CRN 24-mo final report

Gene transfer studies for cystinosis

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November 2009

Initial specific aims

I) Validate *in vitro* gene transfer results on primary murine hepatocytes by *in vivo* gene transfer to the liver

II) Generate clinically relevant vectors (HD CAV-2 and AAV8) expressing *CTNS*

III) Conduct *in vivo* corneal-targeted gene transfer studies

IV) Refine characterisation of the CNS anomalies in *Ctns*$^{-/-}$ mice and determine whether cysteamine can cross the blood-brain barrier

Our initial proposal was to obtain a salary for technical help. We hired Sandy Ibanes (a research assistant) in December 2007. Since this time, we successfully accomplished most of the aims outlined in our original grant application and published an additional article. This final report will provide an overview of our results for the 2-year funded period.

Abbreviations

GFP	green fluorescent protein
IRES	internal ribosomal entry site
CTNS-IRES-GFP	expression cassette containing *CTNS* and *GFP* separated by an IRES sequence
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
ΔE1	deleted for the E1 region
ΔE3	deleted for the E3 region
AAV8	adeno-associated virus serotype 8
AAV-GFP	adeno-associated virus vector containing the gene *GFP*
AAV-CIG	adeno-associated virus vector containing the CTNS-IRES-GFP expression cassette
Ad5	human adenovirus serotype 5
AdGFP	human adenovirus vector expressing the gene *GFP*
AdCTNS	human adenovirus vector expressing the gene *CTNS*
AdCTNSGFP	human adenovirus vector expressing the gene *CTNS* fused to the gene *GFP*
CAV-2	canine adenovirus serotype 2
CAVGFP	canine adenovirus vector expressing the gene *GFP*
CAVCTNS	canine adenovirus vector expressing the gene *CTNS*
HD CAV-2	helper-dependent canine adenovirus vector (devoid of all viral genes)
HD CAV-GFP	HD canine adenovirus vector containing the gene *GFP*
HD CAV-CIG	HD canine adenovirus vector containing the CTNS-IRES-GFP expression cassette
Ctns$^{-/-}$	homozygous deletion of the mouse *Ctns* gene

I) *In vivo* gene transfer studies

Background:

We initially performed *in vitro* gene transfer studies using fibroblast cell lines (the only cystinotic cell line then available) from affected and normal individuals. Following transduction with a ΔE1 CAV-2 vector expressing *CTNS* (CAVCTNS), cystine levels were significantly reduced in *CTNS*$^{-/-}$ cells as compared to control cells. These results showed that *in vitro CTNS* gene transfer could reduce lyososmal storage. We then validated these results using cystinotic cells that were more readily transducible than fibroblasts by generating primary hepatocyte cultures from *Ctns*$^{+/+}$ and $^{-/-}$ mice. CAVCTNS transduction of hepatocytes from young mice significantly reduced cystine levels (5-fold) compared to controls. CAVCTNS transduction of older mice resulted in a lower (2-fold) but significant decrease in cystine levels. This work suggested that the efficiency of cystine clearance by *CTNS* gene transfer could be age-dependent in hepatocytes. Subsequently, in vivo gene transfer studies targeting the liver of *Ctns*$^{-/-}$ mice confirmed these observations: One week after injection with ΔE1/ΔE3 Ad vectors expressing cystinosin (AdCTNS) or cystinosin fused to GFP (AdCTNSGFP), we significantly reduced hepatic cystine levels as compared to controls (AdGFP-transduced or non-transduced mice). In contrast, we did not significantly reduce cystine levels beyond that observed with AdGFP in older mice. Taken together, our results showed that it was feasible to reduce lysosomal cystine levels *in vivo* by viral-mediated gene transfer and strongly suggested that the efficiency of cystine clearance over a short transduction period is age-dependent in the liver.

