CRF 6-mo progress update (fellowship)

Gene transfer studies for cystinosis

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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 fluorescence-activated 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 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 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 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.

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 non-integrating 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 CAV-CIG (targets neurons) and AAV8-CIG (targets glial cells) in *Ctns*$^{-/-}$ mice and assay cystine levels post-transduction.