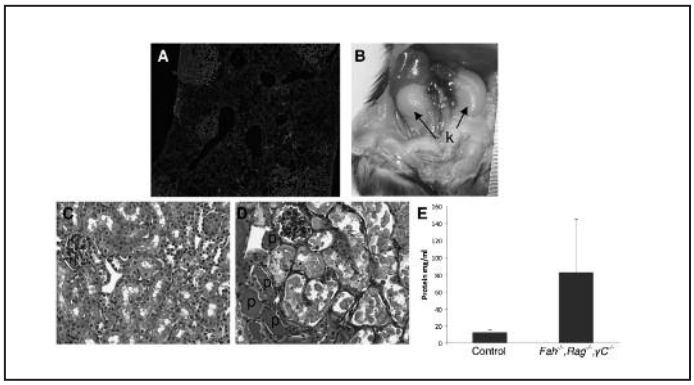


Figure1. Kidney damage in Fah-/-,Rag2-/-,C -/- mice transplanted with Hgd-/-,Fah+/+ hepatocytes. A. Clusters of FAH-expressing hepatocytes (red) in liver two months after transplantation. B. Picture showing massive enlargement and pale appearance of kidneys (k) in the same mouse. C-D. Sections of kidney tissue stained with Periodic-Acid-Schiff (PAS) from control healthy mouse (C) and Fah-/-,Rag2-/-,C -/- mice with liver repopulation by Hgd-/-,Fah+/+ hepatocytes (D). Note the injured epithelial cells in cortical tubules and protein deposits (p) caused by proteinuria. E. Comparison of total urine protein levels in control healthy mice and Fah-/-,Rag-/-,C-/- mice with liver repopulation by Hgd-/-,Fah+/+ hepatocytes.

PROGRESS REPORT

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

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

Date: 12/20/09

The overall goal of this research grant is to establish the feasibility of therapy of Fanconi syndrome due to nephropathic cystinosis with kidney progenitors derived from parthenogenetic embryonic stem cells (pESC). We have taken several steps towards realization of this goal. First, we have generated a mouse model hat recapitulates the early-onset kidney injury characteristic for the severe infantile form of human cystinosis. Second, we have obtained proof-of-principle for the ability of pESC to differentiate into renal proximal tubular cells with normal functional and proliferative capabilities in vivo. Previously, we adopted a protocol for the differentiation of mouse embryonic stem cells (ESC) into kidney progenitors in vitro.

Our current results support the feasibility of therapy of renal Fanconi syndrome with kidney progenitors derived from pESC but also other cells sources such as induced pluripotent stem (iPS) cells. In the remaining part of this research project, we aim at rescuing kidney function in the new renal Fanconi syndrome mouse model with pESC-derived kidney progenitors.