Results:

Long-term (1 mo) gene transfer experiments: A possible explanation for the age-dependent efficiency of cystine clearance was that a longer duration of cystinosin expression was required to reduce the higher cystine levels in older mice. Therefore we prolonged transgene expression from 1 wk to 1 mo using an immunosuppressive protocol. Consistent with our short-term *in vivo* transduction data, we significantly reduced cystine levels by AdCTNS- or AdCTNSGFP-transduction in young but not older mice. Thus our long-term transduction data continue to show an age-dependent reduction of cystine clearance and suggest that this phenomenon is due to a factor other than duration of cystinosin expression.

Kupffer cell degeneration and regeneration affects cystine levels: Contrary to our expectations, cystine levels were more efficiently reduced in short-term as opposed to long-term transduction experiments. Furthermore, AdGFP non-specifically reduced cystine levels in both short- and long-term experiments but the decrease was more pronounced at 1-wk post-injection. This suggested that, in the absence of *CTNS*, cystine levels increased over time. Intravenous adenovirus vector entry destroys Kupffer cells, which could account for the decrease in cystine levels observed in short-term AdGFP-transduction experiments. In turn, we hypothesised that a long post-transduction period may result in the regeneration of Kupffer cells and hence cause a rise in cystine levels. To test this hypothesis, we artificially depleted Kupffer cells from young and older mice using an anti-macrophage agent. At 1-wk post-depletion cystine levels were greatly reduced whereas at 1 mo cystine levels had increased to almost initial levels. We correlated these results to the absence and presence of Kupffer cells on histological sections at both time-points respectively.

Taken together, we showed that Kupffer cell degeneration (following AdGFP injection) and regeneration causes a non-specific decrease and increase in cystine levels, respectively. In contrast, we demonstrated that cystine levels remain reduced in hepatocytes transduced with a *CTNS*-expressing vector. Our observations also suggested that the cystine content of the Kupffer cells, which represent only 10% of the liver cell population, accounts for 75% of hepatic cystine levels in the mouse model.

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

Transgene expression: We detected *GFP* expression in AdGFP-injected mice and *CTNS* expression in AdCTNS-injected mice at 1-wk and 1-mo post-injection. Interestingly, we only detected cystinosin-GFP expression from AdCTNSGFP-injected mice at 1-wk post-injection (pi). Taken together, these results suggested that GFP-tagged cystinosin is degraded more rapidly or is more immunogenic and should be avoided for viral-vector constructs.

Manuscript: The ensemble of this work represented the culmination of a project begun in 2003. Our work comprising the first *in vitro* and *in vivo* viral vector-mediated gene transfer studies for cystinosis was published in August 2008:

Hippert, C., Dubois, G., Morin, C., Disson, O., Ibanes, S., Jacquet, C., Schwendener, R., Antignac, C., Kremer, E.J. & Kalatzis, V. (2008) Gene transfer may be preventive but not curative for lysosomal storage due to a defective transporter. *Mol. Ther.* 16: 1372-1381.

II Generation of clinically relevant vectors (HD CAV-2 and AAV-8) expressing *CTNS*

Background:

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

Results:

- **HD CAV-2 production**

Over the last two years due to the indispensable work of Sandy Ibanes:

i) We optimised the production protocol of HD CAV-2 vectors by producing a control HD CAV-2 vector (referred to here as 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*.

ii) We produced a HD CAV-2 vector expressing a CTNS-IRES-GFP cassette (HD CAV-CIG). The production of HD CAV-CIG was less 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 gradient. 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. We produced an initial stock of HD CAV-CIG but there was a high percentage of helper contamination. 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.

iii) We verified that the expression cassette of HD CAV-CIG is functional *in vitro*: we detected both cystinosin and GFP expression by immuno- and epi-fluorescence studies of transduced cells as well as by western blot analyses, and reduced cystine levels by 70% in *CTNS*$^{-/-}$ fibroblasts.

iv) In parallel, we returned to our initial 30-kb HD CAV-CIG plasmid to remove 2 kb of sequence by restriction enzyme digestion. Our rationale was that this smaller vector would separate more efficiently from the helper than HD CAV-CIG-32 kb. In parallel, we produced a new helper vector (CAV-Cherry) that has a delayed packing time as compared to the HD vector, which should thus decrease the rate of helper contamination. Furthermore, CAV-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.

