

CRF 18-mo progress report

Title of proposal: Gene transfer studies for cystinosis

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Initial specific aims

- I) Validate preliminary *in vitro* gene transfer studies on primary murine hepatocytes by *in vivo* gene transfer to the liver.
- II) Generate viral vectors (helper-dependent canine adenovirus serotype 2 and adeno-associated virus serotype 8) expressing *CTNS* and perform eye-targeted gene transfer studies to correct the corneal anomalies of cystinosis.
- III) Finish characterising the CNS anomalies in *Ctns*^{-/-} mice, and begin CNS-targeted gene transfer to correct these anomalies.

Abbreviations

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

I) *In vivo* gene transfer studies

Background:

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

Results:

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

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

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

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

Background:

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

Results:

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

With regards to AAV-8 vector production, we received a first stock of AAV-CTNS-IRES-GFP and AAV-GFP (vehicle control) from the Vector Production Platform at the Centre of Biotechnology and Animal Gene Therapy (Barcelona, Spain) in July 2007. Unfortunately, due to a problem during the vector purification steps, we were provided with small amounts of each vector at a low titre. Thus it was impossible to use these vectors for *in vivo* experiments. We requested a second batch that arrived in December 2007. Although, we received an appropriate volume of the vectors at a reasonable titre, the stocks arrived thawed due to a delay in the shipping process. Thus, although we began testing these vectors *in vivo*, see section IIb below, we are prudent as to the interpretation of the results as we do not know if the thawing affected vector viability. To avoid the risk of another shipping accident, Claire Hippert went to Barcelona in June 2008 and brought back a third (frozen) stock of AAV-CTNS-IRES-GFP, which we just began testing *in vivo*.

IIb) *In vivo* corneal-targeted gene transfer studies

Background:

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

Results:

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

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

Background:

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

Results:

We first set out to isolate individual cell types from the brain to assay their respective cystine levels. As our previous work evoked a hippocampal defect primarily, we are first concentrating on this structure. The procedure we are currently using involves labelling individual cell types with fluorescent-labelled cell markers and isolating these cells via FACS. To discriminate between cell types, we have selected antibodies directed to specific cell surface markers: NGF receptor for neurons; the glutamate transporter EAAT1 for astrocytes; myelin oligodendrocyte glycoprotein for oligodendrocytes, and CD11b for microglial cells. For our pilot experiments, we have concentrated on the isolation of microglial cells as we have prior experience with the anti-CD11b antibody. We isolated the hippocampi from an 8 mo-old mouse and enzymatically dissociated the tissue by incubating with the digestive enzyme papain. We then mechanically dissociated the tissue by trituration with a 1-ml pipette and collected the cells after passage through a cell strainer. We labelled the cells with the fluorescent anti-CD11b antibody and sorted by FACS. We were able to see various populations in our cell suspension with a clearly separate, labelled population corresponding to the microglial cells. Following this encouraging and exciting data, we will now repeat the experiment with all four markers and determine whether we are able to clearly differentiate the other three populations. We will then determine the number of cells we can isolate from one hippocampus to know whether we need to upscale in order to have enough cells for the cystine assay. Once the optimisation steps are finished, our plan is to isolate individual cell types from different-aged mice, sort the cells by FACS, and assay their respective cystine levels. We hope this will give us direct information about which cell type is most affected in the cystinotic brain.