- **AAV8 vector production:**

We cloned the CTNS-IRES-GFP cassette into a transfer plasmid to initiate the steps of AAV-CIG vector production. We then subcontracted AAV-CIG and AAV-GFP vector production to the “Vector Production Platform” at the “Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain)”. Upon receiving our first stock (over the last 2 years we have ordered 4 production stocks of each vector to meet

our needs), we tested the expression of *CTNS* and *GFP in vitro* by immuno- and epi-fluorescence studies. We then initiated our *in vivo* studies in the liver (the target organ of choice for AAV8 vectors) of *Ctns*$^{-/-}$ mice to test for a reduction of cystine levels. However, due to the difficulty of obtaining elevated titres of AAV vectors, we were never able to accomplish a satisfactory transduction rate of this organ. Nevertheless we had some puzzling results when we assayed the cystine levels. When we assayed cystine levels 1, 2, 3 or 4 d pi with either AAV-CIG or AAV-GFP vector, we did not reduce cystine levels. In contrast, when we assayed cytine levels 5 or 7 d pi, we reduced cystine levels with both vectors. Lastly, if we assayed cystine levels 3 wk pi, we did not reduce cystine levels (or rather cystine levels had likely increased like the situation in the liver with adenovirus vectors). Curiously though, we were unable to clearly correlate these observations with the presence or absence of Kupffer cells either by immunohistochemical studies or by transmission electron microscopy. Consistently, depletion of Kupffer cells prior to administration of AAV vectors did not increase transduction efficiency.

III - *In vivo* corneal-targeted gene transfer studies

Background:

Our gene transfer studies to the liver using adenovirus vectors provided the proof-in-principal that viral vector-mediated gene transfer could reduce lysosomal cystine levels *in vivo*. The next step was to perform gene transfer studies to the cornea, a tissue that is more clinically relevant for cystinosis. For the corneal gene transfer studies, we planned to use more stable viral vectors (HD CAV-2 and AAV8) to be as clinically relevant as possible. As a prelude to this work, we had previously characterised, spatially and temporally, the ocular anomalies in the backcrossed C57Bl/6 mice (Kalatzis *et al.* 2007).

Results:

i) Nicolas Serratrice, a Ph.D. student in our lab, showed that intra-stromal injection of CAV 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 wk *ex vivo*, 2 wk *in vivo*). We went on to test the HD CAV vectors and found that they showed the same kinetics even though they are less immunogenic. Thus we think that the non-integrating HD CAV vectors are likely eliminated from the cornea due to an apoptosis/proliferation repair mechanism following injection. We evaluated the possibility of using the intravitreal and intracamaral routes to reach the corneal stroma. However, these routes of administration preferentially transduced the corneal endothelium.

ii) We then tested our AAV vectors by intra-stromal injection. We injected mouse corneas with AAV-GFP and followed expression by *in vivo* microscopy and histological studies: Forty-eight h after AAV-GFP injection, we detected expression in the corneal epithelium (Figs. 1A & B) likely due to the needle traversing this tissue. This expression disappeared by 1 wk pi, probably due to the 7-day turnover of this tissue. We began to see GFP expression in the corneal stroma around 4 wk pi. This expression persists over time (Figs. 1C & D) and can still be seen today at 11-mo pi. Like CAV, AAV vectors are theoretically "non-integrating" thus we don't know why they are able to escape the fate of CAV vectors following corneal repair. It is possible that **i)** the vector genome remains encapsidated and capsid disassembly only occurs following cell division, **ii)** the AAV vector genome integrates into stromal keratocytes or **iii)** the extra-chromosomal genome forms > 50 kb concatamers that are not lost during cell division.

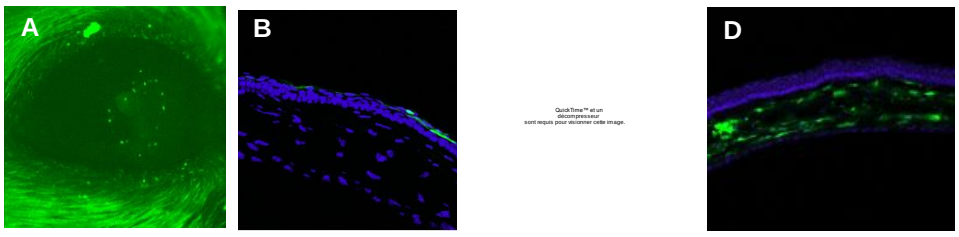

Figure 1: GFP expression following intra-stromal AAV-GFP injection by *in vivo* microscopy (A & C) and histological studies (B & D). (**A** & **B**) GFP expression is observed in the corneal epithelium 48 h pi (**C** & **D**) GFP expression persists in the corneal stroma 6-mo pi.

iii) 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 not yet expressed), we provoked GFP expression in the stroma

(Fig. 2). This expression rapidly decreases after 24 h and disappears by 1 wk. 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). To understand the basis of these observations, we recently performed qPCR studies to follow the fate of viral mRNA and DNA. We detected a peak (60-fold higher) in viral mRNA expression levels at 24 h post-PBS injection (correlating with strong GFP expression), which were no longer detectable at 1 wk (correlating with the loss of GFP). In terms of viral DNA, levels decreased by 50% at 24 h, and 75% at 1 wk, post-PBS injection. We can propose the following hypothesis to explain these results: following initial AAV injection, the encapsidated AAV particles find refuge in the cell (most likely in the nucleolus) and avoid evacuation following corneal repair. However, following the second injection, the corneal reparation mechanism (including release of cytokines) likely causes mobilisation and disassembly of the AAV particles and synthesis of the second DNA strand, which then results in high mRNA expression levels and strong GFP expression. However, concomitantly, cell death would take place causing removal of the green cells and repopulation of the cornea by proliferating keratoblasts. This would explain the disappearance of viral mRNA and the progressive diminution observed in viral DNA levels. Furthermore, a certain quantity of AAV particles still remain in the corneal cells as a second PBS injection again results in GFP expression, although at a lower level.

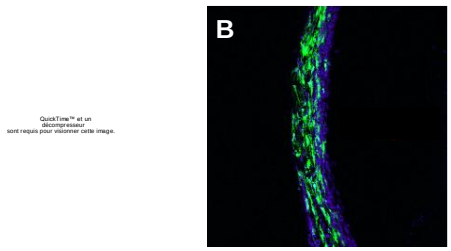

Figure 2: GFP expression 24 h after PBS re-injection. GFP expression is observed throughout the corneal stroma by *in vivo* microscopy (**A**) and on histological sections (**B**).

iv) 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. 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. We then repeated this experiment but assayed cystine levels 3 wk post PBS injection and we observed increased and similar levels for both vectors. These results are reminiscent of our observations with AAV in the liver, thus, for an as yet unknown reason, injection of AAV appears to cause a non-specific decrease in cystine levels.

v) Finally, we recently complemented this study by assaying the tropism of AAV8 in human corneal explants (Fig. 3). We showed that a strong GFP expression from AAV8-GFP appears 5 d pi and persists until 8 wk (longest period we were able to keep the explants in culture). We compared the profile of AAV8-induced GFP expression to that of three other AAV vectors, serotypes AAV1, -2 and -5: AAV1 showed a similar profile to AAV8 however GFP was expressed at lower levels; in contrast, GFP expression from AAV2 and 5 was weak and short-lived. These studies identified AAV8 as the most efficient serotype to transduce human corneal keratocytes via intra-stromal injection.

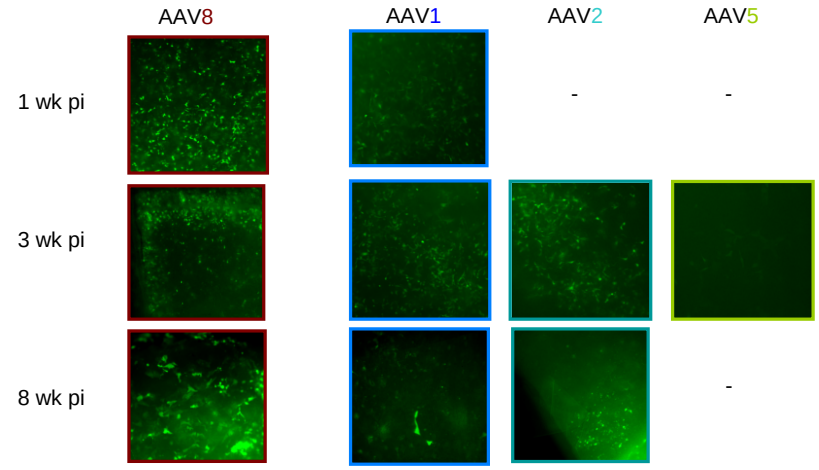

Figure ~~2~~3: GFP expression in the stroma of human corneal explants following injection with an AAV8 vector compared to AAV1, -2 and -5 vectors. The strongest GFP expression can be seen following injection with AAV8 and this expression persists for > 8 wk pi.

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For cystinosis, corneal gene therapy could be a feasible alternative if we can achieve long-term gene expression to avoid the need for multiple injections. Along this line, we have shown that AAV vectors could be promising vectors for corneal gene therapy via intra-stromal injection as they avoid the initial repair mechanism to give long-term gene expression. However, their low transduction efficiency is a limiting factor and we believe it is the reason why we haven't been able to detect cystinosin-driven reduction of cystine levels. The production of vectors of a higher titre would allow us to inject a higher number of viral particles into the eye for the same volume, which would result in a higher number of transduced cells and thus likely allow us to detect a cystinosin-specific effect.

IV) Refine characterisation of the CNS anomalies in *Ctns*$^{-/-}$ mice

Background:

At the time of our initial proposal in 2007, we had performed behavioural studies of young and middle-aged *Ctns*$^{-/-}$ mice and showed that the middle-aged mice have marked spatial and working memory defects, reminiscent of those seen in some patients, which were most likely hippocampal in origin (Maurice *et al*. 2009). 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 fluorescence-activated cell sorting (FACS), and assay each cell type for their respective cystine levels. In parallel, we also performed stereotaxic injections to target the hippocampus in mice.

Results:

This project was more challenging than we first anticipated and thus we did not accomplish all of our goals. 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 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. These results remain to be confirmed.

In parallel, we performed stereotaxic injections in the brain of wild-type mice to determine the correct coordinates to consistently reach the region of the hippocampus (CA1) likely affected in the *Ctns*$^{-/-}$ mice. This is a technically challenging approach however, after multiple efforts, Sandy Ibanes succeeded in correctly targeting the CA1 region (Fig. 4). Our next goal is to inject HD CAV-CIG (targets neurons) and AAV8-CIG (targets glial cells) in *Ctns*$^{-/-}$ mice and assay cystine levels post-transduction. These *in vivo* gene transfer studies should also help validate our *in vitro* data as to the cell type most affected in cystinosis.

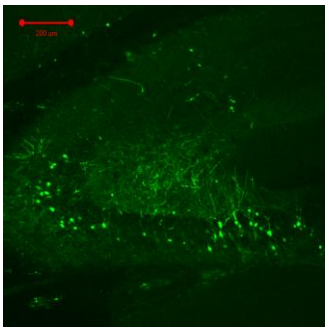

Figure ~~2~~4: GFP expression in the mouse brain. Following stereotaxic injection of CAVGFP into the mouse brain, GFP expression can be seen in the neurons of the CA1 region of the hippocampus.